A urinary metabolite ratio that reflects systemic caffeine clearance

Systemic caffeine clearance and urinary metabolite profiles were determined in 15 subjects with diverse exposure histories to cytochrome P-450 inducers (cigarette smoke) and inhibitors (oral contraceptive steroids). A correlation was observed between caffeine clearance and a urinary ratio based on the molar recovery of paraxanthine 7-demethylation products relative to a paraxanthine 8-hydroxylation product ($r = 0.91$; $P < 0.001$). Analysis of urinary metabolites was undertaken in a larger population to assess the effects of gender, age, oral contraceptives, and smoking on the ratio. No gender differences were observed in either adults or children; children ($n = 21$) showed a higher ($P < 0.001$) mean metabolite ratio than adults ($n = 61$), oral contraceptive users ($n = 9$) had lower ($P < 0.05$) ratios than women not taking oral contraceptives ($n = 30$), and smokers ($n = 26$) had higher ($P < 0.001$) ratios than nonsmokers ($n = 61$). The data indicate that a urinary metabolite ratio based on paraxanthine 7-demethylation/8-hydroxylation products reflects systemic caffeine clearance and likely monitors cytochrome P-450 activity inducible by polycyclic aromatic hydrocarbons. (CLIN PHARMACOL THER 1987;42:157-65.)

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Considerable circumstantial evidence exists concerning the involvement of polycyclic aromatic hydrocarbon (PAH)–inducible cytochrome P-450 (presumably a P_1-450 analogue, also known as aryl hydrocarbon hydroxylase [AHH]) in the biotransformation of caffeine in animals and humans.[1-6] This inducible enzyme is of toxicologic and pharmacologic importance because of its role in the activation of many xenobiotics into more toxic intermediates.[7,8]

Animal studies suggest a target tissue–specific association between increased AHH activity and biologic injury, including mutagenesis, carcinogenesis, and teratogenesis.[9-11] Corresponding studies in humans have had to await the development of safe methods of assessing AHH activity in large populations. Notable in this regard is the development of the caffeine breath test,[3,6] which is a proven indicator of caffeine clearance and likely a good indicator of P_1-450 activity in humans.[4] In contrast to the caffeine breath test, which depends on the use of isotopically labeled caffeine, our aim is to develop a urinary test based on metabolite ratios derived from unlabeled caffeine to monitor PAH-inducible P-450 activity because of its potential as a convenient and relatively inexpensive method applicable to studies in biochemical epidemiology.

We have recently provided several lines of evidence[12,13] indicating that all *N*-demethylations from caffeine and its dimethylxanthine products, paraxanthine, theophylline, and theobromine, are mediated in large part by PAH-inducible P-450 in human liver microsomes. In contrast, the corresponding methylxanthine C_8-hydroxylations are in large and variable part mediated by another enzyme(s) that is apparently not inducible by PAHs.[12,13]

In humans, caffeine 3-demethylation to paraxanthine is the dominant pathway (Fig. 1) among initial *N*-demethylations, and paraxanthine's subsequent urinary products 1-methylxanthine (1X), 1-methyluric acid (1U), 5-acetylamino-6-formylamino-3-methyluracil (AFMU), and 1,7-dimethyluric acid (17U) account for 30% to 55% of the administered dose of caffeine 24 hours after ingestion.[14] Based on previous experimental observations of the in vitro metabolism of paraxanthine by human liver microsomes, we proposed that the molar ratio of paraxanthine 7-demethylation products (AFMU + 1U + 1X) to a paraxanthine

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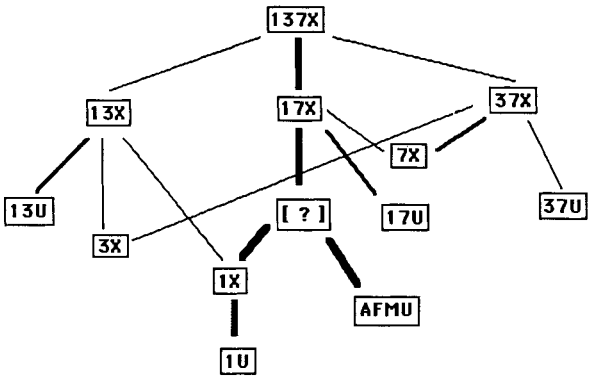

Fig. 1. Proposed pathway of caffeine (137X) metabolism in humans. (137X, caffeine; 13X, theophylline; 17X, paraxanthine; 37X, theobromine; 13U, 1,3-dimethyluric acid; 37U, 3,7-dimethyluric acid; 3X, 3-methylxanthine; 7X, 7-methylxanthine.)[14]

8-hydroxylation product (17U) could provide an index of PAH-inducible cytochrome P-450 activity.[12] We tested this proposal in this investigation by comparing systemic caffeine clearances with AFMU + 1U + 1X/17U urinary ratios in a group of subjects selected for their diverse exposure to known P-450 inducers (cigarette smoke) and inhibitors (oral contraceptive steroids).

MATERIAL AND METHODS

Subjects and drug administration

Comparison of caffeine kinetic parameters obtained from saliva, plasma, and whole blood samples. With a single subject, the determination of caffeine kinetic parameters was compared by four different but simultaneous sampling procedures. Saliva, whole blood (via venipuncture), plasma (via venipuncture), and whole blood (via duplicate finger pricks) samples were obtained 0, 1.5, 3, 5, 7, 9, 11, 13, 15, 17, and 26 hours after caffeine ingestion (5 mg/kg) after an overnight fast. Whole blood samples, whether venous or finger prick, contained 400 μl 2.5% sodium oxalate solution per 100 μl blood. Corresponding blank blood samples were collected to which pure caffeine could be added subsequently for the generation of standard curves.

Comparison of caffeine clearances and urinary metabolite ratios. Fifteen healthy, nonmedicated (except for use of oral contraceptives in five subjects) white subjects participated in this study and were selected to include five nonsmoking oral contraceptive users, five nonsmokers, and five smokers (approximately 20 cigarettes per day). Subjects abstained from methylxanthine-containing foods and beverages for 48 hours before and for the duration of the study. After an overnight fast, each subject received an oral caffeine dose (4 mg/kg). Total urine volume was collected for 40 hours at the following intervals: 0, 0 to 8, 8 to 16, 16 to 24, 24 to 32, and 32 to 40 hours. Urine samples were frozen immediately after voiding. On retrieval of the samples (within 2 days), they were thawed and volumes were measured and aliquots acidified to pH 3.5 with concentrated HCl and stored at −20° C until analyzed (within 3 weeks). In addition, eight of these subjects provided random urine samples after routine dietary caffeine intake. Random urine samples were collected on 3 separate days within 1 week of the experimental period.

Samples of mixed saliva (2 ml, unstimulated) collected at 0, 2, 5, 8, 11, and 16 hours were frozen immediately and stored at −20° C until analysis. Duplicate blood samples (via finger prick) were collected at two different times to coincide with saliva collection, to permit determination of a saliva/blood caffeine ratio. For each finger prick blood sample, precisely 100 μl whole blood was drawn with a glass capillary tube, added to 400 μl 2.5% sodium oxalate solution in a test tube, capped, and frozen at −20° C until analysis.

Multiple-dose study. Two subjects participated in a multiple-dose study in which liberal dietary caffeine consumption preceded the experimental period, followed by regulated caffeine ingestion. Subject 1 was a 32-year-old woman (smoker, not using oral contraceptives), and subject 2 a 35-year-old man (smoker). Each subject received a dose of oral caffeine (1 mg/kg) at 0, 2, 4, 6, 24, 26, 28, and 30 hours. Total urine volume was collected at 2-hour intervals (overnight at an 8-hour interval) for 48 hours, acidified to pH 3.5, and stored frozen at −20° C until analysis.

Comparison of urinary metabolite ratios in different populations. The AFMU + 1X + 1U/17U urinary metabolite ratios were compared in smokers, children, whites, Orientals, and women taking oral contraceptive steroids. For the smoker population, the majority of whom routinely drank three to four cups of coffee per day, a late afternoon urine sample was collected after repeated dietary caffeine intake estimated to be equivalent to about 300 mg caffeine. The smokers (16 men and 11 women) ranged in age from 21 to 52 years (mean age 28 years) and were generally light drinkers (<3 drinks per week) and moderate to heavy smokers (mean cigarette consumption 19 per day; range 10 to 40 per day).

In the case of the children, many of whom did not

routinely ingest dietary caffeine, overnight urine samples were collected to maximize the collection period. The children were healthy, urban, nonmedicated, and prepuberty (age 4 to 11 years, mean age 7 years; seven female and 14 male). Morning urine samples (based on a 6- to 10-hour overnight collection interval) were collected after repeated ingestion of 10 to 20 ounces of Coca Cola (containing 30 to 70 mg caffeine) throughout the afternoon of the previous day.

The data for the white and Oriental populations and four of the oral contraceptive users (Fig. 2) were derived from a previously described study[14] in which subjects ingested 300 mg caffeine, and pooled urine was collected 24 hours thereafter.

The data for the other five oral contraceptive users depicted in Fig. 1 where obtained from the detailed 15-subject study described in the section on caffeine clearances and urinary metabolite ratios. All oral contraceptive users in the study were nonsmokers.

Sample analysis

Urinary caffeine metabolites were quantified as described previously.[14] Briefly, 200 μl urine was added to a centrifuge tube containing approximately 120 mg ammonium sulfate and vortexed 15 seconds. After adding 200 μl internal standard solution (120 mg *N*-acetyl-*p*-aminophenol/L chloroform) and 6 ml chloroform/isopropyl alcohol (90:10, v/v)–extracting solvent, the mixture was vortexed 30 seconds and centrifuged 5 minutes at 2500 rpm. The organic phase was transferred and dried under N_2 (at 40° to 45° C), resuspended in HPLC mobile-phase solvent (0.05% acetic acid/methanol, 88:12, v/v), and injected onto a Beckman Ultrasphere ODS (5 μm, 25 cm by 4.6 mm) analytical column (Beckman Instruments, Inc., Palo Alto, Calif.). Urinary metabolites were eluted at 1 ml/min, detected by ultraviolet absorbance at 280 nm, and quantified by comparison with blank urine spiked with pure standards.

Caffeine levels in saliva, whole blood, and plasma were quantified as above with the following modifications: Saliva samples were first centrifuged 10 minutes, and 200 μl of the supernatant was used in the analysis. For caffeine analysis, 50 μl β_7-hydroxypropyl theophylline (20 mg/L in H_2O) was used as the internal standard. Separate standard curves were prepared for each of saliva, whole blood, and plasma, in which pure standard was added to the corresponding blank biologic fluid, and taken through the same extraction procedure as the samples. The extracted, dried residue containing caffeine was resuspended in 0.05% acetic acid/methanol (88:12, v/v), injected onto the same HPLC analytic column as for urine analysis, and eluted at 1.3 ml/min and a column temperature of 40° C. Caffeine was detected by ultraviolet absorbance at 273 nm.

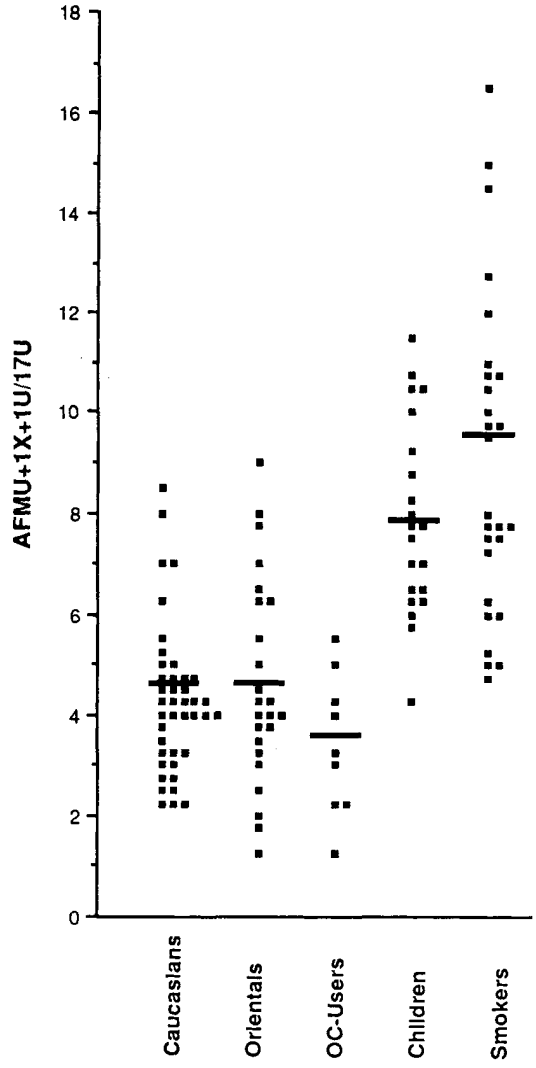

Fig. 2. Comparison of AFMU + 1U + 1X/17U ratios in five different populations. Horizontal bars mark population means. (*OC*, oral contraceptive.)

Chemicals

Caffeine (137X), *N*-acetyl-*p*-aminophenol, and β_7-hydroxypropyl theophylline were obtained from Sigma Chemical Co., St. Louis, Mo.; 1X from Pfaltz and Bauer, Waterbury, Conn.; and 1U and 17U from Adams Chemical, Round Lake, Ill. AFMU was isolated and purified by Dr. B. K. Tang, Department of Pharmacology, University of Toronto[15]; chloroform and

Table I. Comparison of caffeine kinetic profile based on plasma, venous blood, pinprick and saliva sampling

Sample	C_o *(mg · L⁻¹)*	k_e *(hr⁻¹)*	$t_{1/2}$ *(hr)*	*AUC (mg · hr · L⁻¹)*	V_c *(L · kg⁻¹)*	*CL (ml · min⁻¹ kg⁻¹)*
Plasma	10.65	0.173	4.02	61.6	0.470	1.35
Venous blood	10.33	0.170	4.07	60.8	0.483	1.37
Finger prick blood	10.77	0.163	4.24	66.1	0.462	1.26
Saliva	9.76	0.166	4.22	58.8	0.519	1.42
Plasma (calculated)*	10.66	0.166	4.22	64.2	0.475	1.30

*These plasma parameters calculated from saliva analysis based on experimentally determined saliva/plasma caffeine ratio of 0.916 ± 0.028 (SE) (n = 10).

Table II. Kinetic profile of caffeine after oral ingestion (4 mg/kg)

	Gender	*Age (yr)*	*Weight (kg)*	k_e *(hr⁻¹)**	$t_{1/2}$ *(hr)*	*Clearance (ml · min⁻¹ kg⁻¹)†*	*S/B (±SE)*
OC users							
1	F	21	59	0.083	8.3	0.91	1.03 ± 0.02
2	F	23	57	0.057	12.1	0.68	0.94 ± 0.02
3	F	25	64	0.077	9.0	0.94	0.93 ± 0.01
4	F	30	50	0.081	8.6	1.05	1.07 ± 0.02
5	F	24	59	0.086	8.1	0.78	0.91 ± 0.04
Mean ± SE				0.077 ± 0.005	9.2 ± 0.7	0.87 ± 0.06	
Nonsmokers							
1	F	26	64	0.073	9.5	0.73	0.91 ± 0.02
2	F	24	54	0.115	6.0	1.02	0.99 ± 0.01
3	F	25	64	0.093	7.5	0.82	0.68 ± 0.06
4	F	24	75	0.123	5.6	0.96	0.78 ± 0.06
5	M	26	63	0.128	5.4	1.18‡	ND
Mean ± SE				0.106 ± 0.010	6.8 ± 0.8	0.94 ± 0.08	
Smokers							
1	F	22	59	0.132	5.3	1.77	0.55 ± 0.04
2	F	21	59	0.312	2.2	2.59	0.80 ± 0.02
3	F	42	56	0.132	5.2	1.52	1.04 ± 0.03
4	M	35	57	0.193	3.6	2.28‡	ND
5	F	32	57	0.178	3.9	1.56‡	ND
Mean ± SE				0.189 ± 0.033	4.0 ± 0.6	1.94 ± 0.21	

OC, oral contraceptive; S/B, saliva/blood caffeine levels.
*These kinetic parameters, although based on saliva analysis, are expected to be approximately equivalent to those for blood.
†Clearance values listed represent expected CL from blood. Clearances are based on transformation of salivary caffeine levels based on the saliva/blood caffeine ratios determined for each subject.
‡Kinetic parameters based directly on blood analysis.

methanol were from BDH Chemicals, Toronto, Ontario; and all other chemicals were reagent grade from Fisher Chemicals, Toronto, Ontario.

Data analysis

The elimination half-life ($t_{1/2}$) and elimination rate constant (k_e) of unchanged caffeine were calculated by least-squares linear regression of the log-linear decline of drug concentrations in plasma, whole blood, or saliva. Zero–time caffeine concentration (C_o) was calculated by extrapolation of the regression, and AUCs were determined by the trapezoidal rule. Apparent volume of distribution (V_c) and total systemic clearance (CL) were calculated based on the following formulas: $V_c = CL/k_e$ and $CL = dose \cdot f/AUC$, where the bioavailable fraction (f) of the caffeine dose was assumed to be equal to 1.[16]

RESULTS

Comparison of caffeine kinetic parameters obtained from saliva, plasma, and whole blood samples

Table I summarizes caffeine kinetic parameters in a single subject as determined by four different sampling

Table III. Urinary excretion of four major caffeine metabolites*

	AFMU	*1U*	*1X*	*AUX*	*17U*	*Total*	*AUX/17U*
OC users							
1	3.6	18.4	14.2	36.2	6.9	43.1	5.25
2	2.9	10.3	10.3	23.5	6.8	30.3	3.46
3	4.5	15.5	11.8	31.8	7.3	39.1	4.36
4	13.1	15.6	11.7	40.4	7.0	47.4	5.77
5	2.1	8.7	8.4	19.2	4.3	23.5	4.47
Mean ± SE	5.2 ± 2.0	13.7 ± 1.8	11.3 ± 0.9	30.2 ± 3.9	6.5 ± 0.5	36.7 ± 4.3	4.66 ± 0.40
Nonsmokers							
1	11.1	10.1	11.3	32.5	6.3	38.8	5.16
2	4.5	18.4	13.3	36.2	6.0	42.2	6.03
3	5.3	17.5	15.4	38.2	7.8	46.0	4.90
4	7.5	8.6	8.0	24.1	3.5	27.6	6.89
5	14.0	6.9	8.8	29.7	7.6	37.3	3.91
Mean ± SE	8.5 ± 1.8	12.3 ± 2.4	11.4 ± 1.4	32.1 ± 2.5	6.2 ± 0.8	38.4 ± 3.1	5.38 ± 0.51
Smokers							
1	4.4	28.1	30.5	63.0	6.2	69.2	10.16
2	18.4	25.0	18.4	61.8	4.4	66.2	14.04
3	13.3	13.0	12.6	38.9	6.3	45.2	6.18
4†	4.8	13.8	16.9	35.5	3.7	39.2	9.60
5	20.8	16.1	15.2	52.0	6.6	58.7	7.89
Mean ± SD	12.3 ± 7.6	19.2 ± 6.9	18.7 ± 6.9	50.2 ± 5.7	5.4 ± 1.3	55.7 ± 13.1	9.57 ± 2.94

OC, oral contraceptive; AUX, molar sum AFMU + 1U + 1X.
*Molar percent of caffeine dose (4 mg/kg) recovered in 40 hours.
†Based on 24-hour collection period.

methods. Plasma, venous blood, and finger prick blood analysis for caffeine levels yielded the same values for kinetic parameters (coefficient of variation <5%) as did calculated values based on saliva analysis for which the experimentally determined saliva/plasma caffeine ratio of 0.92 was applied. The consistency in clearance values among the different sampling protocols permitted the use of a simplified protocol in our 15-subject study. In the simplified protocol, a combination of saliva and finger prick blood samples was used to estimate CL instead of the more conventional approach based on plasma collection. The utility of salivary caffeine analysis as an accurate predictor of caffeine $t_{1/2}$ in plasma has been established by numerous investigators[5,17-19]; however, the generation of clearance values necessitates in addition the determination of a saliva/blood caffeine ratio.

Comparison of caffeine clearances and urinary metabolite ratios

For this 15-subject study, caffeine kinetic parameters (Table II) and urinary metabolite profiles (Table III) were generally in agreement with published results.[5,14,16,20] Table III shows that the molar fraction of the paraxanthine 8-hydroxylation product (17U) was similar among the 15 subjects, whereas the molar fraction of paraxanthine 7-demethylation products (AFMU + 1X + 1U) was 60% higher in smokers compared with nonsmokers.

Of principal interest in the 15-subject study was the highly significant correlation ($r = 0.91$; $P < 0.001$) between caffeine clearance rates and urinary AFMU + 1X + 1U/17U ratios (Fig. 3). Similarly, k_e and $t_{1/2}$ values were also highly correlated with AFMU + 1X + 1U/17U (k_e vs. urinary ratio, $r = 0.92$, $P < 0.001$; and $t_{1/2}$ vs. urinary ratio, $r = -0.78$, $P < 0.001$).

Dependence of urinary metabolite ratio on collection interval

The AFMU + 1X + 1U/17U ratio varied considerably with the time and length of the urine collection period. Table IV indicates that a 24-hour collection period provided a urinary ratio with the highest correlation to caffeine clearance but that more exhaustive collection did not improve the correlation. Furthermore, Table IV indicates a strong correlation between late afternoon and overnight collection (8 to 16 and 16 to 24 hours after caffeine ingestion) and caffeine clearance, probably as a result of the high metabolite recoveries during this period. Peak metabolite recovery occurred at 12 and 20 hours for smokers and nonsmokers (including oral contraceptive users), respectively.

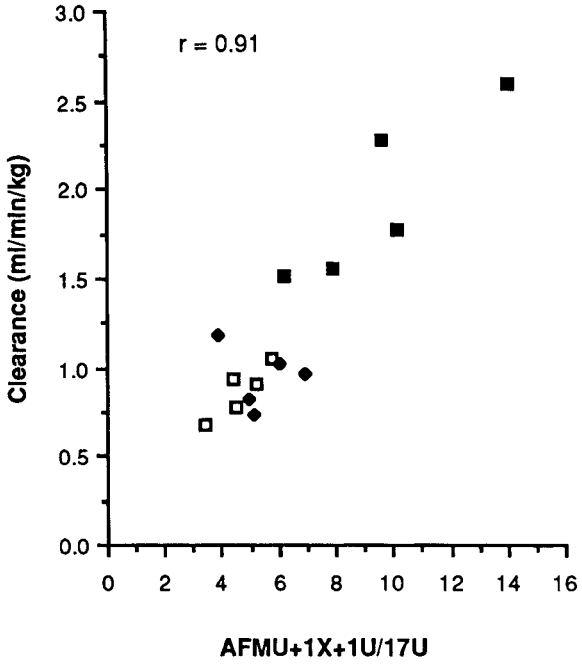

Fig. 3. Correlation between urinary AFMU + 1U + 1X/17U and caffeine CL. Oral contraceptive users (□); nonsmokers (◆); smokers (■).

Fig. 4 demonstrates that AFMU + 1X + 1U/17U ratios determined under a regimen of multiple caffeine administrations were similar to the ratio obtained using a pooled 24-hour urine sample after ingestion of a single caffeine dose. For comparison purposes, Fig. 4 also depicts ratios for AFMU/1X (representative of *N*-acetyltransferase activity)[14] and 1U/1X (representative of xanthine oxidase activity).[21] Xanthine oxidase activity was similar in both subjects whereas acetylation activity was fast in subject 1 and slow in subject 2.

Table V indicates that randomly collected urine samples after chronic dietary caffeine intake may be adequate for the assessment of AFMU + 1X + 1U/17U ratios in population studies, particularly when routine dietary intake patterns approach steady-state conditions. Of interest is that the mean coefficient of variation was only 12% (range 4% to 21%) among AFMU + 1U + 1X/17U ratios determined for four samples that included a 24-hour pooled urine and three random urine samples (Table V).

Comparison of urinary metabolite ratios in different populations

Urinary AFMU + 1U + 1X/17U ratios displayed a high degree of interindividual variability (about 2000%) among different populations (Fig. 2; Table VI).

Table IV. Effect of urine collection period on correlation between AFMU + 1U + 1X/17U and caffeine clearance

Collection interval (hr)	*r**	*Collection interval (hr)*	*r**
0-8	0.85	0-8	0.85
8-16	0.88	0-16	0.89
16-24	0.90	0-24	0.91
24-32	0.79	0-32	0.90
32-40	0.63	0-40	0.91

*n = 15; r (correlation coefficient) >0.64 is significant ($P < 0.001$).

No gender differences were observed in either adults or children. With respect to ethnic origin, mean values were not statistically different between whites and Orientals. In this investigation, children showed significantly higher mean AFMU + 1X + 1U/17U ratios than did adults ($P < 0.001$), smokers showed higher mean ratios than did nonsmokers ($P < 0.001$), and oral contraceptive users showed lower mean ratios than did nonusers ($P < 0.05$).

DISCUSSION

The highly significant correlation between caffeine clearance rates and urinary AFMU + 1U + 1X/17U ratios in 15 subjects with diverse xenobiotic exposure histories demonstrates that urinalysis after caffeine intake can provide a noninvasive procedure for assessing the effect of modulating agents, such as cigarette smoke and oral contraceptives, on caffeine clearance in humans. Since caffeine clearance reflects PAH-inducible cytochrome P-450 activity,[1,3] it appears that the urinary AFMU + 1U + 1X/17U ratio is therefore also a good in vivo index of PAH-inducible cytochrome P-450 activity. Corroborating evidence comes from our previous investigations of methylxanthine metabolism by human liver preparations in which we demonstrated that all three primary demethylations of caffeine are apparently mediated by PAH-inducible P-450[13] and that the ratio of paraxanthine 7-demethylation to 8-hydroxylation products correlated with cytochrome P_1-450 activity (as determined by ethoxyresorufin *o*-deethylation rates).[12]

Of relevance is the detailed study of caffeine metabolism by Callahan et al.[20] If it is assumed that the two acetylated uracil metabolites that these authors refer to as A_1 and A_2 are approximately equivalent to our AFMU levels as determined by a different analytic method, it is seen that their mean 'AFMU' + 1U + 1X/17U ratios among male and female subjects and oral contraceptive users correlate highly with corresponding clearance values ($r = 0.91$; $P < 0.05$). Furthermore,

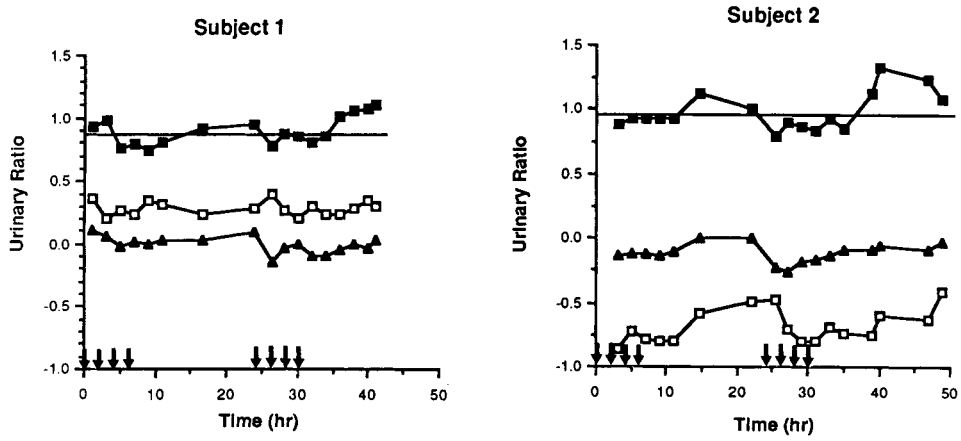

Fig. 4. Time course of urinary metabolite ratios in two subjects under regimens of multiple caffeine administration. The arrows mark the time of caffeine ingestion (1 mg/kg/dose). Logarithms of urinary metabolite ratios are as follows: AFMU + 1X + 1U/17U (■); AFMU/1X (□); and 1U/1X (▲). The horizontal line represents the AFMU + 1X + 1U/17U ratio obtained from a 24-hour pooled urine sample after a single caffeine dose (4 mg/kg).

their numeric relationship between 'AFMU' + 1U + 1X/17U ratios and CL values is completely consistent with our present observations.

The validity of urinary AFMU + 1U + 1X/17U as an indicator of PAH-inducible P-450 activity rests on several assumptions.

First, although 1X formation from caffeine is the sum of theophylline 3-demethylation and paraxanthine 7-demethylation (Fig. 1), it is assumed that the proportion of 1X formed via theophylline is negligible, given that <10% of caffeine is metabolized to theophylline and that only about 15% of theophylline is metabolized to 1X.

Second, it is assumed that the urinary ratio will be unaffected by normal variations in urine flow. The polar nature of AFMU, 1X, 1U, and 17U means that these compounds are rapidly excreted without reabsorption by the kidney. Although a metabolite ratio of AFMU + 1U + 1X/17U should in theory correlate with PAH-inducible P-450 activity, the strong urine flow dependence of 17X[14] makes this ratio inaccurate and highly variable (Campbell, unpublished observations). In contrast, AFMU, 1U, 1X, and 17U formation show no dependence on urine flow,[14] and consequently a ratio based on these metabolites would show no dependence on urine flow.

Third, the validity of the AFMU + 1U + 1X/17U ratio is based on the assumption that the high ratios among smokers, and the low ratios among oral contraceptive users, are the result of inductive or inhibitory effects specific to PAH-inducible P-450. Alternatively,

Table V. Comparison of AFMU + 1U + 1X/17U ratios derived from pooled and randomly collected urine samples

Subject	*24-Hr pooled urine**	*Random urine samples (n = 4)†*	
		Mean ± SE	*CV%‡*
OC-1	5.0	5.4 ± 0.4	14
OC-4	5.7	4.7 ± 0.5	21
OC-5	4.2	4.2 ± 0.1	4
NS-3	4.6	4.6 ± 0.3	12
NS-4	6.3	5.3 ± 0.3	10
NS-5	3.9	4.0 ± 0.3	12
S-4	9.6	10.3 ± 0.7	13
S-5	7.9	8.0 ± 0.3	7

OC, oral contraceptive user; NS, nonsmoker; S, smoker.
*After single caffeine dose (4 mg/kg).
†After routine caffeine intake on 3 separate days, compared with 24-hour pooled urine.
‡Coefficient of variation based on mean of three random urine samples plus 24-hour pooled urine.

it is possible, particularly in the case of cigarette smoke, which contains hundreds of different compounds, that one or more compounds specifically inhibit 17U production and thereby result in a high ratio; however, the results of the 15-subject study would argue against this possibility. In our 15-subject study, the molar excretion of 17U was similar among subjects, whereas AFMU + 1X + 1U excretion was greatly increased among smokers, consistent with induction of PAH-inducible cytochrome P-450 activity as represented by

Table VI. Comparison of mean AFMU + 1X + 1U/17U ratios in different populations

Aspect	*Population 1 (n) (mean ± SE)*	*Population 2 (n) (mean ± SE)*	*P value**
Gender	Female adults (30) 4.7 ± 0.3	Male adults (30) 4.8 ± 0.3	0.45
	Female children (7) 7.6 ± 0.8	Male children (14) 8.1 ± 0.5	0.38
Age (yr)	Children: age 3-7 (13) 8.3 ± 0.5	Children: age 7-11 (8) 7.4 ± 0.7	0.24
	Children: age 3-11 (21) 7.9 ± 0.4	Adults: age 18-65 (61) 4.7 ± 0.4	<0.001†
	Adults: age 18-30 (42) 4.4 ± 0.2	Adults: age 30-65 (15) 4.7 ± 0.4	>0.25
OC use	OC users (9) 3.6 ± 0.5	Women not using OC (30) 4.7 ± 0.3	0.03†
PAH exposure	Smokers (26) 9.4 ± 0.7	Nonsmokers (61) 4.7 ± 0.2	<0.001†
Ethnic origin	Oriental adults (26) 4.6 ± 0.4	White adults (42) 4.7 ± 0.4	>0.05

OC, oral contraceptive.
*Tests of significance based on Mann-Whitney nonparametric test.
†Significant.

the specific increase in excretion of paraxanthine 7-demethylation products. The consistency in the relative rank of mean AFMU + 1X + 1U/17U ratios among adults,[20,22] children,[4,11,23] oral contraceptive users,[20,24,25] and smokers[3,5] with published clearance parameters further reinforces the validity of the ratio.

It should be noted that the current investigation involved only overtly healthy subjects with no suspicion of impaired liver or kidney function. We speculate that liver disease such as cirrhosis could affect both paraxanthine 7-demethylation and 8-hydroxylation pathways equivalently, resulting in no change in metabolite ratio despite massive reductions in normal liver function. In such an instance, the urinary ratio AFMU + 1X + 1U/17U would be a poor indicator of caffeine clearance but might nonetheless remain a good discriminator of PAH induction.

Furthermore, it is possible that renal disease might differentially affect the excretion of caffeine metabolites dependent on active transport mechanisms and thereby alter the AFMU + 1X + 1U/17U ratio in a manner that no longer reflects caffeine CL or enzyme induction. Consequently, caution should be used in interpreting urinary ratios from individuals suspected of suffering from liver or kidney disease.

The best protocol for accurate assessment of the AFMU + 1X + 1U/17U ratio entails a complete 24-hour recovery of urine after caffeine ingestion. However, in population studies in which the interindividual variation of ratios is high, two short-cut methods of assessing this ratio appear to be adequate: overnight urine collection and random urine collection after chronic dietary caffeine intake. For studies based on dietary caffeine intake, it is prudent to discourage excessive caffeine ingestion (more than three cups of brewed coffee) on the day of collection of samples to avoid possible saturation kinetics and consequent shifts in metabolic pathways, although published data are not consistent on this matter. Using the caffeine breath test, Kotake et al.[3] demonstrated saturation kinetics at a caffeine dose of 3 mg/kg, whereas Bonati et al.[16] demonstrated linear kinetics up to 10 mg/kg.

Our urinary test is suitable for large-scale population studies because it requires only dietary intake of a popular and safe substance—caffeine—and because it relies on the simple collection of urine rather than on multiple blood sampling. Furthermore this urine test is inexpensive relative to the caffeine breath test because it foregoes the need for isotopically labeled caffeine. Another advantage of this urine test is that the same urine sample provides an assessment of xanthine oxidase[14] and *N*-acetyltranferase[21] activity.

In summary, our results indicate that a urinary metabolite ratio of AFMU + 1U + 1X/17U reflects systemic caffeine clearance and likely monitors PAH-inducible cytochrome P-450 activity. This urinary test is suitable for studies in biochemical epidemiology because it is noninvasive, relatively inexpensive, convenient, and analytically simple to determine with standard HPLC equipment.

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