

Five Caffeine Metabolite Ratios to Measure Tobacco-induced CYP1A2 Activity and Their Relationships with Urinary Mutagenicity and Urine Flow¹

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Abstract

To choose a sensitive protocol to discriminate populations exposed and not exposed to inducers, five urinary metabolite ratios (MRs) [MR1 (17X + 17U)/137X, MR2 (5-acetylamin-6-formylamino-3-methyluracil [AFMU] + 1X + 1U)/17U, MR3 (17X/137X), MR4 (AFMU + 1X + 1U + 17X + 17U)/137X, and MR5 (AFMU + 1X + 1U)/17X] were calculated in 4–5 h and 0–24 h urine samples after caffeine intake. One hundred twenty-five healthy volunteers (59 nonsmokers and 66 smokers) were included in the study. All ratios showed a log-normal distribution. MR2 in the two time intervals was the only ratio nondependent on the urine flow. Differences between nonsmokers and smokers could be detected with all ratios at 4–5 h. However, only MR2 and, to a lesser extent, MR5 allowed the discrimination of higher cytochrome P450 1A2 (CYP1A2) activity in smokers in the 0–24 h sample. Although smokers had increased urinary mutagenicity in relation to nonsmokers, a significant association between MRs and urine mutagenicity was observed only with MR2 in the 4–5 h interval; this ratio/time schedule being that of higher association with tobacco consumption. The most flow-dependent ratios, MR1, MR3, and MR4, were closely correlated with each other at the two intervals. The flow dependency profile of each ratio may explain their different power to indicate both tobacco exposure and tobacco-derived mutagenicity. In conclusion, MR2 in the period of 4–5 h after caffeine intake seems preferable, especially at high urine flow rates.

Introduction

The human cytochrome P450 isoform CYP1A2³ is involved in the activation of environmental procarcinogens, such as arylamines, heterocyclic amines, and aflatoxin B1 (1–3). CYP1A2 activity may be considerably increased by xenobiotics, including cigarette smoke (4), and polycyclic aromatic or halogenated hydrocarbons from other sources (5). Accordingly, as the expression levels of the enzyme increases, so does the metabolism of certain procarcinogens to DNA-reactive products, leading to a higher risk of cancer development. On the other hand, inhibition of CYP1A2 by quinolone antibiotics (6), serotonin reuptake inhibitors (7), verapamil (8), oral contraceptives (4), and propafenone (9) may produce adverse reactions when these agents are administered concomitantly.

Methods for measuring CYP1A2 activity *in vivo* may be important tools to assess the relationship between exposure to chemicals and cancer incidence (10) or to demonstrate changes in enzyme activity produced by exposure to inducers or inhibitors. Standard methods include the caffeine breath test (11, 12) and the caffeine plasma clearance (13, 14). Another approach increasingly used is based on the determination of metabolites in urine after caffeine intake, as a noninvasive method.

The main metabolic routes of caffeine are illustrated in Fig. 1. In the complex metabolism of caffeine (137X), CYP1A2 is solely responsible for the caffeine 3-demethylation to paraxanthine (17X) and overwhelmingly for the 7-demethylation of 17X (15). Thus, 17X is both a product and a substrate of CYP1A2. 17X later undergoes two reactions, one of which is 8-hydroxylation by other cytochromes, including CYP2A6 and also CYP1A2 (16). Another reaction, 7-demethylation via CYP1A2, leads to an unstable intermediate (Z). This ring-opened compound gives two competing products: 1X and the acetylated product (AFMU). 1X suffers 8-hydroxylation to 1-methyluric acid (1U), mediated by xanthine oxidase with a little contribution of CYP1A2 (16, 17). The polymorphic NAT2 mediates the step toward AFMU (17). CYP1A2 catalyzes the *N*-demethylation of 137X at two other sites (N1 and N7) with the participation of CYP2E1 to produce theobromine and theophylline, respectively (17, 18).

Several urinary MRs have been proposed to assess CYP1A2 activity (4, 19–22). However, both the optimal MR and the adequate urine collection interval remain to be clarified.

Received 6/23/98; revised 11/3/98; accepted 11/9/98.

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¹ This study was supported by grants from the Fondo de Investigación Sanitaria (PB 94/1549) and the Dirección General de Investigación Científica y Técnica (PB 94/0573).

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³ The abbreviations used are: CYP1A2, cytochrome P450 1A2; 137X, 1,3,7-trimethylxanthine (caffeine); 137U, 1,3,7-trimethylurate; 17X, 1,7-dimethylxanthine (paraxanthine); 13X, 1,3-dimethylxanthine (teophylline); 37X, 3,7-dimethylxanthine (theobromine); 17U, 1,7-dimethylurate; 13U, 1,3-dimethylurate; 37U, 3,7-dimethylurate; 1X, 1-methylxanthine; 3X, 3-methylxanthine; 7X, 7-methylxanthine; 1U, 1-methyluric acid; AFMU, 5-acetylamin-6-formylamino-3-methyluracil; AAMU, 5-acetylamin-6-amino-3-methyluracil; GST, glutathione S-transferase; NAT2, *N*-acetyltransferase; PAH, polycyclic aromatic hydrocarbon; MR, metabolite ratio.

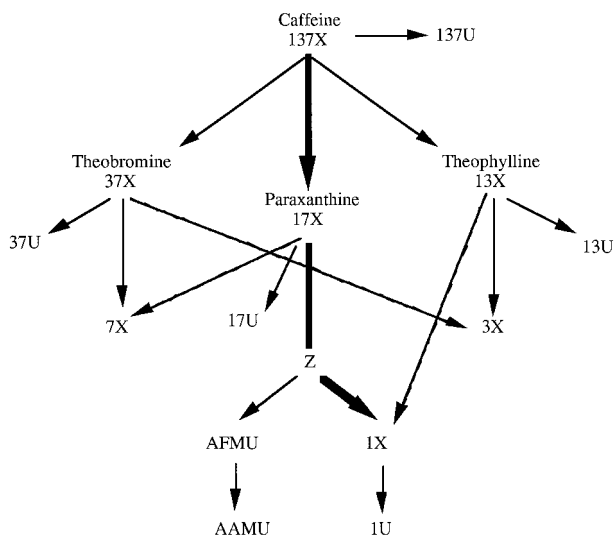


Fig. 1. Pathways of caffeine metabolism.

In this regard, the debate is focused on several points: (a) caffeine metabolism is subject to different competing pathways; (b) paraxanthine, which is used in most of the ratios, is both product and substrate of CYP1A2; and (c) the renal excretion of some metabolites may be differently affected due to their distinct polarity. At the center of this controversy is whether or not caffeine concentrations in urine are urine-flow dependent. In particular, Butler *et al.* (20) and Tang *et al.* (23) report opposite results. The bimodal or trimodal frequency distributions of CYP1A2 activity obtained with Butler's ratio, which has been used in studies of cancer epidemiology (24, 25), contrast with the unimodal distributions found with the other ratios. On the other hand, in a recent report (26), it has been suggested that urinary MRs are probably inaccurate to assess the distribution of CYP1A2 within populations.

Cigarette smokers excrete mutagenic compounds in their urine at significant higher levels than nonsmokers (27–30). Many compounds in tobacco smoke are bioactivated to electrophiles by oxidation, the most representative being benzopyrene (31), which is metabolized by aryl hydrocarbon hydroxylase (AHH). This enzyme belongs to the CYP1A1 subfamily of the cytochrome P450, which is known to be induced by polycyclic aromatic hydrocarbons contained in tobacco smoke (32–35).

Bearing in mind the points mentioned above, as well as the known inducibility of CYP1A2 by PAHs contained in tobacco smoke, we compared five MRs obtained at two different time intervals in urine from smokers and nonsmokers, with the aim to select the most sensitive MR/timing schedule to discriminate populations nonexposed and exposed to inducers of CYP1A2 activity. The association between each individual ratio and urinary mutagenicity, as well as the relative influence of the urinary flow, is also investigated in this work.

Patients and Methods

Chemicals. Caffeine (137X), paraxanthine (17X), 17U, 1X, 1U, and *N*-acetyl-*p*-aminophenol (internal standard), were purchased from Sigma Chemical Co. (St. Louis, MO). AFMU was obtained from Drs. W. Kalow and B. K. Tang, University of Toronto (Toronto, Ontario, Canada). Ammonium sulfate

Table 1 Caffeine urinary MRs used to explore CYP1A2 activity		
Abbreviation	Quotient	Reference
MR1	(17X + 17U)/137X	Butler <i>et al.</i> (20)
MR2	(AFMU + 1X + 1U)/17U	Campbell <i>et al.</i> (4)
MR3	17X/137X	Kadlubar <i>et al.</i> (19)
MR4	(AFMU + 1U + 1X + 17U + 17X)/137X	Carrillo and Benitez (22)
MR5	(AFMU + 1U + 1X)/17X	Grant <i>et al.</i> (21)

(HPLC grade) was from Merck (Barcelona, Spain). Chloroform, isopropanol, acetic acid, and methanol (HPLC grade) were from Panreac (Barcelona, Spain). Water was filtered by a Milli-Q water system from Millipore Ibérica S.A. (Madrid, Spain).

Subjects. One hundred twenty-five unrelated healthy volunteers (63 males and 62 females) were included in the study. These individuals were undergraduate medical students from the University of Zaragoza (Zaragoza, Spain) or members of the professional or technical staff. All individuals filled in a detailed questionnaire on smoking habits, diet, coffee, and drug consumption. Diets or cooking procedures known to induce CYP1A2 activity or to increase the urinary mutagenicity, were avoided (*e.g.*, cruciferous vegetables; watercress; meat char-broiled, grilled, or cooked at a high temperature; smoked foods; and toasted bread). During the entire study, all participants abstained from consuming these kinds of food. Passive smokers were excluded, and only smokers of blond tobacco were recruited. Smoking exposure was similar along the study (4 days). Subjects with idiosyncratic reactions to caffeine or histories of chronic diseases or recent illnesses were excluded from the study, as well as those who were taking any medication, including oral contraceptives. Regular alcohol drinkers were also excluded. The average age was 26.16 ± 9.04 (mean ± SD) years, ranging from 18 to 57. Fifty-nine individuals were non-smokers, and 66 were smokers of 17.72 ± 8.69 (mean ± SD) cigarettes/day. To avoid the influence of the dietary factors on urinary mutagenicity, we chose all participants from the same hospital to minimize the differences in diet-derived mutagen exposure. Thus, all of them followed the same diet, a controlled Mediterranean diet, throughout the urine collection period. Coffee or caffeine containing beverages were allowed on the day of urine collection. In no case was the daily caffeine consumption greater than the equivalent of 4 cups of coffee. For the mutagenicity study, 115 subjects of the total 125 participants were included (61 smokers and 54 nonsmokers). Written informed consent was obtained from each subject. The study was approved by the Ethic Committee for Clinical Research of the Hospital Clínico of the University of Zaragoza.

Study Protocol. Subjects refrained from consuming alcoholic drinks and foods or beverages containing methylxanthines for 48 h before and during the day of urine collection for MR analysis. After emptying their bladder, subjects ingested an instant coffee beverage containing 200 mg of caffeine in 250 ml of water. Consumption of liquids was not restricted during the study. All urine formed in the 0–4, 4–5, and 5–24 h intervals was collected in bottles preloaded with 1 mol/liter citric acid phosphate buffer, pH 3. Urine volumes were recorded. After separating a 2-ml aliquot from the 4–5 h urine sample, the urine of the three intervals was added together to be analyzed, as a 0–24 h urine sample. Total urine volume was recorded, and a 2-ml aliquot was taken. The two aliquots (0–24 and 4–5 h) were immediately frozen and stored at –80°C until analysis.

Table 2 Characteristics of CYP1A2 MRs in the sampling period of 0–24 h

MR	Mean \pm SD	Range	Coefficient of variation (%)
MR1			
Total	9.06 \pm 5.27	0.64–30.34	58.23
Nonsmokers	9.06 \pm 4.84	1.50–30.34	53.48
Smokers	9.05 \pm 5.67	0.64–25.99	62.59
MR2			
Total	7.06 \pm 3.05	0.99–18.08	43.20
Nonsmokers	5.94 \pm 2.06	2.58–12.47	34.73
Smokers	8.07 \pm 3.43	0.99–18.08	42.57
MR3			
Total	3.73 \pm 2.01	0.27–13.61	54.03
Nonsmokers	3.72 \pm 1.84	0.89–10.76	49.59
Smokers	3.73 \pm 2.17	0.27–13.61	58.07
MR4			
Total	45.18 \pm 31.90	2.50–165.83	70.60
Nonsmokers	40.22 \pm 24.17	3.06–125.62	60.09
Smokers	49.61 \pm 37.11	2.50–165.83	74.81
MR5			
Total	9.69 \pm 5.06	0.71–26.66	52.24
Nonsmokers	8.46 \pm 4.17	0.71–20.13	49.32
Smokers	10.79 \pm 5.54	3.13–26.66	51.39

Table 3 Characteristics of CYP1A2 MRs in the sampling period of 4–5 h

MR	Mean \pm SD	Range	Coefficient of variation (%)
MR1			
Total	8.38 \pm 7.63	0.86–58.68	91.04
Nonsmokers	6.00 \pm 3.58	0.86–19.78	59.55
Smokers	10.51 \pm 9.49	1.66–58.68	90.24
MR2			
Total	5.82 \pm 2.67	2.54–18.24	45.77
Nonsmokers	4.70 \pm 1.64	2.58–9.88	34.91
Smokers	6.83 \pm 2.99	2.54–18.24	43.83
MR3			
Total	3.59 \pm 2.91	0.50–21.24	81.18
Nonsmokers	2.57 \pm 1.25	0.50–6.05	48.68
Smokers	4.50 \pm 3.61	0.90–21.24	80.26
MR4			
Total	40.16 \pm 55.87	2.10–423.11	139.09
Nonsmokers	22.54 \pm 19.04	2.10–121.59	84.49
Smokers	55.92 \pm 71.39	3.60–423.11	127.65
MR5			
Total	7.36 \pm 4.93	0.80–30.83	66.92
Nonsmokers	6.15 \pm 3.81	0.80–17.57	61.96
Smokers	8.44 \pm 5.55	2.00–30.83	65.74

Analysis of Caffeine and Metabolites. Caffeine and its metabolites were quantified according to the procedure described by Grant *et al.* (21) with some modifications. Modifications included the use of chloroform/isopropanol 95:5 (vol/vol) as extracting solvent. Caffeine and its metabolites were separated by a Waters Novapak C18 reverse-phase column (4 μ m particle size, 25 cm \times 4.6 mm internal diameter) (Millipore Ibérica S.A.), which was eluted isocratically with a mobile phase containing acetic acid/methanol/water (0.5:90:905.5 vol/vol/vol) at a flow rate of 1 ml/min and a pressure of 1500 psi.

The compounds were detected by UV absorbance at 280 nm. The chromatograph was an LC Module I Plus equipped with a Millennium 2010 software (Waters Corp., Madrid, Spain). Calibration curves were performed with known amounts of metabolites in a range from 5 to 80 μ g/ml, added to blank urine samples, and then processed as described above. 1U was dissolved under basic conditions (pH 9) by the addition of 10 N sodium hydroxide and then neutralized to pH 7 with 12 N hydrochloric acid, according to Tang *et al.* (36). For data analysis, caffeine and its metabolites excreted in the two urine sampling periods, 0–24 and 4–5 h, were expressed as mmol/liter. Table 1 shows the five MRs used to assess CYP1A2 activity.

Several authors proceed to deformylate AFMU to AAMU by adjusting the urine to pH 10 prior to analysis. This is due to both the observation that AFMU deformylates to AAMU in alkaline conditions *in vitro* (17) and the suggestion that AFMU may spontaneously deformylate in the bladder when the urine is basic or neutral (36). However, we preferred to measure AFMU in acidified urine (pH 3.5) because of the recovery of AFMU when deformylated to AAMU in previous experiments was always below 30% (37). In addition, as reported by Butler *et al.* (20), AFMU did not decompose either during storage at pH 8 or after the consumption of sodium bicarbonate.

To assess whether or not any urinary constituent could co-migrate with caffeine or caffeine metabolites used in the ratios, we reanalyzed 37 urine samples using a Waters 996 photodiode array detector, and no peak impurities were observed when compared with the spectral libraries of standards.

However, we did not examine different columns or column conditions to unequivocally exclude this possibility.

Urine Mutagenicity. For the mutagenicity assay, a 24 h urine sample was collected from each individual 4 days prior to the caffeine study and frozen at -80°C until analysis. The method of Yamasaki and Ames (38) was followed. An aliquot of 75 ml of each sample was filtered and then passed through a glass column with XAD-2 resin. The concentrate was eluted with 20 ml of acetone and dried on rotavapor (R110) at 45°C , and the residue was rediluted in 400 μ l of DMSO.

The microsuspension procedure of Malaveille *et al.* (39) and Kado *et al.* (40) was used with some modifications. Each assay consisted of incubation for 90 min at 37°C in a bath, with shaking, of 100 μ l of concentrate bacterial culture medium ($3\text{--}4 \times 10^9$ cells) of *Salmonella typhimurium* TA 98, 20 μ l of urinary extract (equivalent to the mutagens contained in 3.75 ml of urine), and β -glucuronidase (1118 units/ml; Sigma). Then, after addition of 2 ml of histidine-poor soft agar, the mixture was plated onto minimal glucose agar as described by Maron and Ames (41). The revertants colonies were counted after 3 days of incubation at 37°C , and mutagenicity was expressed as number of revertants colonies, after subtracting the spontaneous revertants in the urine of 24 h.

Statistical Analysis. The characteristics of the distributions of the variables were investigated by measuring Skewness, Kurtosis, and Wilk-Shapiro statistic of normality by using an SPSS statistical program. Variables (x) were transformed to $\log x$ or the square root of $\log x$ to obtain a better symmetry. Differences of means were analyzed by the Student *t* test. Correlations between transformed variables were calculated by linear regression analysis. Spearman rank correlation test was used to determine the association between nonparametric data.

Results

Because of the frequency distribution of the five metabolite indexes in the two time intervals were significantly skewed, logarithms of the molar ratios (MR1, MR2, MR4, and MR5) were taken. Because the logarithm of MR3 resulted in a dis-

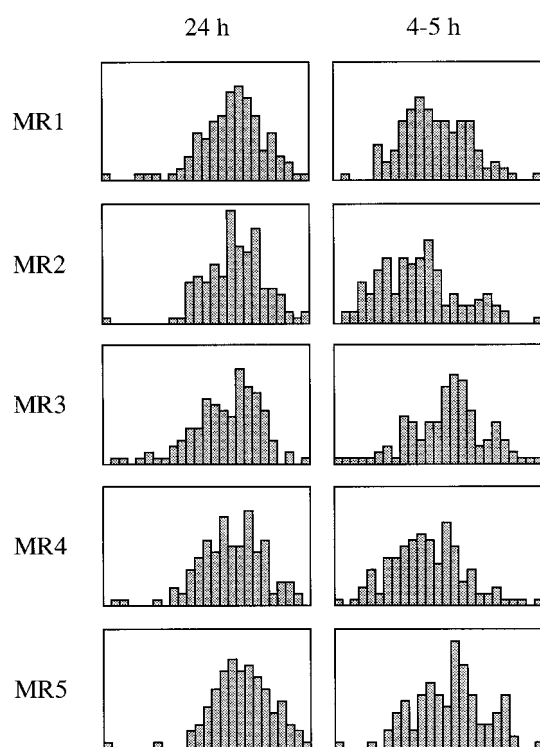


Fig. 2. Frequency distributions of the transformed metabolic ratios.

Table 4 Means \pm SDs of the transformed MRs: comparison between nonsmokers and smokers

	0–24 h interval	4–5 h interval
Log MR1		
Nonsmokers	0.90 \pm 0.22	0.70 \pm 0.26
Smokers	0.86 \pm 0.30 ^a	0.88 \pm 0.34 ^b
Log MR2		
Nonsmokers	0.74 \pm 0.15	0.64 \pm 0.13
Smokers	0.86 \pm 0.20 ^c	0.79 \pm 0.17 ^d
$\sqrt{\text{Log MR3}}$		
Nonsmokers	1.87 \pm 0.45	1.55 \pm 0.39
Smokers	1.85 \pm 0.54 ^a	1.99 \pm 0.71 ^c
Log MR4		
Nonsmokers	1.52 \pm 0.29	1.22 \pm 0.33
Smokers	1.57 \pm 0.34 ^a	1.51 \pm 0.44 ^c
Log MR5		
Nonsmokers	0.86 \pm 0.24	0.71 \pm 0.27
Smokers	0.97 \pm 0.21 ^b	0.84 \pm 0.28 ^b

^a NS, not significant.

^b $P < 0.01$.

^c $P < 0.001$.

^d $P < 0.0001$.

tribution significantly skewed, it was necessary further transformation of this variable to the square root to obtain a well modeled normal distribution. Tables 2 and 3 summarize the mean values, ranges, and coefficients of variation for each of the five ratios obtained at the two urine collection times 0–24 and 4–5 h. Fig. 2 shows the frequency distributions of the transformed ratios.

Comparisons of means from the transformed variables between nonsmokers and smokers are reflected in Table 4. In

Table 5 Correlation coefficients between daily cigarette consumption and MRs at 0–24 h and 4–5 h

	<i>r</i>	
	0–24 h interval	4–5 h interval
Log MR1	0.03	0.13
Log MR2	0.26 ^a	0.39 ^b
$\sqrt{\text{Log MR3}}$	0.03	0.14
Log MR4	0.15	0.18
Log MR5	0.28 ^a	0.33 ^c

^a $P < 0.05$.

^b $P < 0.001$.

^c $P < 0.01$.

the 0–24 h interval, higher values in smokers in relation to nonsmokers could be detected only with MR2 ($P < 0.001$) and, to a lesser extent, with MR5 ($P < 0.05$). In contrast, by comparing the mean values obtained in the sampling period of 4–5 h, higher values in smokers were observed with the five MRs. Nevertheless, the level of significance was not the same for each individual ratio; MR2 had the highest discriminating power ($P < 0.0001$), followed by MR3 and MR4 ($P < 0.001$). Although MR1 and MR5 were also able to distinguish between nonsmokers and smokers, the differences were of a lower order of magnitude ($P < 0.01$).

MR2 in the urine sample of 4–5 h was the best correlated ratio with the number of cigarettes/day ($r = 0.39$; $P < 0.001$), whereas MR1, MR3, and MR4 were not significantly associated with the tobacco consumption (Table 5).

Although among nonsmokers, males had higher values of the MRs than females at the two intervals, there were not statistically significant differences for any ratio ($P > 0.05$). Among smokers, MR2 at 4–5 h was the only ratio significantly higher in males ($n = 33$) than in females ($n = 33$) (7.88 ± 3.33 versus 5.78 ± 2.20 ; $P < 0.001$). At this point it is important to note the sex differences in tobacco exposure (cigarettes/day): 21.21 ± 12.30 and 14.94 ± 8.85 for males and females, respectively ($P < 0.05$).

Both AFMU and the sum of 1X and 1U ($1X + 1U$) are primarily products of the CYP1A2 pathway leading to the intermediate (Z) from 17X. However, the intervention of the polymorphic NAT2 in the formation of AFMU may introduce a certain loss of the association between the values of the molar excretion of AFMU and those of $1X + 1U$. When analyzed this association, a lower correlation coefficient was obtained in the 0–24 h urine ($r = 0.39$) than in that of 4–5 h ($r = 0.75$).

Correlation analysis showed MR1, MR3, and MR4 to be acceptably well correlated to each other at the two time intervals, whereas MR2 and MR5 appeared to be independent of the other ratios (Tables 6 and 7). Although a certain association between MR2 and MR5 was obtained, the coefficient of correlation was insufficient to allow the prediction of individual values of MR2 based on those of MR5 and *vice versa*. Correlations between the two urine samples for each MR were weak, as shown in Table 8.

As expected, smokers had a higher mean value of urinary mutagenicity than nonsmokers: 4.01 ± 0.46 and 3.66 ± 0.46 , respectively ($P < 0.001$). A positive association existed between urine mutagenicity and tobacco consumption, measured as cigarettes/day ($r = 0.41$, $P < 0.01$).

Table 9 shows the correlation coefficients obtained when correlated the different MRs with the urinary mutagenicity. Although weak, the only statistically significant correlation was

Table 6 Correlations between metabolite ratios in the period of 0–24 h

The values above the diagonal are the Pearson coefficients of correlation between the transformed variables (log MR1, MR2, MR4, and MR5; $\sqrt{\log}$ MR3). The values below the diagonal are the Spearman rank correlation coefficients.

	MR1	MR2	MR3	MR4	MR5
MR1					
MR2	−0.0488				
MR3	0.9076	0.0948			
MR4	0.8956	0.3483	0.7906		
MR5	0.2716	0.5413	0.0209	0.6225	

Table 7 Correlations between MRs in the interval of 4–5 h

The values above the diagonal are the Pearson coefficients of correlation between the transformed variables (log MR1, MR2, MR4, and MR5; $\sqrt{\log}$ MR3). The values below the diagonal are the Spearman rank correlation coefficients.

	MR1	MR2	MR3	MR4	MR5
MR1					
MR2	0.3545				
MR3	0.9207	0.4398			
MR4	0.9557	0.5748	0.8586		
MR5	0.6153	0.5822	0.3636	0.7867	

that between MR2 and mutagenicity in the period of 4–5 h, in both the total group and that of smokers.

Urine flow (ml/min) in the interval of 4–5 h (3.78 ± 3.30) was significantly higher than in that of 0–24 h (1.18 ± 0.60) ($P < 0.001$), probably due to the relatively high volume of the caffeine beverage and possibly also to the ingestion of additional liquids by some individuals. The frequency distributions were highly skewed, showing a nonnormal distribution at the two intervals. However, when the values were logarithmically transformed, the distributions were normal, in spite of the high coefficients of variation of the nontransformed values: 50 and 87% for 0–24 and 4–5 h urine samples, respectively.

Table 10 shows the correlations between ratios and urine flow rates for the two sampling periods. MR2 was the only ratio nondependent on the urine flow rate, even at the 4–5 h interval, when the urine flow was notably high. The coefficients of correlation of MR1, MR3, MR4, and MR5 showed different level of significance; MR1 was the most flow dependent.

Discussion

The differences among the five MRs (Table 1) compared in the present study consist in both the metabolites included in the respective quotients and the reactions underlying the indexes, such as caffeine 3-demethylation (MR1 and MR3), paraxanthine 7-demethylation (MR2 and MR5), or both reactions (MR4).

MR1, MR3, and MR4 behaved in a similar way at the two time intervals (Tables 6 and 7). All three ratios were highly correlated with each other in both the urine of 0–24 h and that of 4–5 h. By contrast, they were not closely associated with either MR2 or MR5 (Table 8). This same pattern of correlations has been reported by Notarianni *et al.* (26), who compared four identical ratios to our MR1, MR2, MR3, and MR5 and another ratio similar to our MR4. These authors concluded that different ratios reflect three distinct entities. On the other hand, MR1, MR3, and MR4 have not been able to demonstrate higher enzyme activity in smokers in the urine of 0–24 h. However, the values of these ratios have been significantly greater in smokers in the 4–5 h urine samples (Table 4).

As indicated by Rostami-Hodjegan *et al.* (42) the corre-

Table 8 Correlations between 0–24 and 4–5 h for each MR

The transformed variables correspond to log MR (MR1, MR2, MR4, and MR5) or $\sqrt{\log}$ (MR3).

	<i>r</i>	
	Transformed variable ^a	Nontransformed variable ^b
MR1	0.4495	0.4001
MR2	0.5569	0.6561
MR3	0.3276	0.3041
MR4	0.5190	0.5195
MR5	0.5339	0.6082

^a Pearson correlation coefficient.

^b Spearman rank correlation coefficient.

lation matrix of the ratios and their sensitivity to indicate higher CYP1A2 activity in smokers can be explained, at least in part, by their pattern of dependency, as well as by the underlying pharmacokinetic basis of each individual ratio.

Renal clearance of caffeine is very sensitive to urine flow (16). Thus, it may be expected that MR1, MR3, and MR4, when 137X is included in the denominator, will depend on the urine flow rate. The influence of the urine flow on caffeine renal clearance has been demonstrated experimentally by Tang *et al.* (23). These authors have suggested that the bimodality found in the renal clearance of 137X implies that ratios containing this compound in the denominator could be bi or trimodally distributed. Bimodal or trimodal distributions have been reported in populations with MR1 (19, 24) or MR3 (20). The important dependence of MR1, MR3, and MR4 on the urine flow found in the present study (Table 10) is in agreement with that above referred and also with the theoretical assessment noted by Rostami-Hodjegan *et al.* (42). The urinary concentration of 17X does not always parallel its plasma concentration, and the flow dependence of caffeine or 17X is not the same (16). This could explain the relatively lower flow dependence of MR5 in relation to MR1, MR3, or MR4 (Table 10).

In a previous work, a urinary constituent was found that co-migrates with caffeine in 20–40% of urine samples (25). The flow dependency of this compound led to the authors to suggest that the flow dependency of the ratios containing caffeine is something artifactual, due to the use of detection systems lacking ability to assure peak purities. Nevertheless, in our work, after spectral validation by using a photodiode array detector, we could not find any component masking caffeine peaks in 37 urine samples. This latter result might reinforce the suggestion about the dietary origin of the above-mentioned constituent, because dietary habits differ among different countries.

MR1, MR3, and MR4 use 17X in the numerator. 17X is both product and substrate for CYP1A2. Hence, as emphasized by Kalow and Tang (16), the sampling time is crucial. This topic may explain the poor correlation between the values of each ratio obtained at the two time intervals, and also the lack of discrimination power between nonsmokers and smokers in the 0–24 h urine (Table 4). According to the rational basis of the ratios, 17X must represent its formation rate rather than that of its degradation. Therefore, MR1, MR3, and MR4 should be more valid when caffeine concentrations are high, around one half-life of caffeine (16). As demonstrated by Aramaki *et al.* (43), the urinary quotient 17X/137X is enhanced with time, due to the fact that 17X in plasma is affected by the plasma concentration of 137X. Thus, MR1 and specially MR3 can conceivably change with postadministration time.

Table 9 Correlation coefficients between urine mutagenicity (revertants/24 h urine) and MRs

	Urine mutagenicity (log revertants/24 h urine), <i>r</i>		
	Total group (<i>n</i> = 115)	Smokers (<i>n</i> = 61)	Nonsmokers (<i>n</i> = 54)
0–24 h period			
Log MR1	–0.02	0.01	–0.03
Log MR2	0.09	0.03	–0.09
√Log MR3	–0.01	–0.04	0.06
Log MR4	0.01	0.03	–0.09
Log MR5	0.04	0.10	–0.19
4–5 h period			
Log MR1	0.06	0.03	–0.15
Log MR2	0.34 ^a	0.26 ^b	0.05
√Log MR3	0.14	0.08	–0.05
Log MR4	0.11	0.09	–0.15
Log MR5	0.05	0.09	–0.17

^a *P* < 0.01.^b *P* < 0.05.

The interval of 4–5 h after caffeine intake has been proposed by Butler *et al.* (20) to minimize the impact of paraxanthine conversion to secondary metabolites. In the present study, this interval has allowed the detection of significant differences between nonsmokers and smokers. This latter result is in agreement with that reported by Rost and Roots (37), who found that MR1 and MR3 correlated highly with caffeine systemic clearance and caffeine breath test in the 5–8 h interval, but not in that of 8–24 h.

As in the work of Notarianni *et al.* (26), MR2 and MR5 were not acceptably correlated. These indexes are based on the 7-demethylation of paraxanthine, the only difference being the inclusion of 17U or 17X in the denominator of MR2 or MR5, respectively. Although 17X demethylation has been found to correlate closely (*r* = 0.87) with the formation of 17U from 17X (32), the flow dependency of 17X makes urinary 17U, which is a polar metabolite, a better surrogate marker of plasma 17X concentration than 17X in urine. According with this notion, MR5 in the present study showed to be significantly correlated with the urinary flow. This could explain the lack of a stronger correlation between MR2 and MR5 at the two time intervals, as well as the lower discrimination power of MR5. Racial differences in MR5 noted by Relling *et al.* (44) could be related to the interethnic variation in the renal clearance of 17X, as suggested by Tang *et al.* (23).

The greater capacity of MR2 to indicate high enzyme activity in smokers found in the present work may rise from its lack of flow dependency, which is particularly evident at the 4–5 h interval, because the urine flow rates have been especially high (Tables 4 and 10). This seems particularly important to compare different populations, as in the present paper because, as noted by Rostami-Hodjegan *et al.* (42), one could expect greater variation in confounding factors than when investigating changes in activity within a population. Moreover, MR2 in the period of 4–5 h was the best associated index (*r* = 0.39; *P* < 0.001) with the tobacco consumption, measured as number of cigarettes/day (Table 5). The statistical significance of this association is similar to that previously found by Kalow and Tang (33) (*r* = 0.62; *P* < 0.01) in a group of 19 smokers. The lack of a stronger association between cigarettes/day and MRs could be explained, at least in part, by the influence of factors, such as the different patterns of tobacco consumption (frequency and depth of aspirations) or the different content in

Table 10 Correlations between urinary flow (ml/min) and metabolite ratios in the periods of 0–24 and 4–5 h

	<i>r</i>				
	Log MR1	Log MR2	√Log MR3	Log MR4	Log MR5
Log urine flow (0–24 h)	–0.50 ^a	0.16 ^b	–0.43 ^a	–0.42 ^a	–0.20 ^c
Log urine flow (4–5 h)	–0.47 ^a	0.12 ^b	–0.27 ^d	–0.39 ^a	–0.25 ^d

^a *P* < 0.001.^b Not significant.^c *P* < 0.05.^d *P* < 0.01.

PAHs, depending on the kinds of cigarettes. Higher sensibility of MR2 in relation to other ratios has been reported. Lower CYP1A2 activity in contraceptive users could be demonstrated by caffeine clearance and MR2 but not by MR1 (23). In addition, differences between nonsmokers and smokers in some populations could not be detected by MR1 (20).

MR2 has shown a slightly greater discriminatory power between nonsmokers and smokers in the period of 4–5 h than in that of 0–24 h (Table 4). This is consistent with the results of Rost and Roots (37), who found better correlations between MR2 and caffeine clearance, caffeine breath test, or changes induced by omeprazole in the 5–8 h urine sample than in that of 8–24 h. The formation of IX, possibly mediated by CYP1A2, competes with the polymorphic NAT2 pathway leading to AFMU (Fig. 1). It is possible that at higher concentrations of substrate 17X, occurring at 4–5 h in relation to 0–24 h interval, the CYP1A2 activity may obscure the competitiveness of the NAT2. In fact, the correlations between urinary concentrations of AFMU and (1X + 1U) in the two sampling periods seem to indicate a lower influence of the NAT2 on the CYP1A2 activity in the 4–5 h interval. This could account for the higher correlation of MR2 or MR5 at 4–5 h with the tobacco exposure (Table 5) and also for the higher discriminatory capacity of MR2 between nonsmokers and smokers in this interval in relation to that of 0–24 h (Table 4).

Although the correlations of MR2 and MR5 values between the two periods has been higher than those of the other three ratios (Tables 6 and 7), the relatively low coefficients of correlation corroborate the notion that the time of the urine collection is also important for MR2 and MR5. Because CYP1A2 catalyzes both the formation and degradation of paraxanthine, variations in the timing of the urine collection can change the values of MR2 and MR5. In addition, the influence of the urinary flow on 17X in a slide of time may be greater than in accumulative samples (45). Thus, this could account for the lower correlation of MR5 between 0–24 and 4–5 h in relation to that of MR2. In fact, in the present study, a slightly lower correlation between MR5 and urinary flow was observed in the 0–24 h sample in relation to that of 4–5 h (Table 10).

The influence of sex on CYP1A2 activity is conflicting. The absence of gender differences among nonsmokers in the MRs found in this work, is in agreement with the results obtained by Horn *et al.* (46) and in contrast with those of Nakajima *et al.* (47). Among smokers, only MR2 at 4–5 h was able to show increased values in men in relation to women. Bearing in mind that tobacco exposure was higher in males, this result seems to reinforce the idea about the higher sensitivity of this index to indicate tobacco-induced CYP1A2 activity.

Among the different bacterial mutagenicity assays, we selected the TA 98 strain of *S. typhimurium* for three reasons:

(a) it has been found to be the one of highest sensitivity to cigarette smokers' urine (38, 48). (b) The mutagens in smokers' urine are a complex mixture of metabolites originated from tobacco pyrolysis, such as PAHs, aromatic amines, and heterocyclic amines (49, 50). The use of different strains is useful to reflect the mutation spectrum dominance of one class of chemical mutagen. Although TA 100 seems to be slightly more specific for PAHs than TA 98 (51), TA 100 is more efficient in detecting caffeine derived mutagens than TA 98 (52), and this could have represented a source of confusion for our study. (c) Urinary mutagenicity on TA 98 has been found to correlate with the levels of urinary PAHs metabolites (53, 54). In our study, metabolic activation by addition of S9 mix was avoided, because our objective was to measure only those mutagens produced by endogenous bioactivation. Because the PAH metabolites suffer conjugation with glucuronic acid, the presence of β -glucuronidase enhances the assay sensitivity (54). The microsuspension method used in this work has been reported to be the most sensitive in detecting the mutagenicity of smokers' urine (40, 54). The mutagenicity has been expressed as revertant colonies of *S. typhimurium* TA 98 strain in urine collected in a 24-h interval, to avoid the variation in creatinine excretion, which has been observed to be a source of confusion (27), as well as to correct the differences in urinary flow between individuals (48). In addition, the creatinine excretion is known to decrease with tobacco exposure (27, 55).

MR2 at 4–5 h was the only ratio with a statistically significant correlation with the urinary mutagenicity (Table 9); this association was relatively weak ($r = 0.3$) and lower than that observed between tobacco consumption (cigarettes/day) and mutagenicity ($r = 0.4$). Some possibilities that could explain the weakness of this association cannot be excluded: aromatic compounds, such as PAHs or arylamines, are known to be responsible for most of the mutagenicity, at least under the conditions used here to measure urine mutagenicity in smokers (27). Nevertheless, tobacco smoke contains other mutagenic substances on TA 98, such as tobacco-specific nitrosamines *N'*-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (27), which are also metabolized by other cytochromes, including CYP2E1 and the polymorphic CYP2D6 (56, 57). In addition, the polyfunctional induction produced by PAHs through Ah receptor may derive in an enhancement of both bioactivating and detoxifying enzymes. In this regard, PAHs are mostly metabolized by CYP1A1, and CYP1A1 differs from CYP1A2 not only in substrate specificity but also in induction specificity. The Ah receptor seems to be a more important determinant in the *CYP1A1* gene regulation than in that of *CYP1A2* (58). With regard to detoxifying enzymes, a certain correlation, although nonstrict, has been reported between urinary thioethers and mutagenicity in smokers (30, 59). Thioethers may be considered not only as markers of human exposure to electrophiles but also as indicators of detoxification capacity, because they are products of GST. GST *Ya* class genes belong to the Ah battery, which is transcriptionally activated by PAHs (60). Moreover, the smokers who are genetically deficient in GST M1 (around 50% of Caucasians) present higher urine mutagenicity than those without this hereditary trait (61). Therefore, the genetically determined differences in enzymes among individuals could account for important differences in the balance between bioactivation and detoxification pathways in smokers, thus leading to the low association between CYP1A2 activity and urine mutagenicity, as described above.

In summary, this study suggests that MR2 ratio is the best indicator of smoking induction of CYP1A2 at the 4–5 h inter-

val. Nevertheless, it is important to consider that MR2 ratio includes AFMU in its numerator, which is a product of the NAT2. Although NAT2 is noninducible, it is known to be polymorphic in the population. The levels of AFMU, and thus of the MR2 ratio, can be influenced by the NAT2 activity in subjects. This limitation of MR2 in comparing different groups, as in the present work, may be avoided when investigating changes within the same individuals (e.g., in studies of drug interactions). In this kind of studies, MR2 at 4–5 h may be a more specific ratio to assess CYP1A2 activity, especially at high urine flow rates.

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Five Caffeine Metabolite Ratios to Measure Tobacco-induced CYP1A2 Activity and Their Relationships with Urinary Mutagenicity and Urine Flow

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Cancer Epidemiol Biomarkers Prev 1999;8:159-166.

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