Validation of urine caffeine metabolite ratios with use of stable isotope-labeled caffeine clearance

Objectives: A number of caffeine metabolite ratios have been proposed to measure CYP1A2 activity in vivo. The data to validate these ratios are scanty. The objective of this study was to validate urine caffeine metabolite ratios versus stable isotope–labeled caffeine clearance under different caffeine dosing conditions.

Study design: Two experiments, one with nine nonsmoking subjects and the other with 12 cigarette smokers, were performed. We explored the relationship between caffeine clearance, measured by means of intravenous infusions of stable isotope–labeled caffeine, and a number of caffeine metabolite ratios during administration of different single or multiple doses doses of caffeine to smokers and nonsmokers on three different occasions over a 2-week period, using different durations of urine collections, including spot urines. The stable isotope technique allowed simultaneous oral dosing of caffeine and measurement of caffeine metabolite ratios and caffeine clearance, the latter reflecting CYP1A2 activity.

Results: The caffeine metabolite ratio of AAMU+1U+1X/17U (5-acetylamino-6-amino-3-methyluracil + 1-methyluric acid + 1 methylxanthine/1,7-dimethyluric acid) maintained a significant correlation with caffeine clearance for all the above conditions (r^2 range, 0.4 to 0.9) except for dose. With high doses of caffeine (12 mg/kg), a significant relationship was not observed. AAMU+1U+1X/17U also correlated with the formation clearance of paraxanthine ($r^2 = 0.6$, $p = 0.002$). Other reported caffeine metabolite ratios did not display the same robust correlation with caffeine clearance under all these different conditions.

Conclusions: We conclude that AAMU+1U+1X/17U measured from a single spot urine collection is a valid measure of CYP1A2 activity except at very high levels of caffeine dosing. The validity of the other proposed caffeine metabolite ratios is questionable. (CLIN PHARMACOL THER 1996;59:284-96.)

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The tobacco smoke–inducible P450 enzyme CYP1A2 is thought to have an important role in the activation of carcinogens,[1,2] and its role in human disease is the subject of current research. To facilitate studies of CYP1A2 activity and human disease, a test has been sought that can easily be performed in a large number of subjects. Caffeine is metabolized primarily by *N*-3-demethylation to paraxanthine. The enzyme responsible is CYP1A2.[3] Therefore various measures of caffeine clearance and/or metabolism have been used to estimate CYP1A2 activity.[4] However, the metabolism of caffeine is complex (Fig. 1) and dose dependent,[5,6] and direct measurement of caffeine clearance is cumbersome. Therefore measurement of metabolite ratios of caffeine has been proposed as a noninvasive and easy to use method of indirectly assessing CYP1A2 activity. Because metabolite ratios relate concentrations of

metabolites to one another, they are essentially independent of total recovery from the urine,[7] and short collections of urine after a dose of caffeine may be all that is required.

A variety of such ratios has been proposed, but whether they accurately reflect CYP1A2 activity is open to question. In some studies, ratios with caffeine in the denominator and its dimethylxanthine products in the numerator have been used.[8,9] Validations of these ratios were never performed and one would expect urine flow changes to unduly influence these ratios.[10,11] Another proposed ratio is AFMU+1U+1X/17X (5-acetylamino-6-formylamino-3-methyluracil + 1-methyluric acid + 1-methylxanthine/1,7-dimethylxanthine [paraxanthine]).[7,12] However, this ratio varies thirtyfold in the adult population[7] and some of the variability could again be the influence of urine flow on paraxanthine excretion. A number of other demethylation ratios have been proposed and used without any published verification in the investigation of the maturity of demethylation pathways in the neonate.[13] Similarly, the paraxanthine/caffeine test (17X+17U/137X [paraxanthine + 1,7-dimethyluric acid/1,3,7-trimethylxanthine (caffeine)]) has also been used,[14] but its validity has been questioned.[4]

Finally, the ratio of AAMU+1U+1X/17U (5-acetylamino-6-amino-3-methyluracil + 1-methyluric acid + 1-methylxanthine/1,7-dimethyluric acid) has been proposed as an in vivo index of CYP1A2 activity.[15] Although the ratio does correlate to caffeine clearance after single doses of caffeine, a number of concerns have been raised. It is likely that when CYP1A2 is highly induced, this ratio loses its sensitivity[3]; therefore it may not be a good measure in smokers. Because the metabolism of caffeine is dose dependent with apparent end-product inhibition,[5] the size of the dose and the effect of multiple doses of caffeine on a ratio reflecting CYP1A2 activity also requires further exploration.

Any simple and practical test should be robust under the variety of conditions that would be encountered if such a test was to be applied to a large population of subjects. This study explores the relationship between caffeine clearance and a number of caffeine metabolite ratios during administration of different single or multiple doses of caffeine to smokers and nonsmokers on three different occasions over a 2-week period, using different durations of urine collections.

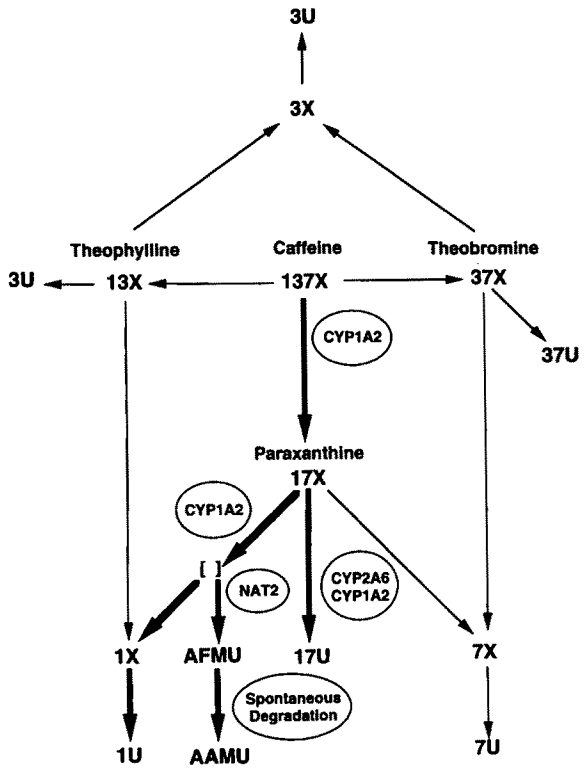

Fig. 1. Primary pathways of caffeine metabolism. *Heavy arrows* indicate major pathways, and adjacent to these arrows are the enzymes thought to catalyze the pathway. 137X, 1,3,7,-Trimethylxanthine (caffeine); 17X, 1,7-dimethylxanthine (paraxanthine); 37X, 3,7-dimethylxanthine (theobromine); 13X, 1,3-dimethylxanthine (theophylline); 17U, 1,7-dimethyluric acid; 13U, 1,3-dimethyluric acid; 1X, 1-methylxanthine; 3X, 3-methylxanthine; 7X, 7-methylxanthine; 1U, 1-methyluric acid; 3U, 3-methyluric acid; 7U, 7-methyluric acid; 137U, 1,3,7-trimethyluric acid; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; AAMU, 5-acetylamino-6-amino-3-methyluracil.

METHODS

Subjects and experimental protocol

Correlation of caffeine clearance and metabolite ratios at different doses of caffeine (experiment 1). The experimental protocol for this section has been described previously.[5] In brief, nine healthy nonsmokers who were regular consumers of caffeine were randomized in a crossover fashion to three blocks, each lasting 5 days. In each block, subjects consumed daily, at regular intervals during the day, six cups of decaffeinated coffee with added caffeine. Caffeine doses for each block were placebo, low (4.2 mg/kg per day), and high (12 mg/kg per day), re-

Table I. Gradient changes

Time (min)	*% Solvent A*	*% Solvent B*
0	97.5	2.5
21	97.5	2.5
22	85	15
38	85	15
39	50	50
49	50	50
50	97.5	2.5

spectively. To measure the clearance of caffeine, 25 mg of stable isotope–labeled caffeine ([2-^{13}C, 1,3,-$^{15}N_2$]caffeine) was given on the third day of each block by intravenous injection, and blood was sampled before dosing and at 5, 15, 30, and 45 minutes and at 1, 2, 3, 5, 8, 12, 24, and 36 hours after the dose. On the fifth day of every block, urine was collected for 24 hours for the measurement of caffeine and its metabolites. Two subjects did not receive isotope-labeled caffeine because of the unavailability of this compound at the time that they were entered into the study.

Correlation of caffeine clearance and metabolite ratios in smokers consuming multiple or single doses of caffeine (experiment 2). Twelve healthy male smokers (15 to 40 cigarettes per day), 33 to 64 years of age (mean age, 43 years), who regularly consumed caffeine participated in a baseline and then a follow-up study. On day 1 of the baseline study, subjects were given 25 mg of stable isotope–labeled caffeine by intravenous injection and blood was collected at 5, 15, 30, and 45 minutes and at 1, 2, 3, 5, 8, 12, and 24 hours for the measurement of caffeine clearance. During day 1, subjects consumed multiple beverages that contained caffeine at regular intervals throughout the day. The time they consumed their caffeine beverage and the amount of caffeine consumed approximately matched their intake the day previously except that decaffeinated coffee or tea with added caffeine was substituted for their regular beverage. The dose of caffeine that subjects consumed ranged from 3.1 to 9.9 mg/kg per day (mean, 5.6 mg/kg per day). Urine was collected for 24 hours. This was divided into 0- to 6-, 6- to 12-, and 12- to 24-hour collections, and single spot urine samples were also collected. The spot samples were taken at 8 AM on the morning of day 1 (this sample was not necessarily the first urine from overnight), at 5 PM in the afternoon of day 1, and the next morning at 8 AM on day 2 after the 12- to 24-hour collection was completed (again, not a first morning urine).

For the next 2 days, subjects received no further caffeine and were given a standard hospital diet except that the consumption of alcohol or other methylxanthine-containing beverages or foods was prohibited. On day 4, after an overnight fast, subjects were given a single dose of oral caffeine in the morning. The dose was either 2 or 4 mg/kg and no further caffeine or methylxanthine-containing beverages or food were given. Caffeine clearance was measured and urine collected as described for day 1. The mean recovery of caffeine and its metabolites in the urine collected for the 24 hours after the single dose of caffeine was 64% ± 13% (SD). The following week, eight subjects returned for the follow-up study, which was identical to day 1.

Analytic methods

The acquisition of chemicals and assays for plasma-labeled caffeine and urine caffeine concentrations are described in a previous article.[5] Metabolites of caffeine in the urine were measured with use of HPLC. The method for methylxanthines and methylurates is a modification of the method of Tang-Liu and Riegelman[16] in that (1) 1,8-dimethylxanthine was used as an internal standard; (2) 270, 280, and 290 nm wavelengths were used to measure metabolites and to check for peak purity using peak ratios at 270:290, 280:290, and 270:280 of pure standards for comparison; and (3) a mobile phase was used varying in both percent of methanol and in pH to provide well-separated peaks without the need for the counterion tetrabutyl-ammonium phosphate in either the mobile phase or reconstitutive solution. The mobile phase comprises the following: solvent A: 8.7 mmol/L aqueous acetic acid adjusted to pH 4.8 to 5.0 with 4N sodium hydroxide; solvent B: 8.7 mmol/L acetic acid in 50:50 water/methanol with no adjustment of pH (apparent pH, 3.4). The gradient changes are given in Table I.

The column oven temperature was 40° C. The samples were reconstituted with 200 μl solvent A mobile phase plus 10% methanol, and 50 to 75 μl were injected. Unlike the method of Tang-Liu and Riegelman,[16] our methanol gradient was accompanied by a decreasing pH gradient from 4.9 to 4.35 and, despite the lack of counterion, we were able to achieve excellent separation of metabolites with retention times as follows: 7-methyluric acid (7U), 10.1; 1U, 13.4; 7-methylxanthine (7X), 16.0; 3-methylxanthine (3X), 19.4; 1X, 22.4; 1,3-dimethyluric acid (13U), 28.6; 3,7-dimethylxanthine (theobromine, 37X), 30.1; 1,8-dimethylxanthine (18X), 32.6 (internal

standard); 17U, 34.0; 17X, 38.3; 1,3-dimethylxanthine (theophylline, 13X), 39.9; 1,3,7-trimethyluric acid (137U), 41.0; and 137X, 42.0 minutes. Because of the good separation of peaks, we later tested direct injection of urine samples without preliminary extraction, after filtering and addition of internal standard, and we obtained good correlation with results from the extracted urines. This direct injection method saved time, and peak purity could be checked with the peak ratios from the three detecting wavelengths. When problems of over-lapping peaks occurred or were suspected from peak ratios deviating from standard values, the extraction method was the preferred method.

AFMU in each sample was converted to AAMU by adjusting the urine to pH 10 for 10 minutes. AAMU was separated on a BioGel TSK-20 gel chromatography column (BIORAD, Richmond, Calif.) according to the method of Tang et al.[17]

Pharmacokinetic analysis

A one- or two-compartment model with extended least-squares regression (MKMODEL, Biosoft, Milltown, N.J.) was used to approximate the decline in plasma concentrations of isotope-labeled caffeine after intravenous injection. Area under the plasma concentration–time curve (AUC) was computed by the log-linear trapezoidal rule, steady-state volume of distribution (V_{ss}) was calculated with use of the area under the moment curve,[18] and clearance (CL) was calculated as Dose/AUC. Formation clearance (CL_f) of the major metabolite paraxanthine was approximated by use of the equation: Ae_m/AUC,[19] in which Ae_m represents the total amount of paraxanthine and its metabolites in urine collected for 24 hours (assuming steady state), and AUC is the AUC of caffeine. This equation is valid only if there is minimal first-pass effect, which is the case for oral caffeine.[20] The CL_f is an approximation because some of the metabolites of paraxanthine measured in the urine come from other pathways of caffeine metabolism and other minor metabolites such as 6-amino-5-[*N*-formylmethylamino]-3-methyluracil were not measured. However, because the fraction of caffeine metabolized to metabolites other than paraxanthine is so small,[21] the estimate of CL_f of paraxanthine should be a good one. It also assumes that there are no further unidentified pathways of paraxanthine metabolism.

Statistical analysis

Hypothesis testing used repeated measures of ANOVA, and the Tukey posttest was used for multiple comparisons when there were three or more conditions. When two conditions were compared, either a two-tailed paired *t* test or a Wilcoxon paired test was applied. Linear and stepwise multiple regression was used to examine the relationship between caffeine metabolite ratios and caffeine clearance and the coefficient of correlation (or r^2) was corrected for the degrees of freedom. Comparison of the intraindividual and interindividual variation for caffeine metabolite ratios and caffeine clearance was carried out with use of the theory of generalizability.[22]

Metabolite ratios of caffeine

More than 30 different caffeine metabolite ratios were examined for their ability to correlate with caffeine clearance. These ratios either originated from the literature or were derived empirically. The ratios that are described in the results section of this article are a subset of the original data analysis, and this subset includes ratios for which significant correlations of caffeine clearance were observed regularly, ratios that have been described in literature and thought to accurately reflect CYP1A2 activity, and other well-established ratios of caffeine metabolites that are used to describe the activity of enzymes other than CYP1A2. These other ratios include the acetylation ratio of caffeine AAMU/AAMU+1U+1X; the 1U/1X ratio, which is thought to describe xanthine oxidase activity; and the 17U/17X ratio, which has been postulated to reflect the activity of enzymes involved in hydroxylation of paraxanthine.[4,7] Hydroxylation of 17X is thought to be carried out in part by CYP1A6 and in part by CYP1A2.[23] The use of these non-CYP1A2 or "control" ratios has two purposes: (1) to examine possible correlations seen between non-CYP1A2 ratios and caffeine clearance and (2) to see whether a combination of ratios describing the activity of different enzymes involved in the metabolism of caffeine would correlate better to total caffeine clearance than a single metabolite ratio said to describe CYP1A2 activity.

RESULTS

Table II shows the mean and median values of a number of selected ratios measured during experiment 1 from subjects given low (4.2 mg/kg/day) and high (12 mg/kg/day) multiple doses of caffeine. Also displayed are their correlations to both caffeine clearance and the formation clearance of paraxanthine. The values of the ratios change only slightly

Table II. CMRs in nonsmokers during different multiple-dosing conditions and regression with caffeine clearance and formation clearance of paraxanthine: Experiment 1

No.	Ratio	Dose level	Mean*	Median*	CV %	Regression†							
						L versus CL		*H versus CL*		*L versus CL_f-17X*		*H versus CL_f-17X*	
						p	r^2	*p*	r^2	*p*	r^2	*p*	r^2
1	AAMU+1U+1X / 17X (paraxanthine demethylation ratio)	L	8.60	7.80	27								
		H	8.22	7.81	31	0.14	0.26	0.20	0.17	0.51	0.00	0.38	0.00
2	AAMU+1U+1X / 17U (paraxanthine demethylation ratio)	L	8.84	8.86	28								
		H	8.18	8.69	28	0.01‡	0.73	0.14	0.26	0.04	0.42	0.22	0.10
3	17X+17U / 137X (*N*-3-demethylation ratio)	L	8.19	7.51	37								
		H	5.50	5.76	32	0.37	0.00	0.88	0.00	0.02	0.50	0.81	0.00
4	17U / 17X (paraxanthine hydroxylation ratio)	L	1.00	1.08	24								
		H	1.05	1.02	34	0.59	0.00	0.70	0.00	0.21	0.11	0.72	0.00
5	AAMU / AAMU+1U+1X (acetylation ratio)	L	0.35	0.35	35								
		H	0.36	0.40	40	0.60	0.00	0.75	0.00	0.35	0.00	0.13	0.20
6	1U / 1X (xanthine oxidase ratio)	L	1.66	1.65	17								
		H	1.46	1.42	14	0.64	0.00	0.31	0.04	0.97	0.00	0.65	0.00
7	(DX+DU)+2(MX+MU+AAMU) / 3(DX+DU+MX+MU+AAMU+TX+TU) (total demethylation ratio)	L	0.58	0.59§	3								
		H	0.57	0.57	4	0.04	0.52	0.05‡	0.49	0.15	0.17	0.18	0.13
8	(17X+17U)+2(1X+1U+AAMU) / 3(17X+17U+1X+1U+AAMU) (modified total demethylation ratio)	L	0.60	0.60	2								
		H	0.60	0.60	3	0.02	0.61	0.06	0.44	0.11	0.23	0.22	0.10
9	17X+17U+1X+1U+7X+7U+AAMU / DX+DU+MX+MU+AAMU+TX+TU (*N*-3-demethylation ratio)	L	0.90	0.90§	2								
		H	0.87	0.87	3	0.62	0.00	0.06	0.44	0.56	0.00	0.03	0.45
10	13X+13U+1X+1U+3X+3U+AAMU / DX+DU+MX+MU+AAMU+TX+TU (*N*-7-demethylation ratio)	L	0.76	0.71§	6								
		H	0.67	0.67	5	0.56	0.00	0.12	0.29	0.91	0.00	0.03	0.45
11	37X+3X+7X+3U+7U+37U / DX+DU+MX+MU+AAMU+TX+TU (*N*-1-demethylation ratio)	L	0.13	0.11§	37								
		H	0.18	0.18	19	0.18	0.19	0.29	0.07	0.17	0.14	0.57	0.00

CMRs, Caffeine metabolite ratios; CV, coefficient of variation; L, low dose (4.2 mg/kg/day); CL, caffeine clearance per kilogram of body weight (L/hr/kg); H, high dose (12 mg/kg/day); CL_f-17X, formation clearance of paraxanthine (L/hr/kg); *p*, level of significance for regression ($p < 0.05$ is significant); r^2, coefficient of determination adjusted for degrees of freedom; AAMU, 5-acetylamino-6-amino-3-methyluracil; 1U, 1-methyluric acid; 1X, 1-methylxanthine; 17X, 1,7-dimethylxanthine (paraxanthine); 17U, 1,7-dimethyluric acid; 137X, 1,3,7-trimethylxanthine (caffeine); DX, all dimethylxanthines; DU, all dimethyluric acids, MX, all monomethylxanthines; MU, all monomethyluric acids; TX, caffeine; TU, 1,3,7-trimethyluric acid; 7X, 7-methylxanthine; 7U, 7-methyluric acid; 13X, 1,3-dimethylxanthine (theophylline); 13U, 1,3-dimethyluric acid; 3X, 3-methylxanthine; 3U, 3-methyluric acid; 37X, 3,7-dimethylxanthine (theobromine); 37U, 3.7-dimethyluric acid.

*For mean and median, $n = 9$.

†For regression, $n = 7$.

‡Best ratio identified in stepwise multiple regression to explain the variation in caffeine clearance.

§Significant difference between low and high dose with Wilcoxon paired test.

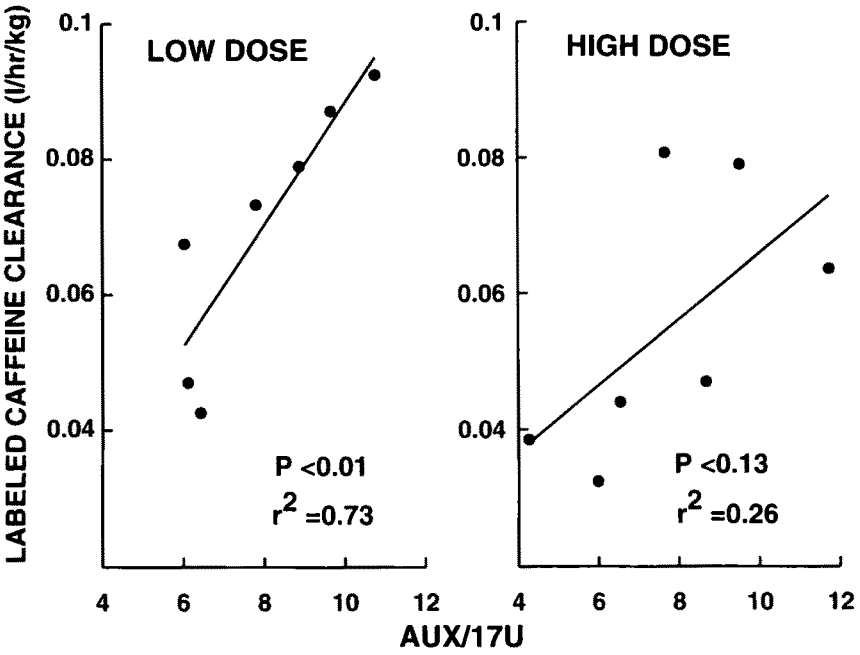

Fig. 2. AUX/17U versus caffeine clearance in experiment 1. AUX represents the sum of AAMU+1U+1X.

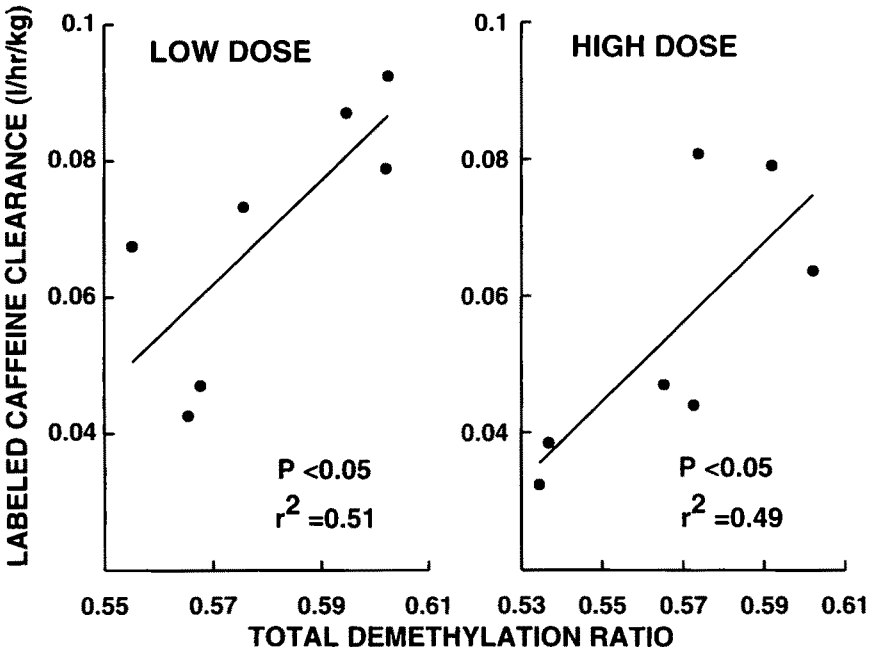

Fig. 3. Total demethylation ratio versus caffeine clearance in experiment 1.

with the change in dose. The AAMU+1U+1X/17X ratio did not show any significant relationship to caffeine clearance. AAMU+1U+1X/17U was highly correlated to caffeine clearance in the low-dose group but not in the high-dose group (Fig. 2). Total demethylation ratio and a modified version of this ratio (just using the major metabolites) did show a significant correlation for both dosing periods

Table III. Stable isotope caffeine pharmacokinetic parameters in 12 healthy smokers

Date	V_c *(L/kg)**	$t_{1/2}\alpha$ *(min)**	V_{ss} *(L/kg)*	*CL (all) (L/hr/kg)*	*CL (8 subjects) (L/hr/kg)*	$t_{1/2}$ *(hr)*
Day 1						
Mean	0.20	7.10	0.65	0.14	0.13	4.25
CV	33%	56%	23%	48%	38%	44%
Day 4						
Mean	0.24	6.78	0.67	0.15	0.16†	3.80
CV	56%	46%	28%	52%	51%	42%
Follow-up day‡						
Mean	0.25	5.80	0.61	0.11	0.11†	4.83
CV	37%	49%	24%	48%	48%	48%

V_c, Volume of central compartment; V_{ss}, volume of distribution at steady state; $t_{1/2}\alpha$, distribution phase half-life; $t_{1/2}$, elimination half-life; CL (all), clearance in all subjects; CL (eight subjects), clearance of same eight subjects who participated in both baseline and follow-up study.
*Two-compartment fitting was possible only in some subjects: six subjects on day 1; three subjects on day 4; and four subjects on the follow-up day.
†Identified by repeated measure ANOVA as statistically significant, $p < 0.05$.
‡Only eight of 12 subjects completed follow-up trial.

(Fig. 3). Significant differences were seen between low and high dose for the demethylation ratios but not for AAMU+1U+1X/17U. Stepwise multiple regression clearly identified just the single ratio with the highest r^2 value for each dosing period as the best ratio that accounts for most of the variation in caffeine clearance, and no combination of ratios was found to be superior than the single ratios identified. AAMU+1U+1X/17U for the low-dose group did correlate with the formation clearance of paraxanthine, but not as well as with caffeine clearance.

The pharmacokinetic parameters of stable isotope–labeled caffeine measured in experiment 2 are shown in Table III. The distribution phase could be characterized in six subjects on day 1 of the baseline study, and this was quite short. Clearance on day 4 of the baseline study was higher than observed on the other days and significantly different from the first day of the follow-up study.

Table IV shows the relationship between caffeine metabolite ratios and caffeine clearance in 12 smokers given multiple doses of caffeine on day 1 of the baseline study in experiment 2. These ratios were calculated from the full 24-hour collection of urine. AAMU+1U+1X/17U and the total demethylation ratio still show a significant relationship to caffeine clearance, and especially AAMU+1U+1X/17U, when these data are combined with the low-dose group from experiment 1 (Table V, Fig. 4). These relationships, shown in Tables II, IV, and V, were maintained even if clearance was not adjusted for weight or corrected for lean body mass.

Table VI reaffirms the good correlation of AAMU+1U+1X/17U after a single dose of caffeine with caffeine clearance and the formation clearance of paraxanthine. The demethylation ratios were poorly correlated with caffeine clearance but better with paraxanthine formation clearance. However, with the follow-up study, only AAMU+1U+1X/17U maintained a good correlation with caffeine clearance ($p = 0.04$, $r^2 = 0.47$). AAMU+1U+1X/17U maintained a good correlation with caffeine clearance even when urine collections were of short duration or with just spot urine samples (Table VII). Other ratio correlations to caffeine clearance with shorter collections of urine were highly variable.

Twelve subjects had metabolite ratios measured on two occasions; eight of these had ratios measured on three occasions. Table VIII provides the average data and the intersubject and intrasubject standard deviations for various ratios. For most ratios, the intraindividual variation was much smaller than the interindividual variation. For example, for AAMU+1U+1X/17U the interindividual coefficient of variation averaged 44%, whereas the intraindividual coefficient of variation averaged 25%.

DISCUSSION

The best measure of CYP1A2 activity in humans is not clear. Liver biopsy is an impractical method, and even then measurement of CYP1A2 function in vitro or the amount of messenger ribonucleic acid of CYP1A2 contained per gram of liver tissue may not accurately predict CYP1A2 functional capacity in vivo. It has been argued that the caffeine partial clearance by paraxanthine formation is the primary standard in vivo method,[4] but equally the paraxanthine partial clearance by 1X and AFMU formation may be just as good or superior. However, formation clearance of paraxanthine has not been previously

Table IV. CMRs in healthy smokers given multiple doses of caffeine and regression with caffeine clearance on day 1 of baseline study: Experiment 2

No.	Ratio	No. of subjects	Mean	CV (%)	Regression CL	
					p	r^2
1	$\frac{AAMU+1U+1X}{17X}$ (paraxanthine demethylation ratio)	12	8.63	40	0.10	0.17
2	$\frac{AAMU+1U+1X}{17U}$ (paraxanthine demethylation ratio)*	12	9.98	39	0.00	0.68
3	$\frac{17X+17U}{137X}$ (*N*-3-demethylation ratio)	12	8.84	34	0.31	0.01
4	$\frac{17U}{17X}$ (paraxanthine hydroxylation ratio)	12	0.93	47	0.39	0
5	$\frac{AAMU}{AAMU+1U+1X}$ (acetylation ratio)	12	0.36	36	0.95	0
6	$\frac{1U}{1X}$ (xanthine oxidase ratio)	12	1.86	38	0.36	0
7	$\frac{(DX+DU)+2(MX+MU+AAMU)}{3(MX+MU+DX+DU+TX+TU+AAMU)}$ (total demethylation ratio)	12	0.58	4	0.02	0.40
8	$\frac{(17X+17U)+2(1X+1U+AAMU)}{3(17X+17U+1X+1U+AAMU)}$ (modified total demethylation ratio)	12	0.60	3	0.01	0.43
9	$\frac{17X+17U+1X+1U+7X+7U+AAMU}{MX+MU+DX+DU+TX+TU+AAMU}$ (*N*-3-demethylation ratio)	12	0.93	3	0.04	0.31
10	$\frac{13X+13U+1X+1U+3X+3U+AAMU}{MX+MU+DX+DU+TX+TU+AAMU}$ (*N*-7-demethylation ratio)	12	0.73	9	0.04	0.31
11	$\frac{37X+3X+7X+3U+7U+37U}{DX+DU+MX+MU+AAMU+TX+TU}$ (*N*-1-demethylation ratio)	12	0.09	48	0.71	0

Note that ratios were calculated from 24-hour collection of urine.
CL, Caffeine clearance (L/hr/kg).
*Identified by stepwise regression as the best ratio to explain the variation in caffeine clearance.

used and, because most of caffeine is demethylated by CYP1A2,[23] the clearance of caffeine has remained the "gold standard" to validate simple methods of estimating CYP1A2 activity such as caffeine metabolite ratios. Although the caffeine breath test has been validated,[24,25] it is still an impractical and cumbersome investigation for large numbers of subjects.

Several different caffeine metabolite ratios have been proposed and used to investigate CYP1A2 activity, but with little published validation. Also, the clearance of caffeine is dose dependent and the mechanism of dose dependence is likely attributable to end-product inhibition[5] because CYP1A2 metabolizes all the dimethylxanthines, as well as caffeine.[23] The performance of these caffeine metabolite ratios with different doses of caffeine and under multiple-dose conditions in which there are high levels of

Table V. CMRs and correlation to caffeine clearance in the combined group of smokers and nonsmokers: Experiments 1 and 2 combined

No.	*Ratio*	*Mean*	*CV (%)*	*Regression CL*	
				p	r^2
1	$\frac{AAMU+1U+1X}{17X}$ (paraxanthine demethylation ratio)	8.58	36	0.07	0.13
2	$\frac{AAMU+1U+1X}{17U}$ (paraxanthine demethylation ratio)*	9.23	36	<0.01	0.69
3	$\frac{17X+17U}{137X}$ (*N*-3-demethylation ratio)	8.32	32	0.09	0.11
4	$\frac{(DX+DU)+2(MX+MU+AAMU)}{3(MX+MU+DX+DU+TX+TU+AAMU)}$ (total demethylation ratio)	0.58	4	0.01	0.29
5	$\frac{(17X+17U)+2(1X+1U+AAMU)}{3(17X+17U+1X+1U+AAMU)}$ (modified total demethylation ratio)	0.60	3	0.01	0.34
6	$\frac{17X+17U+1X+1U+7X+7U+AAMU}{MX+MU+DX+DU+TX+TU+AAMU}$ (*N*-3-demethylation ratio)	0.92	3	<0.01	0.46

Note that ratios were calculated from 24-hour collection of urine.
CL, Caffeine clearance (L/hr/kg).
*Identified by stepwise regression as the best ratio to explain the variation in caffeine clearance.

circulating dimethylxanthines requires appraisal. The use of stable isotope caffeine in our studies allowed the measurement of caffeine clearance during various conditions of oral caffeine dosing.

With nonsmokers, the first experiment looked at caffeine metabolite ratios during two different steady-state conditions of caffeine dosing and correlated these ratios to both caffeine clearance and paraxanthine formation clearance. A group of nonsmokers was chosen to optimize the conditions to demonstrate dose dependency. Their low CYP1A2 activity should mean that this enzyme would be more susceptible to end-product inhibition. This would give a better chance of seeing whether dose dependency influenced the relationship between metabolite ratios of caffeine and caffeine clearance. Furthermore, in vitro studies have shown that the AAMU+1U+1X/17U ratio may lose sensitivity in its ability to reflect caffeine clearance and CYP1A2 activity when CYP1A2 is highly induced.[3] This is because CYP1A2 is partially involved in the hydroxylation and demethylation of paraxanthine and thus catalyzes the production of metabolites on both sides of the ratio. Campbell et al.[3] showed that the more CYP1A2 is induced, the more it contributes to the production of 17U. They showed a hyperbolic relationship between CYP1A2 activity and this ratio. This means that at the higher end of the scale of CYP1A2 activity, large changes in CYP1A2 function would not be reflected in the ratio by the same magnitude.

The second experiment was designed to see how robust the caffeine metabolite ratios were in their correlation to the clearance of caffeine by evaluation of their performance in subjects who continued their normal habit of caffeine consumption. Furthermore, smokers were purposely included because they would have higher activity levels of CYP1A2.

Many of the ratios (data not shown) that use caffeine itself or dimethylxanthines did not reliably correlate with caffeine clearance, and the use of such ratios must be questioned. As expected, no relationship was observed between the acetylation, hydroxylation, and xanthine oxidase ratios and the clearance of caffeine. The use of the 17X+17U/137X ratio requires a single dose of caffeine and a collection between 4 and 5 hours and therefore would not be expected to be useful in this setting. Although some

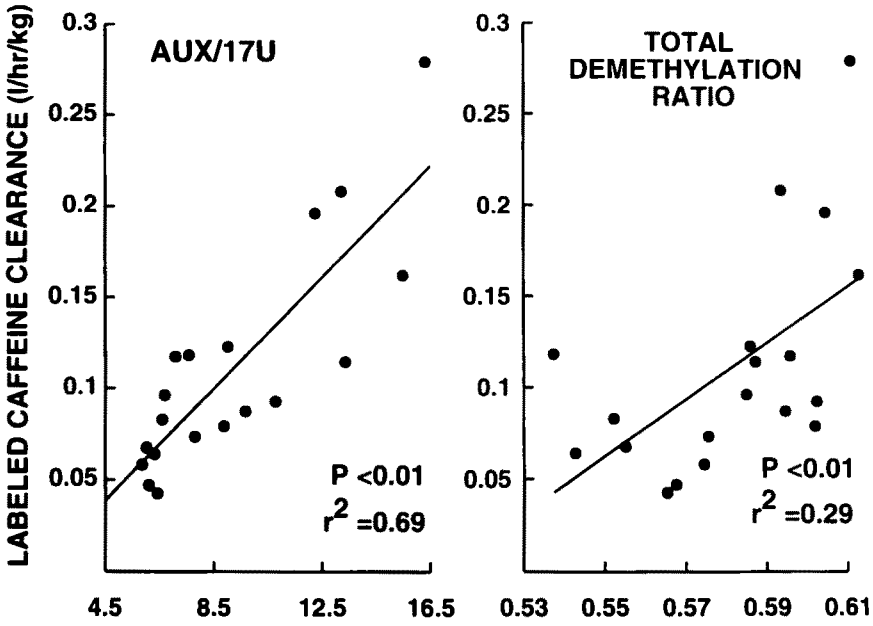

Fig. 4. Caffeine clearance versus AUX/17U and total demethylation ratio in smoking and nonsmoking subjects (experiments 1 and 2 combined). AUX represents the sum of AAMU+1U+1X.

Table VI. Regression of caffeine clearance and paraxanthine formation clearance with CMRs in smokers given a single dose of caffeine: Experiment 2

No.	*Ratio*	*n*	*Mean*	*CV (%)*	*Regression CL*		*Regression CL_f-17X*	
					p	r^2	*p*	r^2
1	$\frac{AAMU+1U+1X}{17X}$ (paraxanthine demethylation ratio)	12	9.12	59	0.92	0.00	0.55	0.00
2	$\frac{AAMU+1U+1X}{17U}$ (paraxanthine demethylation ratio)	12	10.56	49	0.01	0.44	0.002	0.61
3	$\frac{17X+17U}{137X}$ (*N*-3-demethylation ratio)	9	8.76	30	0.31	0.03	0.03	0.44
4	$\frac{(DX+DU)+2(MX+MU+AAMU)}{3(MX+MU+DX+DU+TX+TU+AAMU)}$ (total demethylation ratio)	12	0.58	4	0.25	0.05	0.03	0.35
5	$\frac{(17X+17U)+2(1X+1U+AAMU)}{3(17X+17U+1X+1U+AAMU)}$ (modified total demethylation ratio)	12	0.60	4	0.24	0.05	0.07	0.23
6	$\frac{17X+17U+1X+1U+7X+7U+AAMU}{MX+MU+DX+DU+TX+TU+AAMU}$ (*N*-3-demethylation ratio)	12	0.95	4	0.98	0.00	0.52	0.00

CL, Caffeine clearance (L/hr/kg); CL_f-17X, Paraxanthine formation clearance (L/hr/kg).

Table VII. Regression of caffeine clearance with AAMU+1U+1X/17U calculated from short collections of urine on 3 different days: Experiment 2

	Day 1			*Day 4*			*Follow-up day*		
Urine collection	*Mean*	*p*	r^2	*Mean*	*p*	r^2	*Mean*	*p*	r^2
0-24 hr	9.98	0.00	0.68	10.56	0.01	0.44	8.64	0.04	0.47
0-6 hr	11.03	0.04	0.34	8.16	0.00	0.75	9.03	0.06	0.40
6-12 hr	9.50	0.00	0.72	9.34	0.00	0.57	7.78	0.01	0.72
12-24 hr	11.85	0.00	0.87	11.90	0.00	0.67	9.54	0.02	0.54
Day 1 morning sample	13.21	0.00	0.90	—	—	—	12.63	0.03	0.92*
Day 1 afternoon sample	9.49	0.00	0.64	10.95	0.01	0.52	7.48	0.03	0.60
Day 2 morning sample	12.27	0.01	0.58	—	—	—	10.76	0.14	0.25

*Only four subjects available.

groups have used AAMU+1U+1X/17X[7,12] as a measure of CYP1A2 activity, we found that this ratio does not correlate very well to caffeine clearance and recommend against its use.

Previously, the AAMU+1U+1X/17U ratio has been shown to correlate to caffeine clearance after single doses of caffeine.[15] Our data shows the usefulness of this ratio in subjects consuming multiple doses and in both nonsmokers and smokers, at least at typical levels of caffeine consumption. With high doses of caffeine, the mean and median values of this ratio slightly decreased but did not reach statistical significance. The mean ratio decreased by 7.5%, but caffeine clearance decreased by 22% between the low- and high-dose groups.[5] As a result, the relationship between AAMU+1U+1X/17U and caffeine clearance was not good with the high-dose group (Fig. 2). Although this may be a function of the small numbers of subjects studied, it means at this stage caution must be exercised with any use of this ratio to assess CYP1A2 activity in subjects who consume large quantities of caffeine. It may also mean that the use of serial measures in the same individual to chart changes in CYP1A2 function may require the consumption of roughly the same quantities of caffeine, although the actual magnitude of change in AAMU+1U+1X/17U values from low to high dose was not great. The coefficient of variation for this ratio was 28% in nonsmokers and 39% in smokers, consistent with the variation observed in caffeine clearance (Table III) but less than what is observed in the in vitro studies that directly measure CYP1A2 activity.[4] Although AAMU+1U+1X/17U did correlate with formation clearance of paraxanthine, the correlation was not as great as with caffeine clearance in the first experiment. However, the correlation of AAMU+1U+1X/17U was greater with formation clearance of paraxanthine than with caffeine clearance in the second study (Table VI). The formation clearance of paraxanthine is thought to be the best measure of CYP1A2 activity.[4] Variability in correlation between AAMU+1U+1X/17U and formation clearance of paraxanthine suggests that either the assumptions used to calculate this formation clearance were not entirely correct, that the ratio is only an approximate measure of activity, or that the sample size in the first experiment was too small.

In spot urine samples or urine collections of short duration, the correlation between AAMU+1U+1X/17U and caffeine clearance was also maintained (Table VII). Because the 12- to 24-hour collection would approximate a first morning urine, we purposely chose other times (midmorning and afternoon) for spot urines to be collected so as to mimic real world situations. Although there was some variation, the conclusions from Table VII are that spot samples taken either as a first morning sample or taken in the late afternoon after caffeine consumption would give a reproducible estimate of CYP1A2 activity. Also, Table VIII shows that repeated measures of AAMU+1U+1X/17U in the same individual can be performed with some confidence.

The demethylation ratios—especially total and modified total ratios—seemed initially to be quite robust in reflecting caffeine clearance for both the low- and high-dose groups of experiment 1 (Fig. 3). However, in the second experiment, the correlation to caffeine clearance was highly variable and probably reflects the influence of urine flow on caffeine and the dimethylxanthine excre-

Table VIII. Interindividual and intraindividual differences in urine metabolite ratios and caffeine clearance

No.		*Two measurements (n = 12)*			*Three measurements (n = 8)*		
		Average	*Intersubject SD*	*Intrasubject SD*	*Average*	*Intersubject SD*	*Intrasubject SD*
	CL (L/hr/kg)	0.14	0.07	0.03	0.14	0.06	0.03
1	$\frac{AAMU+1U+1X}{17X}$ (paraxanthine demethylation ratio)	8.88	4.54	2.52	9.43	4.75	2.29
2	$\frac{AAMU+1U+1X}{17U}$ (paraxanthine demethylation ratio)	10.27	4.57	2.54	10.16	4.46	2.54
3	$\frac{17X+17U}{137X}$ (*N*-3-demethylation ratio)	8.35	2.16	1.22	8.73	2.30	1.78
4	$\frac{17U}{17X}$ (paraxanthine hydroxylation ratio)	0.93	0.46	0.16	1.03	0.57	0.20
5	$\frac{AAMU}{AAMU+1U+1X}$ (acetylation ratio)	0.36	0.13	0.03	0.35	0.13	0.03
6	$\frac{1U}{1X}$ (xanthine oxidase ratio)	1.89	0.71	0.62	2.07	0.70	0.58
7	$\frac{(DX+DU)+2(MX+MU+AAMU)}{3(DX+DU+MX+MU+AAMU+TX+TU)}$ (total demethylation ratio)	0.58	0.03	0.01	0.58	0.03	0.02
8	$\frac{(17X+17U)+2(1X+1U+AAMU)}{3(17X+17U+1X+1U+AAMU)}$ (modified total demethylation ratio)	0.60	0.02	0.01	0.60	0.02	0.01
9	$\frac{17X+17U+1X+1U+7X+7U+AAMU}{DX+DU+MX+MU+AAMU+TX+TU}$ (*N*-3-demethylation ratio)	0.94	0.03	0.03	0.94	0.04	0.04

tion.[10,11] Also, their coefficients of variation were remarkably small and therefore unlikely to reflect the true variation in CYP1A2 activity. How well these ratios of total demethylation correlate with *N*-3-demethylation rates remains open to question. The significant differences observed in experiment 1 between the low- and high-dose values for these ratios reflect the low coefficient of variation of these ratios. No statistical difference could be shown between low and high caffeine dose groups for the other ratios.[5] The possibility that a number of ratios together would be superior to a single ratio in the regression with caffeine clearance was examined and eliminated by the use of stepwise multiple regression.

Finally, the caffeine clearance was higher on day 4 of experiment 2 than the other days (Table III). This is further evidence of the dose dependency of caffeine metabolism. On day 4, the subjects had no methylxanthines for the 2 previous days (and therefore little circulating dimethylxanthines) and were given a single dose of caffeine that, in general, was less than the daily dose they consumed on day 1 and in the follow-up study. Therefore one would expect the clearance of caffeine to be higher on this day. AAMU+1U+1X/17U values followed this trend (Table VII).

In conclusion, the AAMU+1U+1X/17U ratio is most consistently correlated to caffeine clearance and paraxanthine formation clearance. AAMU+1U+1X/17U can be measured in subjects while they consume single or multiple doses of caffeine, in nonsmokers and smokers, and with

use of only spot urine samples. High doses of caffeine (12 mg/kg/day) may invalidate the relationship. The relationship of other caffeine metabolite ratios to caffeine clearance was variable; these ratios are less useful than AAMU+1U+1X/17U for estimation of CYP1A2 activity.

The conclusions of this study are based on studies in healthy subjects. Whether these conclusions hold for individuals with hepatic or renal disease remains to be determined.

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