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Evaluation of Caffeine as an In Vivo Probe for CYP1A2 Using Measurements in Plasma, Saliva, and Urine

Carrillo, Juan A.* ; Christensen, Magnus† ; Ramos, Sara I.* ; Alm, Christina† ; Dahl, Marja-Liisa† ; Benítez, Julio*; Bertilsson, Leif†

Author Information

Department of Pharmacology, Medical School, University of Extremadura. E-06071 Badajoz, Spain, †Department of Medical Laboratory Sciences and Technology, Division of Clinical Pharmacology at Karolinska Institute, Huddinge University Hospital. S-141 86 Huddinge, Sweden
Received July 19, 1999; accepted March 13, 2000.
Address correspondence and reprint requests to Juan Antonio Carrillo, M.D., Ph.D, Departamento de Farmacología y Psiquiatría, Facultad de Medicina. Universidad de Extremadura, Avda. de Elvas s/n, E-06071-Badajoz, Spain

Summary:

Twenty-five healthy volunteers were given 100 mg caffeine orally and several estimates of cytochrome P450 1A2 (CYP1A2) activity were evaluated. The validation was performed by correlation of different parameters in plasma, saliva, and urine to two measures of caffeine clearance, CL_{oral} and $CL_{137X->17X}$ that served as standards of reference. Two subjects were excluded because of noncompliance with a caffeine-free diet. In the remaining 23 subjects, both plasma and saliva total clearances of caffeine were highly correlated with each other ($r_s = 0.97$, $p < 0.0001$). The ratio 17X/137X restricted to one sampling point taken 4 hours after dose, showed a high correlation (r_s) with CL_{oral} and $CL_{137X->17X}$ in plasma (0.84 / 0.83) and saliva (0.82 / 0.77) ($p < 0.0001$ for all the correlation values) where 17X is 1,7-dimethylxanthine (paraxanthine) and 137X is 1,3,7-trimethylxanthine (caffeine). Additionally, the ratio (AFMU + 1U + 1X + 17U + 17X)/137X in a 0–24 hours urine sampling showed the highest correlation with $CL_{137X->17X}$ ($r_s = 0.85$, $p < 0.001$) where AFMU is 5-acetylamino-6-formylamino-3-methyluracil, 1U is 1-methyluracil, 1X is 1-methylxanthine, and 17U is 1,7-dimethyluric acid. The major estimates of CYP1A2 activity were significantly less in nonsmoking females, and this probably was related to the use of oral contraceptives in this subpopulation. In summary, among caffeine-based approaches for CYP1A2, the authors recommend either plasma or saliva 17X/137X ratio and the urinary (AFMU + 1U + 1X + 17U + 17X)/137X ratio during a sampling interval of at least 8 hours, starting at time zero since caffeine intake. These indices are simple, reliable, and relatively inexpensive estimates of CYP1A2 activity to be used in the study of human populations.

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Caffeine is one of the most widely and frequently ingested compounds throughout the world. Caffeine is predominantly eliminated via N3-demethylation to 1,7-dimethylxanthine (paraxanthine) (17X). This reaction accounts for 84% of primary caffeine demethylations and is specifically catalyzed by cytochrome P450 1A2 (CYP1A2) (1–3), one of the major cytochrome P450s (CYPs) in the liver, accounting for 15% of the total P450 content (4). Via an unknown intermediate, 17X is converted to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) by the polymorphic N-acetyltransferase type 2 (NAT2) (5). In addition, CYP1A2 also contributes to further degradation of 17X to 1,7-dimethyluric acid (17U) (hydroxylation) and to 1-methylxanthine (1X) (demethylation); the subsequent breakdown of 1X includes hydroxylation to 1-methyluracil (1U) by xanthine oxidase (XO) (6). Because so much is known about the association between caffeine and CYP1A2, caffeine has become popular as a metabolic probe for human xenobiotic metabolizing enzymes such as the aforementioned CYP1A2, NAT2, and XO (3,6). The human cytochrome P450 isoform CYP1A2 plays an important role in the activation of environmental procarcinogens to reactive intermediates that can ultimately trigger carcinogenesis (7,8). Besides caffeine, several clinically important drugs such as theophylline (9), imipramine (10), clomipramine (11), clozapine (12,13), fluvoxamine (14,15), olanzapine (16), tacrine (17), ropivacaine (18) and probably haloperidol (19) and thioridazine (20) are metabolized by this enzyme. Thus, interindividual variability in the CYP1A2 activity may influence individual drug response as well as susceptibility to certain types of chemically induced cancers. Hence, the availability of a reliable probe drug providing a measurement of CYP1A2 activity in vivo is of considerable interest. A number of caffeine-based methods have been proposed, but they have potential shortcomings that deserve further consideration (6,21,22). They include the use of the caffeine breath test, which depends on pulmonary excretion and measurement of ^{13}carbon dioxide following a test dose of caffeine labeled in the N3-methyl group (23). More conveniently, caffeine clearance in either plasma or saliva (as a noninvasive method) has been widely used (21,24). In fact, caffeine clearance is considered a “gold standard” of CYP1A2 activity in humans (6,22). An alternative approach is the use of caffeine urinary metabolite ratios for evaluating CYP1A2 activity, but the search of an optimal ratio is under debate (22,25,26).

To examine the validity and appropriateness of several caffeine-based methods for assessing CYP1A2 activity in plasma, saliva, and urine, we have dealt with those parameters that are commonly used in the literature and seem most adequate for metabolic purposes in large populations.

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SUBJECTS AND METHODS

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Subjects

The population consisted of 25 (12 men and 13 women) healthy volunteers. Among the men, there were eight nonsmokers and four smokers with an average consumption of 12 ± 5 cigarettes per day. Among the women, there were seven nonsmokers and six smokers with an average consumption of 12 ± 4 cigarettes per day. Mean (SD) age of the men was 44.1 (12.3) years (range, 28–68) and mean body weight was 83.7 ± 7.5 kg (range, 74–95). Mean (SD) age of the women was 42.7 (10.3) years (range, 26–59) and mean body weight was 64.5 ± 8.7 kg (range, 45–76). Two women (one nonsmoker and one smoker) were excluded from the evaluation of data because of noncompliance with a caffeine-free diet during the study. All subjects (n = 23) reported only occasional alcohol consumption and were not taking any medication at the time of the study, except three nonsmoking women who were using oral contraceptives (OCs). Thus, volunteer no. 8 used Trionetta (ethinylestradiol 30 or 40 µg + levonorgestrel 50 or 125 µg), no. 15 used Mini-Pe (norethisterone 0.35 mg), and no. 21 used Trivina (estradiol 2 mg + medroxyprogesterone 20 mg). They were otherwise healthy as assessed by a thorough physical examination, ECG, urinalysis, and clinical laboratory panels for chemistry and hematology before admission to the study. They were aware of the purpose and gave informed consent for the study, which was approved by the Human Research Ethics Committee at Huddinge University Hospital, Huddinge, Sweden.

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Study Procedure

Subjects refrained from consumption of methylxanthine-containing foods and beverages for at least 36 hours before and throughout the study. However, because certain subjects who claim to follow a caffeine-free diet do in fact have measurable levels of caffeine (27), blank samples of plasma, saliva, and urine were collected immediately before the administration of the probe drug. These were used to detect compounds that might interfere with the analysis and to evaluate compliance with the caffeine-free diet. After intake of a single 100-mg oral dose of caffeine (Koffein, ACO AB, Helsingborg, Sweden) at 8 AM, blood samples were drawn into Venoject tubes (Terumo Europe NV, Leuven, Belgium) at 1, 2, 3, 4, 6, 8, 10, 24, 32, and 48 hours after dose. The plasma was separated by centrifugation and stored frozen at -20°C until analysis. After caffeine intake, nonstimulated saliva samples were collected at 1, 2, 3, 4, 6, 8, 10, 12, 14, 24, 32, and 48 hours and stored at -20°C until analysis. All urine was collected fractionated in 0–4, 4–8, and 8–24 hours after caffeine intake. A single spot sample was also taken between 4 and 5 hours. Urine volumes were recorded, and to ensure the stability of AFMU (28), the pH of each urine sample was adjusted to 3–3.5 by addition of 5 mol HCl. Aliquots of urine were frozen at -20°C until assay.

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Determination of Caffeine and Metabolites in Plasma and Saliva

Plasma and saliva concentrations of caffeine and its metabolites were measured in one run and in duplicate by a high-performance liquid chromatography (HPLC) method as follows: plasma (500 µL) or saliva (100 µL) was extracted with 4 mL chloroform/isopropanol after addition of 1.5 µg/50 µL internal standard (N-acetyl-p-aminophenol) and 120 mg ammonium sulfate. The organic phase was evaporated to dryness at 45°C under nitrogen. After reconstitution of the residue in 100 µL mobile phase, 50 µL was injected onto an Ultrasphere ODS (75 × 4.6 mm ID, 3 µm, Beckman Instruments, Madrid, Spain) reversed-phase column that was eluted isocratically with acetic acid/acetonitrile/tetrahydrofuran/water (1:30:10:959 v/v). The flow rate was 1.0 mL/min and the compounds were monitored at 280 nm. Calibration curves were linear (coefficients of correlation > 0.99) within the concentration range 0.15–55 µmol. The lowest limit of detection was 0.05 µmol, and the interassay coefficients of variation (CV) were more than 9.5% for all the compounds. The accuracy was between 97% and 103%.

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Determination of Caffeine and Metabolites in Urine

The urinary concentrations of caffeine and metabolites were analyzed by HPLC in a similar manner as for plasma and saliva. Caffeine and its metabolites were extracted simultaneously with 4 mL chloroform/isopropanol after addition of 6 µg/50 µL internal standard (N-acetyl-*p*-aminophenol) and 120 mg ammonium sulfate. The organic phase was evaporated to dryness at 45°C under nitrogen. After reconstitution of the residue in 200 µL mobile phase, 50 µL was injected onto a Ultrasphere-IP (5 µm particle size, 250 × 4.6 mm ID) reversed-phase column (Beckman Instruments, Madrid, Spain) that was eluted isocratically with acetic acid/acetonitrile/tetrahydrofuran/water (1:40:2:957 v/v). The flow rate was 1.0 mL/min and the detection wavelength was 280 nm. Standard curves were made in the range 5.5–221 µmol for AFMU and 0.48–271 µmol for the remaining compounds ($r > 0.99$ for all substances). The limit of detection was 1 µmol for AFMU and 0.1–0.3 µmol for caffeine and the remaining metabolites. The CV was less than 12% at all concentration levels and for all metabolites. The accuracy was between 95% and 106%.

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Equipment

The HPLC equipment for the caffeine assays consisted of a model 126 solvent delivery module, a model 168 diode array detector and a model 507e automatic injector (all from Beckman Instruments, Madrid, Spain). “System Gold” HPLC software (version 8.1) (Beckman Instruments, Madrid, Spain) was used to operate modules and facilitate data management.

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Outline

Pharmacokinetic Analysis of Caffeine in Plasma and Saliva

The following pharmacokinetic parameters were calculated by noncompartmental analysis in plasma and saliva: peak plasma concentration (C_{max}), time to reach C_{max} (t_{max}), area under the plasma or saliva concentration–time curve from zero to infinity ($AUC_{0–[iota]}$), apparent half-life ($t_{½}$) and volume of distribution (Vd). The apparent caffeine clearance (CL_{oral}) was calculated as dose/($AUC_{0–[iota]}$), in which the dose is expressed per kilogram of body weight. The partial caffeine clearance ($CL_{137X->17X}$) via N3-demethylation (17X formation) was approximated by use of the equation (17X + 17U + 1X + 1U+AFMU)/$AUC_{137X,0–24}$ in which the numerator represents the total amount of paraxanthine and its metabolites in urine collected for 24 hours; $AUC_{137X,0–24}$ is the corresponding AUC of caffeine in plasma.

Other apparently simpler estimates used for the assessment of the CYP1A2 activity were the calculation of systemic clearance based on five-point caffeine concentrations from 2–8 hours in plasma, and from 3–10 hours in saliva, CL (2,8 h) and CL (3,10 h) respectively. A plasma and saliva ratio 17X/1,3,7-trimethylxanthine (caffeine) (137X) taken 4h postdose was also calculated.

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Data in Urine

Cumulative excretion (µmol) in the 0–24 hours sampling interval was estimated from urine 17U and caffeine concentrations (µmol · L^{-1}) at different time points (4, 8, and 24 hours) multiplied by urine volume (L) of the respective periods.

Likewise, several urinary caffeine metabolite ratios obtained at different sampling intervals (0–4, 0–8, 8–24 and 0–24 hours) as well as a spot sample between 4 and 5 hours postdose, were evaluated. They are thought to reflect CYP1A2 activity and thereby, commonly used in the literature:

Ratio 1: (AFMU + 1X + 1U)/17U (29)

Ratio 2: 17X/137X (8)

Ratio 3: (17U + 17X)/137X (30)

Ratio 4: (AFMU + 1U + 1X + 17U + 17X)/137X modified from Carrillo and Benítez (31)

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Data Analysis

Data are mean ± SD. Pharmacokinetic data were calculated for caffeine and metabolites using the computer-based "WinNonlin" program (Scientific Consulting, Apex, NC, USA). The existence of metabolic differences in the population was tested with the use of a nonparametric Wilcoxon rank sum test. The correlation studies were performed by the Spearman's rank correlation test. A p value less than 0.05 was regarded as statistically significant. The statistical analysis was carried out using the JMP program package, version 3.1 (SAS Institute, Cary, NC, USA).

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RESULTS

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Pharmacokinetics of Caffeine in Plasma and Saliva

Table 1

Table 2


Fig. 1

The pharmacokinetics of caffeine after a single 100-mg oral dose to 23 healthy volunteers was evaluated in plasma and saliva (Fig. 1 and Table 1). The pharmacokinetic parameters closely resembled each other, and all the correlation indices were significant ($p < 0.0001$ for all the correlation values) (Table 2). Plasma and saliva caffeine CL_{oral} correlated with each other (r_s = 0.97). The standards of reference to validate CYP1A2, total clearance (CL_{oral}) and partial clearance via N3-demethylation ($CL_{137X->17X}$), showed a high coefficient of correlation (r_s = 0.78) (Table 2). The coefficients of correlation (r_s) with CL_{oral} and $CL_{137X->17X}$ were 0.99 / 0.77 and 0.97 / 0.77 for CL (2,8 h) and CL (3,10 h), respectively. CL_{oral} and $CL_{137X->17X}$ also showed a high correlation with the ratio 17X/137X restricted to one sampling point taken 4 hours after dose, in both plasma (0.84 / 0.83) and saliva (0.82 / 0.77) (Table 2). At all times considerably higher plasma concentrations of caffeine were seen in nonsmoking women than in the other subjects (Fig. 1). The limit of determination of plasma caffeine concentration was reached at approximately 32 hours, except for the nonsmoking women, for whom it was noticeably later. Table 1 shows that, compared with smoking women, nonsmoking women had an approximately 2.5 times longer $t_{½}$, a threefold greater $AUC_{0–[iota]}$, and 3.5 times less CL_{oral} of caffeine, all of which were statistically significant. Among nonsmokers, women had an approximately 1.5 times greater maximum concentration, a twofold greater $AUC_{0–[iota]}$, and 1.7 times less CL_{oral} of caffeine than men, all of which were statistically significant. However, no gender-related difference was seen among smokers (Fig. 1 and Table 1).

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Caffeine Urinary Metabolite Ratios

Four different caffeine urinary metabolite ratios (ratio 1->4) were determined within 0–4, 0–8, 8–24, and 0–24 hours after caffeine intake. A spot sample was also taken between 4 and 5 hours after dose. The ratios 1 (AFMU + 1X + 1U)/17U from 8–24 hours and 4 (AFMU + 1U + 1X + 17U + 17X)/137X from 0–8 hours and from 0–24 hours consistently correlated with standards of reference, CL_{oral} and $CL_{137X->17X}$ (Table 3). A comparison of ratio 1 (sampling interval from 8–24 hours) and ratio 3 (spot sample 4–5 hours) yielded a coefficient of correlation of 0.35 ($p > 0.1$). Ratio 4 was highly discriminative at all sampling intervals, and considerably lesser values were found in nonsmoking women when compared with smoking women ($p < 0.05$) and nonsmoking men ($p < 0.05$) (Table 4). Unlike 17U, the cumulative urinary amount (μmol) of caffeine showed a more discriminative pattern of excretion in the different subgroups of the population.


Table 3

Table 4

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DISCUSSION

Probe substrates for CYPs and other enzymes are widely used to assess genetic, environmental, and ethnic differences in the in vivo metabolism of drugs and environmental chemicals. The value of such probes depends on the experimental parameter used to determine the enzyme activity. It has been shown that the high-affinity component of phenacetin O-deethylation is entirely attributable to CYP1A2 (1). As there are ethical problems in giving phenacetin to human populations, today other probe drugs have been considered for in vivo CYP1A2 measurements (6). It is generally accepted that the N3-demethylation of caffeine is the model of choice for assessing CYP1A2 activity in humans in vivo (6), but there is no consensus for accepting a unique, reliable, and simple method. The caffeine breath test has been shown to be useful, but the requirement of labeled caffeine and the need for specialized and expensive equipment for measuring the labeled carbon dioxide in exhaled air are drawbacks (6,23,32). Moreover, approximately one third to one half of the ingested label is lost in transit through the one-carbon pool (6).

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Pharmacokinetics of Caffeine in Plasma and Saliva

Because CYP1A2 activity accounts for more than 95% of the primary caffeine metabolism (3,33), quantitation of total and partial clearances (by 17X formation) of caffeine in plasma are offered as generally accepted standards for validating estimates of CYP1A2 (6). Because the various pharmacokinetic parameters in this study consistently correlated with each other, both biologic fluids plasma and saliva seem to be adequate for evaluating the CYP1A2 metabolic activity using caffeine as a probe drug. Saliva is a noninvasive method. Thus, it has received considerable attention in the literature as an alternative to plasma (34,35). However, gingival contamination is responsible for the higher caffeine concentrations in saliva samples collected earlier than 2 hours after caffeine intake (36). Thus, we have evaluated saliva samples taken no earlier than 2 hours after caffeine intake. In our study, total (CL_{oral}) and partial ($CL_{137X->17X}$) clearances of caffeine showed a significant correlation with two estimates based on five-point concentrations of caffeine in plasma (CL 2,8 h) and saliva (CL 3,10 h) (Table 2). However, collection of repeated samples from each subject for the calculation of systemic clearance is always cumbersome. Hence, it was intriguing to find that the plasma and saliva 17X/137X ratio restricted to one sampling point taken 4 hours postdose in plasma and saliva correlated with the standards of reference ($r_s > 0.77$, $p < 0.0001$) (Table 2). The usefulness of the 17X/137X ratio 3–7 hours postdose has already been reported (24). We found pronounced differences in caffeine metabolism in our population (Table 1, Fig. 1). Although we cannot rule out smoking or sex-related differences in the metabolism of caffeine as shown with other CYP1A2 substrates such as phenacetin (37) and clozapine (38–40), several investigations (41–46) have shown that women who regularly use OCs have a CYP1A2-mediated impairment of caffeine metabolism. In our study, three nonsmoking women reported use of OCs. The presence of ethinylestradiol (47,48) or estradiol (49,50) in OCs seems to account for the inhibition mechanism, but it is not clear whether this is the result of competition or downregulation of the enzyme synthesis (50). Unfortunately, there were too few subjects to consider further stratification into possible subgroups for a more detailed analysis.

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Caffeine Urinary Metabolite Ratios


Fig. 2

Caffeine urinary metabolite ratios have received considerable attention as a relatively simple and noninvasive method to determine the CYP1A2 metabolic activity in large populations (26,29,31,45). Several caffeine urinary metabolite ratios have been proposed as probes for in vivo CYP1A2 activity. However, the results obtained are sometimes contradictory, which raises the question whether they accurately reflect the CYP1A2 activity in humans (6,21,22,24–26). We have evaluated those ratios that have been considered in most publications (26,29–31,45,51). The ratio (AFMU + 1X + 1U)/17U was first ratio suggested as a CYP1A2 index (29). In agreement with data obtained by other authors (23,52), we have found that this ratio was consistently correlated with measures of caffeine clearance in the sampling interval from 8–24 hours. However, the dampening of ratio 1 compared with that of systemic caffeine clearance (6) stems from the fact that the denominator contains the metabolite 17U, which is partly formed by CYP1A2. Although this ratio does correlate to caffeine clearance after single doses (29), it may lose sensitivity in its ability to reflect caffeine clearance and CYP1A2 activity when this enzyme is induced, i.e., by smoking (6). This means that large changes in CYP1A2 function would not be reflected in the ratio 1 in the same magnitude. Also, this ratio showed a poor correlation to the systemic clearance of caffeine when high doses of caffeine were used (26). On the other hand, the ratio 2 17X/137X and ratio 3 (17X + 17U)/137X have been recommended for use in urine collected between 4 and 5 hours after intake of a standard dose of caffeine (8,30). This is based on the measure of paraxanthine excretion at the point when its rates of formation and elimination are similar. However paraxanthine is both a product and substrate of CYP1A2, and small fluctuations in the sampling time after caffeine ingestion appear to affect the values of these indices (52). Unlike other measures of CYP1A2 activity, the ratio 17X/137X (8) was not affected by OCs, which indicates its limited value as an index of CYP1A2 activity (52). The correlation coefficients between ratio 2 and ratio 3 and the standards of reference showed lesser values than those of ratio 1 and ratio 4 (Table 3). Similar to what has been reported by other authors (52), a low correlation coefficient of 0.35 ($p > 0.1$) was found in a comparison study of ratio 1 (sampling interval from 8–24 hours) and ratio 3 (spot sample between 4 and 5 hours). The ratio 4 (AFMU + 1U + 1X + 17U + 17X)/137X, uses all secondary, tertiary, and quarternary caffeine metabolites of the parent drug, making it conform closely to the theoretical ratio of drug:metabolite(s) involved in the major and CYP1A2-dependent metabolic pathway of caffeine. Because 17U is partly formed by CYP1A2, we believe that the inclusion of this metabolite in the numerator of ratio 4 gives a better approach to CYP1A2 activity. Also, a long sampling period from 5–24 hours is recommended (23). Likewise, it is essential to take the first hours after caffeine intake into account because of the inclusion of caffeine in the denominator. Moreover, ratio 4 in the sampling intervals from 0–8 hours and from 0–24 hours showed the best correlation with CL_{oral} and $CL_{137X->17X}$ (Table 3). On the other hand, we have found a high correlation between this caffeine urinary ratio and several markers of the metabolism of the CYP1A2 substrate clozapine (12,53). Furthermore, ratio 4 was highly discriminative with regard to the existence of metabolic differences in the population (Table 4). This could be related to the fact that ratio 4 contains caffeine in the denominator, which also showed a more discriminative pattern of urinary excretion in our population than 17U (Fig. 2). Because most of the ratios showed lesser values in nonsmoking women (Table 4), the aforementioned considerations about the use of OCs and the existence of a CYP1A2-mediated impairment of caffeine metabolism may apply here.

In summary, the clinical importance of cytochrome P450 1A2 (CYP1A2) in the metabolism of xenobiotics is increasingly acknowledged. The CYP1A2 cytochrome shows a large variability in humans. Therefore, it may influence individual drug response as well as susceptibility to certain types of chemically-induced cancers. Caffeine is metabolized by this enzyme to a large extent; thus, a number of parameters related to caffeine metabolism have been used as estimates of CYP1A2 activity in vivo. Pharmacokinetics of caffeine in plasma and saliva correlated with each other in our study. Among indices that appear to be relatively simple and inexpensive to measure, we recommend the plasma or saliva 17X/137X ratio determined 4 hours after dose and the urinary ratio (AFMU + 1U + 1X + 17U + 17X)/137X in a sampling interval of at least 8 hours, starting at time zero since caffeine intake. The existence of pronounced differences in the metabolism of caffeine and hence, in CYP1A2 activity, as reflected by pharmacokinetic parameters and urinary ratios could be related to the use of OCs in the population.

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Key Words: Caffeine; Cytochrome P450; CYP1A2; Pharmacokinetics

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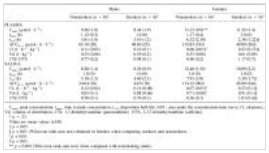

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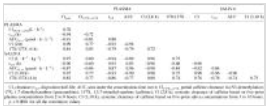

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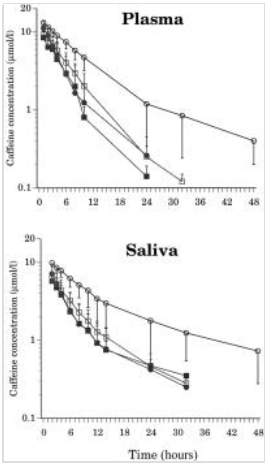
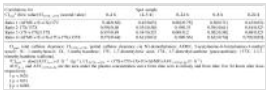


Fig. 1



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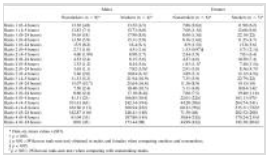

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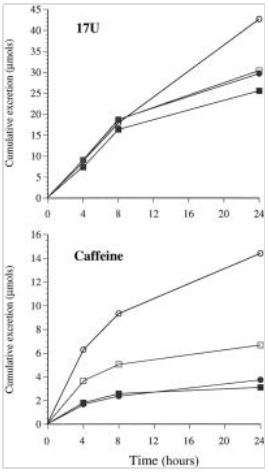

Fig. 2

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