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Influence of the Urine Flow Rate on Some Caffeine Metabolite Ratios Used to Assess CYP1A2 Activity

Blanca Sinués,* Ana Fanlo,* María Luisa Bernal,* Esteban Mayayo,* María Antonia Soriano,* and Enrique Martínez-Ballarin†

*Departments of *Pharmacology and †Physiology, Medical School, University of Zaragoza, Zaragoza, Spain*

Summary: Five established metabolite ratios (MRs) to measure P450 CYP1A2 activity—MR1 (17X + 17U)/137X, MR2 (AFMU + 1X + 1U)/17U, MR3 (17X/137X), MR4 (AFMU + 1X + 1U + 17X + 17U)/137X, and MR5 (AFMU + 1X + 1U)/17X—were calculated in urine 4–5 hours after caffeine intake. First, to assess the potential of omeprazole to induce CYP1A2 activity, a caffeine test was performed in 27 subjects on two occasions: before and after 14 days on omeprazole (20 mg/day). Samples of urine were analyzed by high-performance liquid chromatography (HPLC) to quantify caffeine and metabolites used to calculate the different caffeine MRs. MR1, MR3, and MR4 were enhanced after treatment; the percentage of change was inversely associated with that of the urine flow, with r values of −0.48, −0.49, and −0.47, respectively. However, MR2 or MR5 were not modified. To determine the reason for these contradictory results, the authors analyzed data of metabolites, ratios, and their components (numerators and denominators) from 152 subjects (who underwent one caffeine test) and their relationship with the urinary flow. Caffeine concentration in urine was the only compound nondependent on the urine flow. Consistently, ratios containing caffeine (MR1, MR3, and MR4) were highly influenced by the rate of urine excretion, since the flow dependence of their numerators is not canceled out by that of caffeine in their denominators. The dependency of the caffeine excretion on renal factors may explain the opposite results found with the different ratios in the aforementioned prospective study of drug interaction, the absence of closer correlations of the five MRs to each other, the discrepancies about the type of frequency distribution of the different MRs (either normal or multimodal), and the higher sensitivity of MR2 to detect gender differences in CYP1A2 activity found in this study. In summary, the data clearly emphasize the need for a strict control of the liquid intake to avoid high urine flows when MRs containing caffeine are used to assess CYP1A2 activity, especially in studies of drug interactions. **Key Words:** Caffeine metabolite ratios—CYP1A2 activity—Urine flow.

The human cytochrome P450 isoform CYP1A2 is involved in the metabolism of a number of drugs, such as imipramine (1), clomipramine (2), clozapine (3), fluvoxamine (4), olanzapine (5), tacrine (6), ropivacaine (7), and also in the bioactivation of some environmental carcinogens, such as arylamines, heterocyclic amines, and aflatoxin B1 (8–10). CYP1A2 activity is induced by xenobiotics, including cigarette smoke (11), and polycyclic aromatic or halogenated hydrocarbons from other sources (12) and inhibited by fluvoxamine (13), verapamil (14), quinolone antibiotics (15), oral contraceptives (11), and propafenone (16). Therefore, the changes in CYP1A2 activity may have important therapeutic and toxic consequences. This implies the need for methods that accurately measure this metabolic activity.

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Address correspondence and reprint requests to B. Sinués, Department of Pharmacology, Medical School, University of Zaragoza, Domingo Miral s/n, 50009 Zaragoza, Spain; E-mail: bsinues@posta.unizar.es

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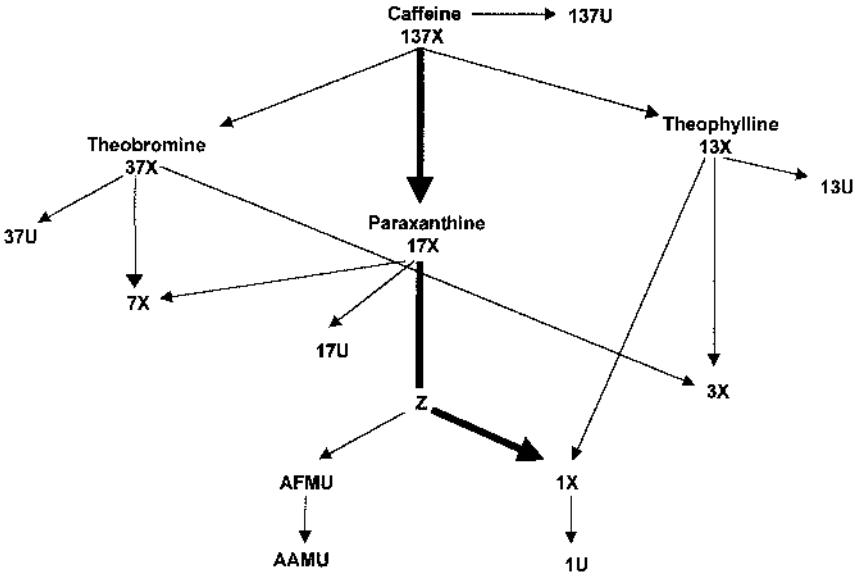

FIG. 1. Pathways of caffeine metabolism.

Among the methods for measuring CYP1A2 activity in vivo, the urinary caffeine metabolite ratios (MRs) after caffeine intake have become popular due to the ubiquitous use of caffeine and also because of its noninvasive method.

The main metabolic routes of caffeine are illustrated in Figure 1. In the complex metabolism of caffeine (137X), CYP1A2 is solely responsible for the caffeine 3-demethylation to paraxanthine (17X) and overwhelmingly for the N-1 and N-7 demethylations leading to theobromine (37X) and theophylline (13X), respectively. In vitro observations in liver microsomes suggest that CYP1A2 catalyzes 94.5% of the three demethylations of caffeine. The contribution of CYP3A4 (17) and CYP2E1 (18) to the caffeine demethylations seems to be negligible in most persons (17,19). 17X later undergoes two reactions, one of which is 8-hidroxylation by other cytochromes, including CYP2D6 and also CYP1A2 (19). 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 undergoes 8-hydroxylation to 1-methyluric acid (1U), mediated by xanthine oxidase with a little contribution of CYP1A2 (19,20). The polymorphic NAT2 mediates the step toward AFMU (20).

Several urinary MRs have been proposed to assess CYP1A2 activity (11,21–24) (Table 1). The more adequate ratio remains to be clarified. At the center of the debate is the urine flow dependency of some metabolites used in these ratios. Opposite results have been reported by Butler et al (22) and Tang et al (25) with regard to the flow dependence of the urinary excretion of caffeine. A report has shown that the different flow dependence of the ratios underlies the differences in sensitivity to indicate CYP1A2 induction (26). Therefore, conclusions of the studies may depend on the choice of one of another ratio. In this regard, a number of authors have reported opposite results about the potential of omeprazole to induce CYP1A2 activity by using different ratios (27–29). Whereas Andersson et al (27) and Rizzo et al (28) show no induction, Rost et al (29) report increments in enzyme activity by using MR1, MR2, and MR3. Butler et al (22), who used MR1, found that CYP1A2 was trimodally distributed in each of three healthy populations. Subjects were divided according to their CYP1A2 "phenotype" in a work of cancer epidemiology (30). In contrast, normal frequency distributions have been found with other ratios (19,24,26). In addition, differences in sensibility to detect exposure to inducers or inhibitors have been reported between the different MRs (22,25,26).

The aim of this study was to investigate the relative influence of the urine flow on the ratios most commonly used (Table1), the metabolites included in the ratios, and the components of each quotient to determine the reasons for the discrepancies found in some results proceeding

TABLE 1. *Caffeine urinary metabolite ratios used to explore CYP1A2 activity*

Abbreviation	Quotient	Reference
MR1	(17X + 17U)/137X	Butler et al. (27)
MR2	(AFMU + 1X + 1U)/17U	Campbell et al. (11)
MR3	17X/137X	Kadlubar et al. (26)
MR4	(AFMU + 1U + 1X + 17U + 17X)/137X	Carrillo and Benitez (29)
MR5	(AFMU + 1X + 1U)/17X	Grant et al. (28)

MR, metabolite ratios.

from studies using one or another ratio. We analyze data extracted from two studies from our group: a prospective study dealing with the potential of omeprazole to induce CYP1A2, and another population-based study including nonsmokers and smokers.

MATERIAL AND METHODS

Subjects

In the population-based study, 152 healthy persons (76 men and 76 women) were included. The average age was 26.9 ± 8.6 (mean ± SD) years, ranging from 18 to 57 years. Of the subjects, 86 were nonsmokers, and 66 were smokers of 18.2 ± 8.5 (mean ± SD) cigarettes/d. In the prospective study, 27 healthy nonsmokers participated (12 men and 15 women). The average age was 30.41 ± 4.77 (mean ± SD) years, ranging from 25 to 43 years. All subjects gave their written informed consent to participate in the study, according to the protocol approved by the Ethics Committee for Clinical Research of the Hospital Clínico of the University of Zaragoza, Spain. Each subject completed a detailed questionnaire on smoking habits, diet, coffee, drug consumption, and lifestyle. Subjects following diets or cooking procedures known to modulate CYP1A2 activity, according to previous works (31,32), were not included in the study. During the entire study, subjects followed a controlled diet. Regular alcohol drinkers were excluded as were subjects taking any medication, including oral contraceptives.

Study Protocol

For the determination of urinary caffeine MRs, subjects refrained from consuming foods and beverages containing methylxanthines for 48 hours before the test (caffeine intake). After emptying their bladders, subjects in the omeprazole study ingested two cups of instant coffee containing about 200 mg of caffeine. In the population-based study, subjects were given instant coffee containing 200 mg of caffeine in 250 mL of water. All urine formed in the 4–5 hours after coffee intake was collected in bottles preloaded with 3 mL of 1 mol/L citric acid phosphate buffer, pH 3.5, to avoid the AFMU deformylation to AAMU (19). Urine volumes were recorded, and aliquots of 2 mL were stored at −80°C until analysis.

Caffeine tests were performed as indicated above at two times: (Day 0) and 14 days after (Day 15) omeprazole treatment at a dose of 20 mg/d. Subjects participating in the population-based study (n = 152) underwent only one caffeine test. The choice of the 4- to 5-hour interval after caffeine intake was due to the fact that this was the time schedule most sensitive to discriminate populations nonexposed and exposed to inducers of CYP1A2 (26). It should be noted that noncomparison with other published methods are made in the present work.

Analysis of Caffeine and Metabolites

Caffeine and its metabolites were determined according to the high-performance liquid chromatography (HPLC) method described by Grant et al (33). Caffeine and its metabolites were separated by a Waters Novapak C18 reverse-phase column (4 μm particle size, 25 cm × 4.6 mm internal diameter; Millipore Ibérica S.A.), which was eluted isocratically with a mobile phase containing acetic acid, methanol, and water (0.5:90:905.5 vol/vol/vol) at a flow rate of 1 mL/min and a pressure of 1,500 psi. The compounds were detected by ultraviolet (UV) absorbance at 280 nm. The chromatograph was an LC Module I Plus equipped with Millennium 2010 software (Waters Corp., Madrid, Spain). Calibration curves were performed with known amounts of metabolites ranging from 5 to 80 μg/mL, which were 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 (34). The limit of detection was 1 μmol for AFMU and 0.1–0.3 μmol for caffeine and the remaining metabolites. For data analysis, caffeine and its metabolites were expressed as mmol/L. Table 1 shows the five MRs used to measure CYP1A2 activity.

To assess whether any urinary constituent could comigrate with caffeine or caffeine metabolites used in the ratios, a Water 996 photodiode array detector was used to compare the peaks with the spectral libraries of standards. Caffeine excretion rate was calculated as the product of caffeine concentration in urine and the urine flow in the sampling time of 1 hour (from 4 to 5 hours after caffeine intake). Renal clearance of caffeine is expressed as excretion rate of 137X over plasma caffeine concentration, and a significant correlation has been observed between caffeine concentration in plasma and urine (35). Therefore, the rate of caffeine excretion can be used as a surrogate marker of caffeine renal clearance.

Statistical Analysis

The characteristics of the distributions of the variables were assessed by the Kolmogorov-Smirnov test. Variables (x) were transformed to log x to obtain a normal distribution of the five MRs. Differences of means were

analyzed by either the Student *t*-test for unpaired data or the *t*-test for paired data. Correlations between transformed variables were calculated by Pearson correlation test when data fitted a normal distribution or by Spearman rank correlation test for nonparametric data. The null hypothesis was rejected when $P < 0.05$.

RESULTS

Table 2 shows mean values of the five MRs before and after omeprazole treatment at a daily dose of 20 mg in 27 subjects. While MR2 and MR5 did not change significantly, the other three ratios were significantly enhanced after treatment.

By comparing the urinary flow before treatment (0.92 ± 0.55 mL/min) with that after treatment (0.77 ± 0.61 mL/min), no significant differences were found.

By analyzing the association between the percentages of change of the five MRs from baseline values to those after omeprazole administration, and the percentages of change in urine flow, the results showed that the changes in MR2 or MR5 do not depend on the variations in urine flow. In contrast, MR1, MR3, and MR4 were dependent on the changes in the rate of urine excretion, with correlation coefficients of –0.48, –0.48, and –0.47 for MR1, MR3, and MR4, respectively ($P < 0.01$ in all cases). By contrast, the correlation coefficients for MR2 and MR5 were –0.11 and –0.14, respectively ($P > 0.05$). This finding led us to analyze in a population-based sample with a larger size (152 subjects) and a high urinary flow (mean ± SD, 3.24 ± 3.2 mL/min) the reason by which urine flow may affect each ratio so differently. First, the correlation analysis between urine flow and MRs showed that MR2 was the only ratio nondependent on the rate of urine excretion. MR5 showed to be weakly associated to the urine flow (Table 3).

Caffeine urinary concentration was not correlated to the urine flow rate, while the metabolite concentrations were importantly influenced (all $P < 0.0001$; Fig. 2).

TABLE 2. *Caffeine metabolite ratios before and after omeprazole treatment*

Log MR	Day 0*	Day 15*
Log MR1	0.56 ± 0.27	0.82 ± 0.19§
Log MR2	0.58 ± 0.20	0.51 ± 0.20
Log MR3	0.21 ± 0.24	0.39 ± 0.19†
Log MR4	1.04 ± 0.32	1.29 ± 0.24‡
Log MR5	0.63 ± 0.28	0.71 ± 0.21

* All values given as mean ± SD.
† $P < 0.05$.
‡ $P < 0.001$.
§ $P < 0.0001$.

TABLE 3. *Pearson correlation coefficients for the relationship between log-transformed values of urine flow and caffeine metabolite ratios*

	r	*P*
Log MR1	–0.57	<0.001
Log MR2	0.064	>0.05
Log MR3	–0.44	<0.001
Log MR4	–0.49	<0.001
Log MR5	–0.16	<0.05

MR, metabolite ratios.

As the flow dependency of the ratios is determined by the relative flow dependency of their components (numerator and denominator), the following analysis was carried out to establish the correlation between each of these components in the MRs and the urine flow rate. There is similar flow dependence of the components in the numerator and denominator of MR2 and MR5, which is in contrast with that of MR1, MR3, and MR4, as can be inferred from the data shown in Figure 2.

The excretion rate of caffeine, expressed as μmol/h, in the urine formed 4–5 hours after caffeine intake, was used as marker of caffeine renal clearance, and correlated highly with the ratios containing caffeine in the denominator; the *r* values were –0.71, –0.64, and –0.68 for MR1, MR3, and MR4, respectively.

The correlation matrix of the ratios (Table 4) showed that MRs containing caffeine correlated highly with each other, while MR2 and MR5 were not acceptably associated either with each other or with the other MRs.

Even though the five ratios were able to distinguish between nonsmokers and smokers, with *P* values for all MRs lower than 0.001, differences between men and women could be detected only by MR2 (Table 5).

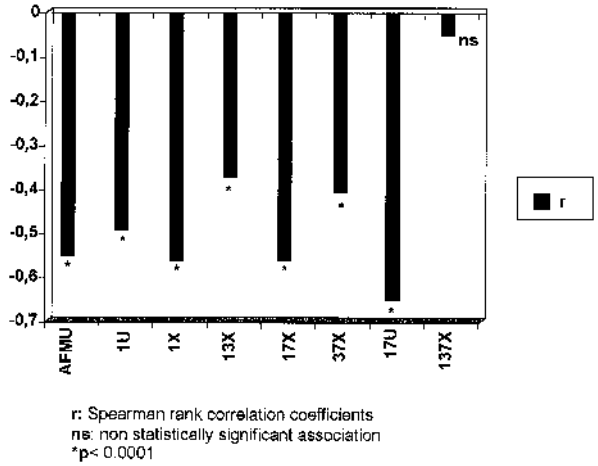

FIG. 2. Effect of urine flow rate on caffeine urinary metabolite concentrations.

TABLE 4. *Correlations between caffeine metabolite ratios**

	MR1	MR2	MR3	MR4	MR5
MR1	1	0.32	0.90	0.90	0.60
MR2		1	0.42	0.53	0.53
MR3			1	0.83	0.34
MR4				1	0.78
MR5					1

* Pearson correlation coefficients between log-transformed MR values.

MR, metabolite ratios.

DISCUSSION

The five MRs compared in the present work differ in the metabolites included in the respective quotients and in the reactions underlying the indices.

Among all metabolites used in the MRs, caffeine behaves in a different way according to its renal excretion. The low renal clearance of caffeine of 1–3 mL/min indicates the importance of the renal tubular reabsorption of caffeine (36–38). In addition, the high correlation coefficient between caffeine concentration in plasma and urine ($r = 0.82$) found by Tang and Kalow (25) reinforces this notion and implies almost complete equilibrium between 137X concentration in plasma and urine, as noted by Birkett and Miners (35). Therefore, the rate of caffeine excretion, i.e., the amount of caffeine eliminated in 1 hour of urine collection, which may be considered as marker of caffeine renal clearance, must be highly dependent on the urine flow rate. The tendency for a diffusion equilibrium between caffeine concentration in renal tubular fluid and blood may explain the lack of a significant negative correlation between urine caffeine concentration and urine flow (Fig. 2) and also the high correlation coefficients found between urinary flow and the MRs containing caffeine in their denominators: MR1, MR3, and MR4 (Table 3).

The different flow dependency of the five MRs appears to depend on the relative influence of the urine flow on the numerator and denominator of each ratio. The flow dependency of MR1, MR3, and MR4 has been high as a result of the lack of flow dependency of caffeine concentration in urine, which is present in their denominators, and the flow dependence of their numerators. Thus, MR1, MR3, and MR4 are expected to increase as the urine flow rate decreases and the contrary in the case of increments in the urine flow rate. In contrast, MR2 and MR5, by containing compounds in their numerators and denominators similarly influenced by the rate of urine excretion, cancel out this influence, and they may provide a better estimation of metabolic activity.

MR1, MR3, and MR4 values are higher after omeprazole treatment (Table 2). In principle, this could indicate that omeprazole causes an induction of CYP1A2 activity. However, the changes in MRs between the two sample times correlated significantly with the changes in urine flow. In contrast, MR2 and MR5 were unchanged by treatment, probably as a result of their independence on the urine flow. Therefore, the choice of one or another ratio can lead to contradictory results in studies of drug interactions, especially when, as in the present work, the urine flow is not the same before and after treatment.

The authors who used MR2 to study the potential of omeprazole to induce CYP1A2 activity have reported lack of drug induction (27,28). By contrast, Rost and Roots (29), who used MR1, MR3, and also MR2, found all three ratios to be enhanced. In this study, doses as high as 40 mg/d or 120 mg/d were given to slow and rapid metabolizers of S-mephenytoin, respectively. It is possible that the induction of CYP1A2 by omeprazole requires higher doses of the drug, as those used by Rost and Roots (29), to be manifested as an increase in MR2. Nevertheless, it is of note that no mention of the urine flow is made in this later study nor in the above referred reports, in which caffeine MRs were used to explore the potential of omeprazole to induce CYP1A2.

Another question is the frequency distribution of CYP1A2 activity measured with distinct MRs. In this work, all five MRs have been log-normally distributed in consonance with previous data (11,32,39). However, Butler et al (27), using MR1, suggested that CYP1A2 enzyme activity was trimodally distributed in each of three populations. Indeed, this index was used in studies of cancer epidemiology to distinguish between CYP1A2 phenotypes with regard to colorectal cancer (30). The dual dependency of MR1 and that of the other ratios containing caffeine in the denominator on metabolic and renal factors could explain any multimodality. Kalow and Tang (19) found a significant correlation ($P < 0.01$) between MR3 and renal clearance of 137X, and these same authors observed a bimodal distribution for caffeine renal clearance, MR3, and urine flow rate.

TABLE 5. *Transformed caffeine metabolite ratios in non smokers**

	Females (n = 43)†	Males (n = 43)†
Log MR1	0.62 ± 0.27	0.69 ± 0.27
Log MR2	0.59 ± 0.18	0.66 ± 0.13‡
Log MR3	0.27 ± 0.24	0.35 ± 0.24
Log MR4	1.11 ± 0.33	1.22 ± 0.34
Log MR5	0.66 ± 0.28	0.71 ± 0.26

* n = 86.

† Values given as mean ± SD.

‡ $P < 0.05$.

Therefore, renal factors controlling the renal excretion of caffeine may introduce a confounding factor in ascribing persons to phenotypic traits of CYP1A2 activity when using MRs containing caffeine.

The correlation matrix of the ratios (Table 4) has shown MR1, MR3, and MR4 to be highly correlated to one another. In contrast, MR2 and MR5 appeared as independent on the other ratios. This same profile was first reported by Notariani et al (40), who concluded that the MRs could be measuring three distinct activities. The flow dependency of 137X, which is present in MR1, MR3, and MR4, may account for the interdependency of these MRs. On the other hand, the lower renal clearance of 17X in relation to 17U (19) could explain the lack of a stronger correlation between MR2 and MR5.

Concerning the sensitivity of the MRs to measure CYP1A2 activity, in the present work all five ratios have been able to detect higher activity in smokers. Nevertheless, MR2 was the only ratio sensitive enough to indicate higher CYP1A2 activity in men than in women (Table 5). This result is in agreement with previous observations (41,42). Higher sensitivity of MR2 in relation to the other MRs has been reported previously. Lower CYP1A2 activity in contraceptive users could be demonstrated by caffeine clearance and MR2 but not by MR1 (25). Differences between nonsmokers and smokers could not be observed by MR1 (22). The absence of flow dependency of MR2 can be of paramount importance in determining its higher sensitivity.

In summary, the results of the current study suggest the need for restricting liquid intake to minimize the influence of the high urine flows when MRs containing caffeine are used to indicate CYP1A2 activity. The flow dependence of the caffeine renal excretion rate could also be the main reason for (1) the lack of a stronger correlation between MRs, (2) the different frequency distributions of CYP1A2 found in some population-based studies, (3) the differences in sensitivity among MRs to indicate CYP1A2 activity, and (4) the controversial results in studies of drug interactions when different MRs are used.

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