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Original article

Caffeine as a probe for CYP1A2 activity: potential influence of renal factors on urinary phenotypic trait measurements

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Two established caffeine-based urinary methods for measuring CYP1A2 activity were compared with each other, and also with the systemic clearance of caffeine which served as a standard of reference for such activity. Following a standardized dose, caffeine (137X) and its metabolites were measured in urine and plasma of 39 healthy subjects. The measurements allowed determinations of: (1) systemic caffeine clearance (CL_{caff}); (2) the caffeine metabolite ratio (AFMU + 1X + 1U)/17U determined in an overnight-urine specimen and referred to as CMR, and (3) the ratio (17X + 17U)/137X measured in urine collected between 4 and 5 h after caffeine intake and referred to as PCUR for 'paraxanthine-caffeine urinary ratio'. The PCUR showed a bimodal distribution and a relatively wide variation, CL_{caff} and CMR were both normally distributed. The correlation between CL_{caff} and CMR was $r = 0.77$ ($p < 0.001$), between CL_{caff} and PCUR $r = 0.46$ ($p < 0.01$), and between CMR and PCUR $r = 0.40$ ($p < 0.02$). The difference between the correlation coefficients 0.77 and 0.46 was statistically significant (z-test; $p < 0.05$). The well established decrease of caffeine metabolism by oral contraceptive use was observed with both CL_{caff} and CMR but not with PCUR.

Examination of possible explanations for the differences between PCUR and CMR led to the finding of a correlation between PCUR and the renal clearance of caffeine (CL_r) with $r = -0.47$ ($p < 0.01$). Further scrutiny demonstrated that a bimodal or non-normal frequency distribution as shown by PCUR was also shown by CL_r and by urine flow rate. It appears that the PCUR method for assessing the *in vivo* activity of CYP1A2 reflects renal factors as much as metabolic function. The assignment of 'phenotypic trait' values to CYP1A2 activity may represent a distortion by renal factors.

Introduction

Butler *et al.* (1992) suggested on the basis of metabolic studies with caffeine that the activity of CYP1A2 was trimodally distributed in each of three healthy populations. They observed an almost three-fold difference between the median values of a urinary excretion trait measurement in healthy nonsmoking subjects in Italy and in the United States (Arkansas); the value for a Chinese population was intermediate. By contrast, an earlier but different procedure, also using caffeine, developed in this laboratory by Campbell *et al.* in 1987 (with subsequent re-evaluations by Denaro & Benowitz, 1991; Kalow & Tang, 1991a, b), indicated an essentially normal frequency distribution of CYP1A2 activity (Kalow & Tang, 1991a, b), with no significant difference observed between Canadian residents of Caucasian and Oriental origin. The differences between the results obtained by the two methods might be disregarded as a trivial discordance were it not for the potential utility of CYP1A2 measurements in studies of cancer epidemiology. Indeed, Butler *et al.* (1992) distinguished between 'CYP1A2 phenotypes' with respect to colo-rectal cancer (Kadlubar *et al.*, 1992).

CYP1A2 is capable of activating many procarcinogens (Guengerich & Shimada, 1991), and it is inducible by polycyclic aromatic or halogenated hydrocarbons present, for example, in cigarette smoke (Campbell *et al.*, 1987; Kalow & Tang, 1991a; Vistisen *et al.*, 1990, 1992). Hence, measurement of CYP1A2 activity may reveal an individual's capacity to bioactivate certain procarcinogens, and may demonstrate unexpected exposure to enzyme inducers, or perhaps genetically variable responsiveness to such inducers.

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†*Glossary*: 137X: 1,3,7-trimethylxanthine, caffeine. 17X: 1,7-dimethylxanthine, paraxanthine. 17U: 1,7-dimethylurate. 37X: 3,7-dimethylxanthine, theobromine. 13X: 1,3-dimethylxanthine, theophylline. 1X: 1-methylxanthine. 1U: 1-methylurate. AFMU: 5-acetylamino-6-formylamino-3-methyluracil. AAMU: 5-acetylamino-6-amino-3-methyluracil, CYP1A2: Cytochrome P4501A2. CYP2E1: Cytochrome P4502E1. XO: Xanthine oxidase. NAT2: Polymorphic *N*-acetyltransferase.

The method proposed by Butler *et al.* (1992) is based on measurements of paraxanthine (17X), its 8-hydroxylated metabolite (17U), and caffeine (137X) (Fig. 1), in urine collected between 4 and 5 h after intake of a standard dose of caffeine. The ratio (17X + 17U)/137X is used as an index of CYP1A2 activity. The essence of the method is to measure paraxanthine excretion at the point in time when its rate of formation and elimination are approximately equal. We will refer to this ratio as PCUR for 'paraxanthine/caffeine urinary ratio'.

The method that originated in this laboratory (Campbell *et al.*, 1987) is based on measuring in urine all four biotransformation products of paraxanthine, that is acetyl-formyl-methyluracil (AFMU), 1-methylxanthine (1X), 1-methylurate (1U), and 17U (Fig. 1). The ratio (AFMU + 1X + 1U)/17U, referred to as 'caffeine metabolic ratio' (CMR), is used as an index of CYP1A2 activity. The method involves measurement of the final metabolic products that arise from caffeine via the paraxanthine pathway, and it is in essence a measure of paraxanthine 7-demethylation. An important point is the fact that paraxanthine (17X), the main initial biotransformation product of caffeine, which is formed by CYP1A2, is also selectively destroyed by CYP1A2 (Gu *et al.*, 1992) (Fig. 1).

As a standard of reference for CYP1A2 activity in this comparative study, we determined the systemic caffeine clearance CL_{caff} by measuring caffeine concentrations in plasma. The justification for the use of caffeine clearance as an index of CYP1A2 activity follows from the observation (Lelo *et al.*, 1986) that the fractional conversion of caffeine into the three dimethylxanthines averages 94.1% of the dose. CYP1A2 catalyses all three demethylations of caffeine (Gu *et al.*, 1992). *In vitro* comparisons with caffeine demethylations by human liver microsomes (Gu *et al.*, 1992) suggest that 94.5% of the 1-, 3-, and 7-demethylations of caffeine are catalysed by CYP1A2. Thus, about 90% of systematic caffeine clearance depends on CYP1A2 activity (Kalow & Tang, 1993).

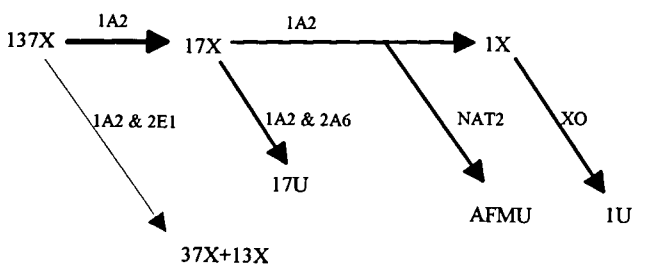

Fig. 1. Major pathways of caffeine biotransformation with primary enzymes shown at each important step. For interpretation of the abbreviations, please see glossary.

Usually less than 3% of the dose appears unchanged in urine (Bonati *et al.*, 1982). There are also contributions to the demethylation of caffeine by CYP1A1 (Tassaneeyakul *et al.*, 1992) and by CYP2E1 (Gu *et al.*, 1992) but these are negligible in the majority of subjects (Kalow & Tang, 1993, for CYP2E1; Tassaneeyakul *et al.*, 1992, for CYP1A1 unless plasma caffeine concentrations are high and above those in the present investigation).

This study compares the two caffeine-based urinary methods with each other and with caffeine's systemic clearance. A critical account of some biological parameters underlying these and other caffeine-based methods has been published recently (Kalow & Tang, 1993).

Materials and methods

Chemicals

Caffeine (137X), paraxanthine (17X), *N*-acetyl-*p*-aminophenol and 7-β-hydroxypropyltheophylline were acquired from Sigma Chemical Co., St Louis, MO; benzyloxyurea was obtained from ICN Pharmaceuticals, Inc., Life Sciences Group, Plainview, NY; 1X was the product of Pfaltz & Bauer Inc., Stamford, CT; 1U and 17U were purchased from Adams Chemical, Round Lake, IL; AFMU was isolated from urine in this laboratory (Tang *et al.*, 1983).

Clinical protocol

Volunteers were recruited from students and staff of the University of Toronto as approved by its Human Experimentation Committee. Forty healthy subjects participated in the study, the majority of whom were students. The data from one subject were excluded because of intake of medication containing acetaminophen and caffeine. The remaining 39 subjects had an average weight of 64.7 ± 11.6 (SD) kg, a height of 169.8 ± 9.1 cm and an age of 23.0 ± 5.5 years. Twenty-six were Caucasians and thirteen Orientals, of which the majority were of Chinese origin except for two of Korean and one of Vietnamese origin. There were 20 female and 19 male subjects. Eight females were taking oral contraceptives (OC), three subjects took antibiotics and one ibuprofen. Those subjects who indicated that they drank alcohol consumed an average of 2.6 ± 1.8 drinks per week, and for those who regularly consumed coffee and tea, the average was 1.7 ± 1.2 cups per day. Only one subject smoked, his maximal number of cigarettes was four per day.

The consent form specified for each subject to abstain from alcoholic drinks and methylxanthine containing food and beverages (i.e. coffee, tea, cola

and chocolate) for one day before and during the study. At 8 a.m. after an overnight fast, each subject drank a cup of instant coffee containing 140 mg of caffeine.

Urine samples were collected for 25 h over the following intervals: 0–4, 4–5, 5–8, 8–16 and 16–25 h after caffeine intake. The urine volume from each period was measured, and a 10 ml aliquot of each sample was titrated to pH 3.5 with concentrated HCl and stored at −20° C until analysed. At 4 and 8 h after caffeine ingestion, 3 ml of blood was drawn via venipuncture into a tube containing 12 mg of heparin (146 units per mg). Plasma was separated by centrifugation and stored at −20° C until analysed.

Analysis of caffeine and its metabolites

Urinary caffeine metabolites were measured by HPLC as previously described (Grant *et al.*, 1983; Tang *et al.*, 1986, 1991) except for a minor modification of the composition of the mobile phase. Specifically, the mobile phase was composed of 1.6% isopropanol and 0.01% phosphoric acid; this improved the peak separation over that achieved with the original solvent system.

The caffeine concentration in plasma was analysed using the method of Campbell *et al.* (1987) with modification of the mobile phase to be 2.8% isopropanol, 0.5% acetonitrile and 0.05% acetic acid.

AFMU was routinely measured by its complete conversion to the more stabilized form AAMU (Tang *et al.*, 1986).

The magnitudes of experimental error were consistent with those previously published; random duplicate determinations were within 8% of the original measurement.

Clearance measurements and statistical procedures

Two point (4 and 8 h) plasma caffeine concentrations were chosen to measure the systemic clearance of caffeine and related parameters. In a previous study, Gu (1992) has shown a significant correlation with $r = 0.98$ between this two-point method and the standard AUC-based measurement.

The elimination rate constant (K_{el}) and the zero time caffeine concentration (C_o) were determined from the log-linear decline of plasma caffeine concentrations (cf Wahllander *et al.*, 1990). The following equations were applied to derive the apparent volume of distribution (V_d) and systemic caffeine clearance (CL_{caff}):

$$V_d = \left(\frac{\text{dose} \cdot f}{C_o} \right) / \text{body weight}$$

and

$$CL_{caff} = K_{el} \cdot V_d$$

where the bioavailability (f) of the oral dose was assumed to be complete i.e. unity (Bonati *et al.*, 1982). 4–5 h and 16–25 h urine samples were used to calculate urine flow rates expressed as urine volume per 60 min. Excretion rates were estimated from urine 137X, 17X and 17U concentrations in the 4–5 hour urine sample, multiplied by flow rate of that period. The renal clearance of caffeine was estimated as follows: CL_r = Amount of caffeine excreted between 4 and 5 h after intake, divided by the caffeine concentration in blood at 4 h.

Urine samples collected from 4 to 5 h after caffeine ingestion were used to measure PCUR, i.e. the ratio (17X + 17U)/137X. Urine samples, collected from 16–25 h, were used for the calculation of CMR (CMR = (AFMU + 1X + 1U)/17U).

Pearson's correlation test was used to assess all correlations. Tests to compare different correlation coefficients utilized z functions. Both histograms and probit plots were plotted using the NTV statistical program (NTV, copyright 1992 by M. Patel, L. Endrenyi). They were used to illustrate and compare the distributions of various parameters, their variances were compared using the *F*-test. Student's *t*-test was applied to compare the mean differences.

Results

Data derived from the two-point measurements of caffeine plasma concentrations for all 39 subjects were: $CL_{caff} = 1.07 \pm 0.48$ (SD) ml min^{-1} kg^{-1}, half life = 6.20 ± 2.49 h, and apparent volume of distribution = 0.52 ± 0.22 l kg^{-1}.

The frequency distributions of CL_{caff}, CMR and PCUR are shown in Fig. 2 in terms of logarithmically transformed values; CL_{caff} and CMR appeared to be normally distributed, while PCUR was not. A nine-fold range in measured raw values was observed for CL_{caff}, five-fold for CMR and twelve-fold for PCUR. As determined by *F*-tests for logarithmically transformed data, there was a significant difference between the variances of PCUR and of CMR ($p < 0.01$), while neither of these variances differed from that of CL_{caff} ($p > 0.05$).

CL_{caff}, CMR and PCUR correlated significantly with each other (Fig. 3) with $r = 0.77$ ($p < 0.001$) between CL_{caff} and CMR, $r = 0.46$ ($p < 0.01$) between CL_{caff} and PCUR, and $r = 0.40$ ($p < 0.02$) between CMR and PCUR. As established by a *z*-test, the correlation coefficients $r = 0.77$ and $r = 0.46$ were significantly ($p < 0.05$) different from each other, indicating that

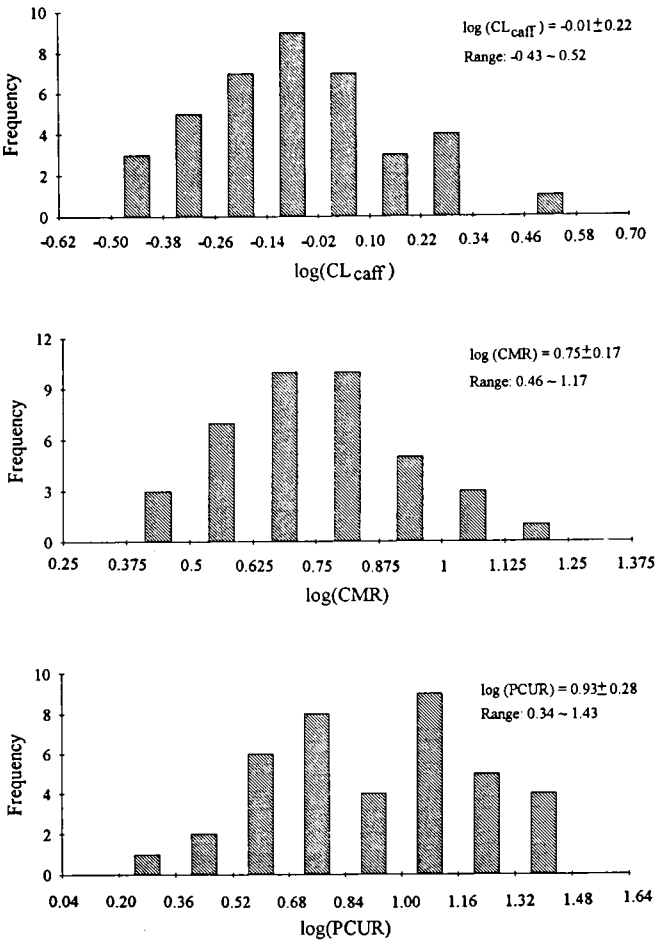

Fig. 2. Frequency distribution histograms of the following measurements in 39 subjects: Systemic caffeine clearance (CL_{caff}) ($ml\,min^{-1}\,kg^{-1}$) the caffeine metabolite ratio (AFMU + 1X + 1U)/17U in overnight urine (CMR), and the paraxanthine/caffeine urinary ratio (17X + 17U)/137X in urine voided 4–5 h after caffeine intake (PCUR). Mean, SD and range for logarithmically transformed values are indicated in each histogram with different scale of abscissa used for each plot.

CMR yielded a better estimate of clearance than did PCUR.

All three frequency distributions were transformed into probit plots on a logarithmic scale to improve the visual comparison of the three measurements (Fig. 4). The bimodality of PCUR is visible as an antimode present in the probit curve; furthermore, this curve is relatively more shallow than the other two.

The CL_{caff} of the eight female oral contraceptive users was significantly different from that of the twelve female non-users (Table 1). The clearance, half life and apparent volume of distribution for the eight female oral contraceptive users were $0.71 \pm 0.20\,ml\,min^{-1}\,kg^{-1}$, 7.26 ± 1.61 h, $0.43 \pm 0.12\,l\,kg^{-1}$, respectively. When CL_{caff}, CMR and PCUR were compared between users and non-users of oral contraceptives (OCs) (Table 1), the PCUR did not show the statistically significant difference recorded by the other two measures.

Excluding the eight oral contraceptive users, the clearance, half life and apparent volume of distribution were $1.16 \pm 0.49\,ml\,min^{-1}\,kg^{-1}$, 5.76 ± 2.46 h and $0.55 \pm 0.24\,l\,kg^{-1}$, respectively. The clearance values of these remaining 31 subjects showed neither a significant gender difference (1.22 ± 0.58 for male *vs* 1.07 ± 0.31 for female, $p > 0.3$) nor a significant ethnic difference (0.99 ± 0.31 for Chinese subjects *vs* 1.27 ± 0.57 for Caucasian subjects, $p > 0.05$).

Auxiliary observations

As pointed out in a recent review paper (Kalow & Tang, 1993), renal factors might play an important part in modulating the fate of caffeine and some of its metabolites *in vivo*. Therefore, we examined the relationship between PCUR and renal clearance of 137X. A significant correlation ($r = -0.47$) was obtained between these two parameters (Fig. 3); as pointed out above, the correlation between PCUR and systemic caffeine clearance was $r = 0.46$. It is evident that PCUR reflects not only liver enzyme activity but also renal function.

We therefore tested separately the distribution of the excretion rates of each of its three components, that is, of 137X, 17X and 17U. When their cumulative frequency distributions were transformed into probit plots, only the 137X data showed both the bimodal distribution and the shallow slope which characterize PCUR (Fig. 5A).

A significant correlation coefficient ($r = 0.82$, $p < 0.001$) (Fig. 6A) was obtained between caffeine concentrations in plasma and in urine with the slope of regression $b = 0.86$. This kind of observation was presumably the basis for developing the PCUR test. However, this relationship also implies substantial renal tubular reabsorption of 137X and almost complete equilibrium between 137X in plasma and urine as described by Birkett & Miners (1991). It follows, therefore, that the characteristics of distribution of the 137X excretion rate that we observed (Fig. 5) must be attributable to urine flow rate. (Since renal clearance of 137X is expressed as excretion rate of 137X over plasma caffeine concentration, renal clearance can be simplified as urine flow rate times a constant; it then is related only to the urine flow rate.)

This inference was supported by comparison of distributions of excretion rate and renal clearance of 137X with urine flow rate (Fig. 5A, B). The

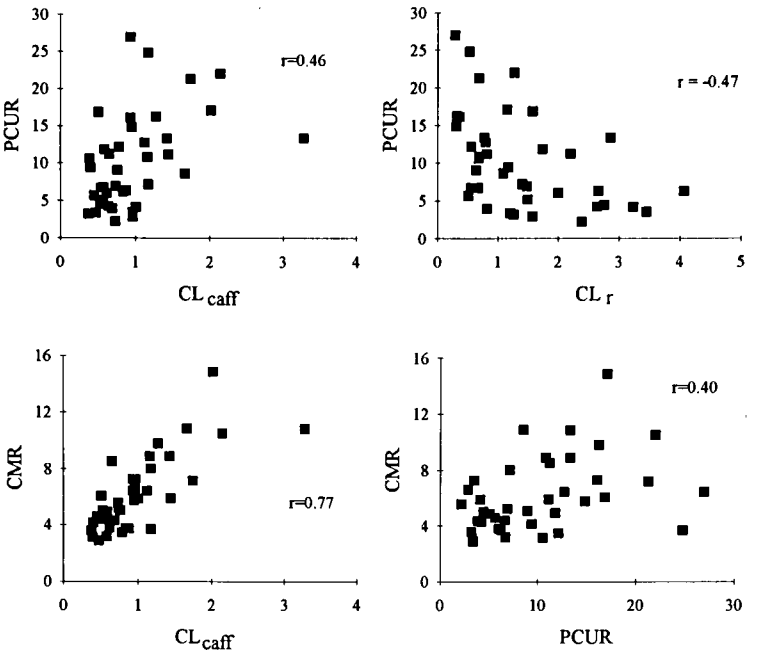

Fig. 3. Correlation plots between CL_{caff} (ml min^{-1} kg^{-1}), CMR and PCUR as well as renal clearance of 137X obtained from 4–5 h urine samples with entry of the correlation coefficients.

distributions seen for excretion rate and renal clearance of 137X were almost identical to that of urine flow rate which also showed the bimodality, consistent with a significant correlation between renal clearance and urine flow rate ($r = 0.77$, $p < 0.001$) (Fig. 6B). Therefore, urine flow rate could impose a multimodality on the excretion rate and renal clearance of 137X. The correlation between renal clearance of 137X and PCUR implied that the latter could also be bi- or trimodally distributed.

Discussion

The results shown in Figs 2 and 4 exhibit the same characteristics that had been observed during previous studies. The comparatively wide variation and the bi-

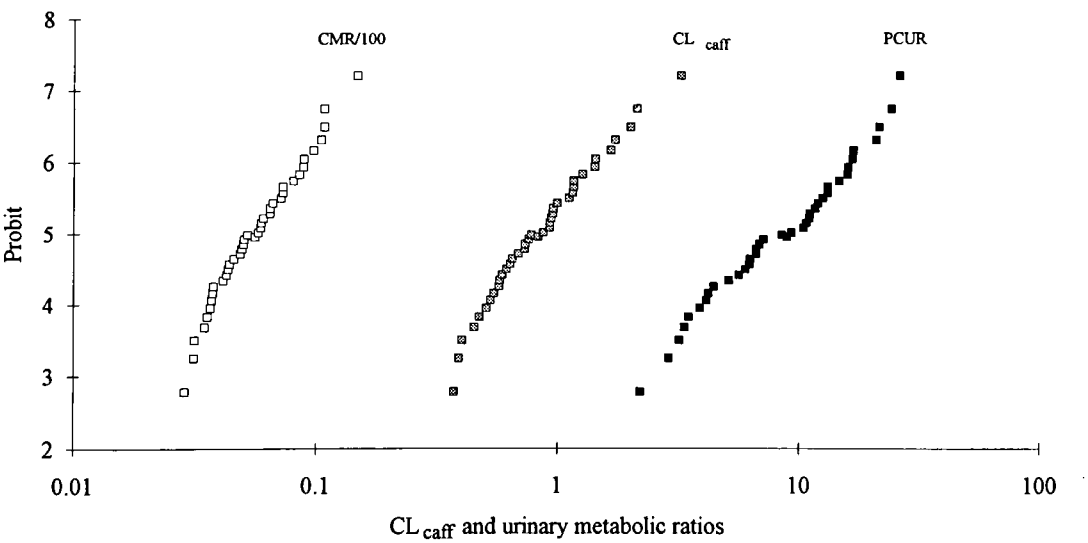

Fig. 4. Probit plots with a uniform logarithmic abscissa showing comparative features of CL_{caff} (ml min^{-1} kg^{-1}), CMR/100, and PCUR. CMR was divided by 100 in order to avoid visual overlap.

Table 1. *t*-test comparing oral contraceptive (OC) users and female non OC users with respect to CL_{caff}, CMR and PCUR

	Category	*n*	*Mean*	*SD*	*p*
CL_{caff}	OC user	8	0.71	0.2	$<0.01^a$
	Non OC user	12	1.07	0.31	
CMR	OC user	8	4.23	0.89	$<0.05^a$
	Non OC user	12	5.99	2.24	
PCUR	OC user	8	10.09	7.25	>0.7
	Non OC user	12	11.55	8.75	

[a]Significant with $p < 0.05$.

or trimodality of log PCUR were points emphasized by Butler *et al.* (1992). The notion of unimodal and normal distribution of log CMR is consistent with previous observations (Kalow & Tang, 1991a, b,

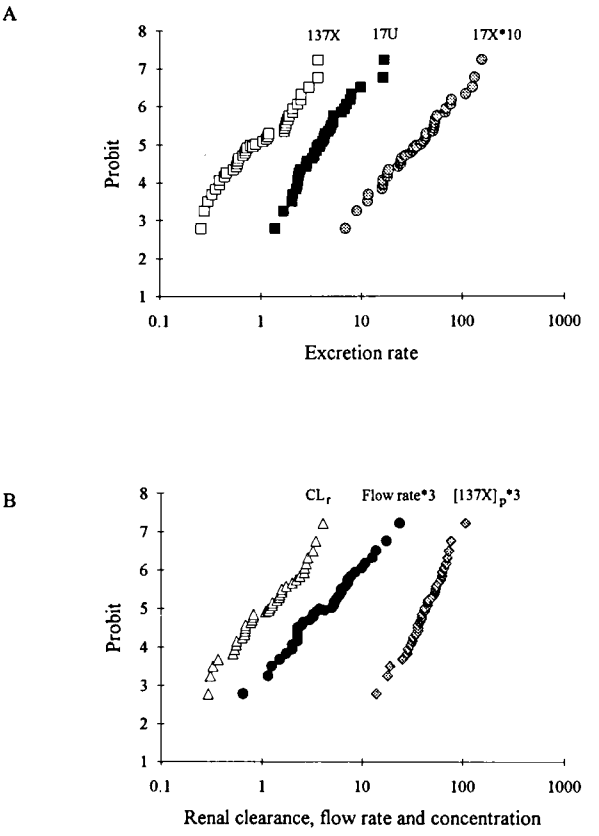

Fig. 5. (A) Probit plots using the equivalent logarithmic scaling of the abscissa as in Fig. 4, showing the distributions of excretion rates of 137X, 17X and 17U ($\mu mol\ h^{-1}$) during the 4–5 h period after 137X intake, and (B) the distribution profiles of renal clearance (CL_r) of 137X ($ml\ min^{-1}$), urine flow rate ($ml\ min^{-1}$) and plasma 137X concentration $[137X]_p$ (μM) derived from 4 h plasma sample after caffeine ingestion. In order to avoid visual overlap between curves, the 17X data in section 5(A), the flow rate and 137X concentration data in plasma in 5(B) were multiplied by 10, 3 and 3, respectively.

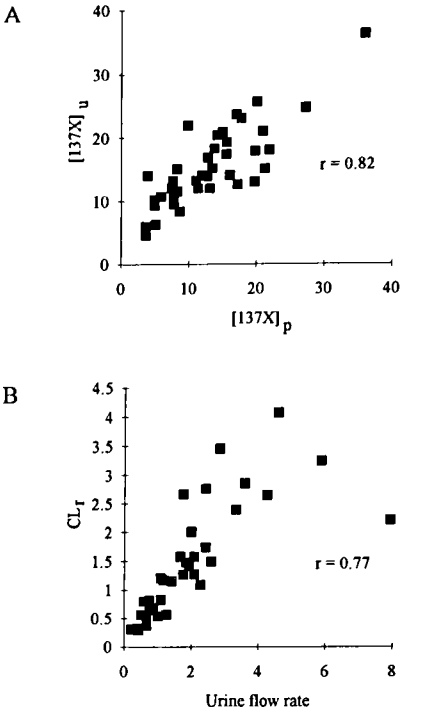

Fig. 6. Correlation plots between 137X concentration in 4–5 h urine sample $[137X]_u$ and in 4 h plasma sample $[137X]_p$ (A), between CL_r of 137X during 4–5 h and urine flow rate of that period (B), with entry of correlation coefficients.

Vistisen *et al.* 1992). In a previous study (Campbell *et al.*, 1987), the correlation between CL_{caff} and CMR was found to be $r = 0.91$; the smaller correlation in the current study is explained by the smaller range of CL_{caff} and CMR values due to the scarcity of smokers in the recently studied population. The dampening of the standard deviation of CMR compared with that of systemic caffeine clearance (see Kalow & Tang, 1993) follows from the fact that the denominator of CMR contains the metabolite 17U which is partly catalysed by CYP1A2 (Gu *et al.*, 1992).

The major question arising from the observations was how to explain the differences and poor correlations of PCUR with caffeine's systemic clearance and with CMR. The explanation turned out to be relatively simple: The appearance of caffeine in urine, the denominator of the PCUR equation, depends on both metabolism and renal function. The observation that a urinary index of enzyme activity can be distorted by kidney function calls for constant caution in developing methods that utilize urinary metabolites.

However, renal factors may not be the only cause of the poor correlation and the discrepancies between the frequency distributions of CL_{caff} and PCUR. The

involvement of CYP1A2 in the catalysis of both the 3-demethylation of 137X (caffeine) and the 7-demethylation of 17X (paraxanthine), may also, in part, be involved, i.e. CYP1A2 catalyses both formation and further metabolism of paraxanthine. A small fluctuation in the timing of the urine collection after caffeine ingestion could conceivably change the value of PCUR. The profiles of urinary metabolites are similar to those in plasma and the concentration-time profiles of 17X, 17U and 137X are not parallel (Bonati *et al.*, 1982; Tang-Liu *et al.*, 1983).

A difference between OC users and non OC users was expected as CYP1A2, and therefore caffeine elimination, is known to be inhibited by oral contraceptive drugs and other steroids (Patwardhan *et al.*, 1980; Balogh *et al.*, 1991; Lane *et al.*, 1992). This difference was shown by CL_{caff} and CMR but not by PCUR. This observation may be taken as an indication of the limited ability of PCUR to reflect the activity of CYP1A2 under different circumstances.

Twenty-four hour abstinence period for caffeine and methylxanthine containing food and drinks is deemed effective since all the subjects claimed to comply with the protocol. In addition, using 4 h blood sample as the first point to calculate the systemic clearance of caffeine, the remnant will be little and negligible after virtually 28 h, which is about 5–6 half lives of caffeine biotransformation *in vivo*.

In summary, two well-established caffeine-based urinary indices were compared in this study. CMR correlated significantly better with systemic clearance of caffeine than did PCUR. The frequency distribution of PCUR was bimodal or trimodal, in contrast to the normal distribution seen for both CMR and CL_{caff}. In addition, the failure of PCUR to distinguish enzyme activity levels in OC users from that in non OC users suggested that PCUR did not always adequately reflect CYP1A2 activity. The flow dependence of the renal excretion rate of caffeine, and most importantly of renal clearance, could be the major causes of the above mentioned differences. Also, PCUR must be extremely vulnerable to errors of the timing of urine sample collection. CMR did not correlate with systemic clearance of caffeine as highly as expected, nevertheless, it was a consistent and suitable probe for CYP1A2 activity (*).

*Occasionally, CMR has been used in form of two modifications: (AFMU + 1X + 1U)/17X designated CMR_{17X}, and (AFMU + 1X + 1U)/(17X + 17U) designated CMR_{17XU}. In this experimental series, the correlation coefficient of CL_{caff} with CMR_{17X} was $r = 0.65$, with CMR_{17XU} $r = 0.78$, and as recalled, with CMR was $r = 0.77$. We have omitted the use of paraxanthine (17X) in our CMR method because we observed earlier that its renal elimination was dependent on urine flow.

PG 4/3—B

Acknowledgement

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