Analysis of Within-Subject Variation of Caffeine Metabolism When Used to Determine Cytochrome P4501A2 and N-Acetyltransferase-2 Activities

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Abstract
Cytochrome P4501A2 (CYP1A2) and N-acetyltransferase-2 (NAT2) are hepatic enzymes that may activate some procarcinogens. Previous reports have determined CYP1A2 and NAT2 phenotypes by quantitating relative amounts of urinary caffeine and metabolites. However, a number of experimental issues with this approach remain. To address these, we measured caffeine and 4 metabolites in urine samples from 20 healthy volunteers on 3 separate occasions at 7-day intervals. Two additional volunteers were studied to measure the pattern of excretion of these analytes in urine over time. The molar ratio of two compounds (1,7-dimethylxanthine/1,3,7-trimethylxanthine) was used to phenotype CYP1A2, while the molar ratio of two other compounds (5-acetylamino-6-formylamino-3-methyluracil/1-methylxanthine) served to phenotype NAT2. Within-subject variation was less than 25% for most participants. In instances when within-subject variation of the metabolic ratio was >25%, metabolite peaks were usually present in one or more control urine samples. Some caffeine metabolites were observed in urine samples at detectable levels up to 48 h after caffeine ingestion. We conclude that: (a) this assay for determining CYP1A2 and NAT2 activities (phenotyping) has an acceptably low within-subject variation over 3 consecutive weeks for most subjects who were caffeine-free for 36 h prior to study; (b) collecting and analyzing urine samples prior to testing can indicate if subjects are excreting caffeine metabolites and will aid in locating metabolite peaks on chromatograms; (c) refraining from caffeine for 48 h before testing is the best compromise between convenience for the subject and obtaining reproducible results; (d) determining metabolite molar ratios in urine collected 4–5 h after ingesting caffeine provides an acceptable time for the measurement; and (e) different ratios of metabolites for determining CYP1A2 phenotype have different advantages.

Introduction
CYP1A2 and NAT2 are hepatic enzymes that have been implicated in the biotransformation of some procarcinogens into carcinogens. Individuals who are extensive metabolizers of substrates of CYP1A2 (e.g., oxidative demethylation of caffeine) or of substrates of NAT2 (e.g., N-acetylation during caffeine metabolism) are suspected of having increased risk for development of some types of urinary bladder and colorectal cancers. This increased risk may be due to increased activation of amines present in cigarette smoke, coal- and shale-derived oils, and agricultural chemicals, or to increased activation of heterocyclic amines present in some cooked foods (1–7). Conversely, poor metabolizers of NAT2 may be at risk of developing some types of urinary bladder cancer(s) if they are slow to inactivate dietary carcinogens (3, 4). Several research groups have described an assay in which the metabolism of the probe drug caffeine by CYP1A2 and NAT2 is measured by HPLC to quantitate urinary caffeine and caffeine metabolites. Measured molar quantities of these analytes are then used to generate molar ratios (metabolic ratios) which can be used to classify human phenotypes for CYP1A2 and NAT2 (2, 7–10). Caffeine is a convenient substrate for phenotyping because it is metabolized in the liver primarily by CYP1A2 and NAT2, and it is widely used, relatively safe, and inexpensive (2, 4, 8, 11).

However, when we reviewed previous work in this field, we found that several issues remained unsettled or incompletely defined: (a) an optimal combination of solid and mobile phases for the best separation and quantitation of peaks of interest was not evident. Some previous papers did not include actual chromatograms or indicate detector sensitivity (1–3, 11, 12), while others included chromatograms but did not completely describe chromatographic conditions such as detector sensitivity (4–7, 9), thus making it difficult to compare new results with data obtained by other groups; (b) no within-subject variation in phenotyping results was presented in some publications (1–3, 6, 8, 11, 12), while in others, preliminary or variable estimates were reported (4, 7, 9, 10); (c) detailed descriptions of the patterns of excretion of caffeine and its metabolites over time were not provided (1–4, 6, 7, 9–11) or...
were presented in preliminary fashion (8, 12). This lack of experimental data made it difficult to formalize a procedure that could be readily reproduced from one laboratory to another. For example, some groups reported the monitoring of subjects in clinical research centers where they were kept caffeine-free for up to 72 h prior to study, while in other protocols, participants were studied as outpatients who refrained from caffeine for just 12 h prior to study (4, 7–11); and in previous studies different molar ratios for phenotyping CYP1A2 activity [e.g., 17X/137X, (17U + 17X)/137X] (2, 4, 7, 10, 12) were used and it was not clear which ratio might be technically most feasible, and which would result in the lowest within-subject variation.

These four issues were addressed in the following ways: (a) several different combinations of columns and mobile phases/gradients were tested until near-baseline separation of analyte peaks of interest was achieved. We have included actual chromatograms and detailed chromatographic conditions to allow comparison of our results to those obtained in other laboratories; (b) 20 healthy volunteers (10 women and 10 men) were studied once a week for 3 consecutive weeks to determine within-subject variation of CYP1A2 and NAT2 phenotypes (for this portion of the study we assumed that all subjects should be caffeine free for 36 h, and that an optimal interval for urine collection was 3–5 h after the dose of caffeine); (c) the pattern of excretion of caffeine and its metabolites was studied over 48 h in 2 healthy male volunteers to address the issues of optimal washout period and optimal time for collection of urine samples; and (d) we determined the phenotype of CYP1A2 in 9 of the women using both 17X/137X and (17U + 17X)/137X as molar ratios and compared the results. By addressing these four issues, we provide additional data that may help other groups implement this technique in a reproducible manner.

Materials and Methods

Protocol for Determining Within-Subject Variation. After receiving permission from our Committee for the Protection of Human Subjects, 20 healthy volunteers (10 women and 10 men; 22–61-years-old) who worked in various capacities within the medical center were recruited for this study. All participants were Caucasian nonsmokers, with no prior histories of caffeine sensitivity, kidney problems, or liver disease. The current medications of each subject were recorded, but we did not track current diets and intercurrent illnesses.

Each volunteer refrained from consuming caffeine-containing foods and beverages for 36 h before each study morning (3 mornings, 1 week apart). Volunteers were also instructed not to ingest acetaminophen on the night before or during each study day because this compound was used as the internal standard. Following an overnight fast of approximately 10 h, each subject discarded her or his first morning urine and drank one glass of water at home. After arriving at the study site at 8:00 a.m., the total second morning urine was collected and the volume was recorded. A 9-ml aliquot of the total urine sample was saved, and the remaining urine was discarded. After consumption of coffee and one or two donuts, the volunteers urinated and samples were obtained at hourly intervals for 8 hours. Drinking water every hour was encouraged, and foods and beverages containing caffeine were allowed after 1:00 p.m. Twenty-three and 47 h after ingesting caffeine, the two subjects voided and discarded their urine. At 24 and 48 h after caffeine consumption, the subjects urinated and the total volumes were measured, and an aliquot of each urine sample was saved.

Chemicals. 1,3,7-Trimethylxanthine (caffeine), 1,7-dimethylxanthine, 1,7-dimethyluric acid, 1-methylxanthine, and NAPAP (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO); ammonium sulfate, hydrochloric and acetic acids, and isopropyl alcohol were purchased from Fisher Scientific (Fair Lawn, NJ); chloroform was purchased from Baxter Diagnostics, Inc. (McGaw Park, IL); AFMU was a gift received from Drs. John Richie and Wayne Kleinman (Division of Nutritional Carcinogenesis, American Health Foundation, Valhalla, NY).

Urine Processing and Extraction. Urine samples were processed and extracted using a protocol modified from Butler et al. (4) and Grant et al. (8). Immediately following collection, 9-ml urine samples were transferred to 10-ml polypropylene tubes (Baxter Diagnostics, Inc.) and the pH of the urines was lowered to 3.5 with approximately 75–150 μl of 6N HCl. Samples were then stored at –70°C until later analysis.

Prior to extraction, a frozen urine sample was thawed at room temperature in low light, vortexed, and then centrifuged for 5 min at 2500 rpm. The supernatant was transferred to a clean 10-ml tube, and a 200-μl sample was added to another 10-ml tube which contained approximately 120 mg ammonium sulfate. After removing the 9-ml urine sample in the freezer, the 200-μl aliquot was vortexed for 2 min, and 50 μl of internal standard solution [125 μg/ml (0.827 mm) NAPAP in 0.05% acetic acid] were added. The sample was then vortexed briefly, followed by the addition of 6 ml of chloroform/isopropyl alcohol (19:1; v/v), vortexing for 1 min, and centrifugation for 5 min at 2500 rpm. A
5.5-mL sample of the lower (organic) phase was removed, transferred to a clean 6-mL glass tube, and evaporated to dryness under a gentle stream of nitrogen in a 40°C water bath. The dry extract was resuspended in 500 µL of 0.05% acetic acid and stored at room temperature in low light, pending analysis.

HPLC. HPLC analysis was performed with a Waters model 680 gradient controller driving two Waters Model 510 solvent delivery systems (Waters Chromatography Division, Millipore Corp., Milford, MA). Sample injections, usually 20 µL, were introduced via a Rheodyne Model 7125 injector equipped with a 100-µL loop, which was connected to a Brownlee RP-18 guard column (5 µm material; 4.6 mm x 3 cm) and a Microsorb RP-18 HPLC column (5 µm material; 4.6 mm x 25 cm; all purchased from Rainin Instrument Co., Woburn, MA). A Beckman Model 160 UV detector (Fullerton, CA) with a 280-nm filter and a sensitivity of 0.02 absorbance unit at full scale, and a Hewlett-Packard Model 3390A recorder-integrator (Wilmington, DE), were used to detect analytes and plot/integrate peaks.

The mobile phase consisted of a linear gradient over 40 min, with solvent A consisting of 0.05% acetic acid and solvent B consisting of 100% methanol alcohol. The gradient profile, which began with 90% A:10% B, was changed in a linear fashion to 50% A:50% B over 40 min, with the flow rate remaining fixed at 1.0 ml/min. Between runs, initial conditions were maintained for 15 min to reequilibrate the HPLC column.

Data Interpretation. The amount of each analyte (in pmol) was calculated by using a method of external standards. Standard curves (pmol injected versus peak area) for each analyte of interest were established each day by injecting known volumes of a standard "sextet" solution of analytes of interest [1.39 µg/mL (6.15 µM) AFMU-0.87 µg/mL (5.24 µM) 1X-2.61 µg/mL (17.3 µM) NAPAP-1.30 µg/mL (6.63 µM) 17U-1.58 µg/mL (8.77 µM) 17X-0.87 µg/mL (4.48 µM) 137X in 0.05% acetic acid] onto the HPLC. A typical day consisted of injecting 20 µL of sextet, 20 µL of each extracted urine sample, 20 µL of NAPAP solution [12.5 µg/mL (82.7 µM) in 0.05% acetic acid], and 50 µL of sextet. To eliminate between-day variation, all six urine samples from each subject were analyzed on the same day.

The addition of the internal standard [50 µL of 125 µg/mL (0.827 µM) NAPAP in 0.05% acetic acid] to the 200-µL urine aliquot prior to extraction served two purposes. The internal standard was an accurate and reproducible time reference point which assisted in the location of caffeine and metabolite peaks and was also used to calculate the percentage recovery of the entire extraction procedure. The theoretical 100% recovery of internal standard was determined as follows: 6.25 µg NAPAP were added to each 200-µL aliquot of urine following ammonium sulfate treatment. After evaporation over nitrogen, each sample was resuspended in 500 µL of 0.05% acetic acid, and 20 µL of the latter was injected; thus, the total amount injected represented (20 µL/500 µL) x 6.25 µg, or 0.25 µg (1.65 nmol) of NAPAP. Complete recovery of internal standard was defined as the peak area obtained when 0.25 µg (1.65 nmol) of NAPAP was injected on the chromatograph [20 µL of 12.5 µg/mL (82.7 µM) NAPAP in 0.05% acetic acid].

Amounts of caffeine and caffeine metabolites (in pmol) were calculated using the sextet-derived standard curves, molar ratios were calculated using Microsoft Excel version 4.0 (Microsoft Corp.), and graphs were constructed using Cricket Graph version 1.2 (Cricket Software, Malvern, PA).

Results

Optimization of Chromatographic Conditions and Showing Representative Chromatograms. The optimal length of the reversed-phase HPLC column to separate caffeine, its metabolites, and the internal standard were investigated. A 25-cm column was superior to a 15-cm column from the same manufacturer (Rainin) and was necessary to provide near-baseline separation of caffeine metabolites from other UV-absorbing peaks in human urine. We also determined that metabolite separation is comparable when using many different guard columns from the same manufacturer (Brownlee). A number of different gradient profiles were also tested for optimization of separation of these substances. The final conditions were a linear gradient from 90% 0.05% acetic acid:10% methyl alcohol (9:1) to 50% 0.05% acetic acid:50% methyl alcohol (1:1) over 40 min, with a flow rate of 1.0 ml/min. The total run time (including washout and column reequilibration) was approximately 50 min.

Fig. 1a shows a typical chromatographic separation of the standard sextet of caffeine metabolites. The approximate elution times for the analytes were: AFMU, 5.10 min; 1X, 11.4 min; NAPAP, 12.0 min; 17U, 16.0 min; 17X, 18.6 min; and 137X, 26.5 min. Standard curves of pmol injected versus peak area units for each of the analytes had typical r² values above 0.998 (data not shown). Extraction efficiency of the internal standard, measured in extracts from 115 urine samples, was 86.9% (+6.3%). As described previously by Butler et al. (4), some small endogenous peaks (<12% full-scale height of chromatogram) were periodically observed coeluting with analytes of interest. Small differences in elution times were also observed for some analytes, but this could probably be accounted for by ambient temperature fluctuations.

Chromatograms of T₀ and T₄₋₅ urines from one male subject (man A) are indicated in Fig. 1, b and c. Man A tended to excrete relatively small amounts of caffeine metabolites in the T₄₋₅ urine sample. The data clearly show the separation of caffeine and its metabolites and indicate the nature of background endogenous peaks found in urine. To determine the reproducibility of the extraction procedure (same day precision), the T₄₋₅ urine sample from Fig. 1c was extracted a total of 10 times on the same day, and the 10 extracts were analyzed on that same day. The percentage CV for the analytes of interest ranged from 2.0–8.2%.

The results are presented in Table 1.

The chromatograms of extracted T₀ and T₄₋₅ urines samples from a second male subject (man B), who tended to excrete large amounts of caffeine metabolites in the T₄₋₅ urine sample, are shown in Fig. 1, d and e. Since the caffeine/metabolite peaks were so large, they were kept on scale by reducing the volume injected to 5 µL (from the usual 20 µL); this in turn made the endogenous peaks appear smaller.

Metabolic Molar Ratios and Within-Subject Variation in CYP1A2 (Using 17X/137X) and NAT2 Phenotypes. Table 2 presents the molar ratios observed in 9 women and 10 men, each of whom was studied 3 times at weekly intervals, as well as their variation in CYP1A2 phenotype (using 17X/137X). Data from woman 4 could not be used for calculating either molar ratio since large endogenous peaks were...
Within-Subject Variation in Caffeine Metabolism

Assuming a cutoff molar ratio for CYP1A2 poor and extensive metabolizers of 2.5 (2), 8 of 9 women and 10 of 10 men would be labeled as extensive metabolizers during all 3 weeks. Only 1 subject (woman 9) changed her metabolizer status within the 3-week test period (from poor to extensive metabolizer), with one result being 2.53 (just over the arbitrary limit of 2.5).

Table 3 presents the molar ratios observed in the same 19 subjects (each of whom was studied three times at weekly intervals), as well as their variation, in NAT2 phenotype (AFMU/1X). In the 19 subjects, 2 exhibited no detectable AFMU or 1X in T0 urine samples for all 3 weeks and had excellent phenotype precision (5.6 and 25% CV). The other 17 subjects had AFMU or 1X peaks present in their T0 urines during 1 or more weeks. The T0 urines in which the AFMU or 1X peaks were present at 5–12% or above 12% full-scale height are marked.

In the 9 women studied, we observed AFMU/1X values ranging from 0.25 to 2.77, with a precision ranging from 6.9 to 34.7% CV. The 10 men displayed molar ratios ranging from 0.27 to 3.54, with a precision ranging from 3.3 to 34.7% CV. The T0 urine from week 2 of man 9 was inadvertently spilled, hence this sample was not considered.

The cutoff for NAT2 poor and extensive metabolizers has been defined as the molar ratio equaling 0.4 (4). Using this definition, 4 of 9 women and 2 of 10 men were labeled as extensive metabolizers, while 4 of 9 women and 5 of 10 men appeared to be poor metabolizers during all 3 weeks. Four individuals changed their phenotype status within the 3-week test period (woman 7; men 2, 5, and 6).

Excretion of Caffeine and Its Metabolites as a Function of Time. The amounts of caffeine and the four caffeine metabolites of interest excreted per hour over 8 hours by men A and B are depicted in Fig. 2, a and b. For both subjects, maximal excretion of caffeine was observed within 1–2 h of ingestion, while the maximal rates of excretion of the metabolites occurred considerably later. For example, excretion rates for AFMU from both subjects did not reach their maximum value for at least 7–8 h after caffeine ingestion (the large amount of 17X excretion at 6 h by man A represents two peaks that could not be resolved and therefore were integrated together). Since the relative amounts of caffeine and its metabolites in urine were changing over time during this 8-h period, so too were the molar ratios of 17X/137X and AFMU/1X changing over time (Fig. 3, a and b). The molar ratios observed in T4-5 urine samples occurred at a time that all analytes of interest were detectable, and caffeine excretion had not yet fallen off dramatically.

Table 1 Within-day precision of measurement of caffeine and its metabolites in T4-5 h urine sample from man A*

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mean pmol</th>
<th>Mean % recovery</th>
<th>Mean molar ratio</th>
<th>SD</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFMU</td>
<td>103.6</td>
<td>8.5</td>
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<tr>
<td>1X</td>
<td>225.9</td>
<td>5.6</td>
<td>2.6</td>
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<td>17U</td>
<td>328.8</td>
<td>7.8</td>
<td>2.4</td>
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<tr>
<td>17X</td>
<td>196.2</td>
<td>7.9</td>
<td>4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>137X</td>
<td>88.3</td>
<td>6.8</td>
<td>7.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAPAP</td>
<td>93.5</td>
<td>1.8</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFMU/1X</td>
<td>0.46</td>
<td>0.04</td>
<td>8.1</td>
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<td></td>
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<tr>
<td>17X/137X</td>
<td>2.23</td>
<td>0.15</td>
<td>6.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17U +</td>
<td>225.9</td>
<td>5.97</td>
<td>6.3</td>
<td></td>
<td></td>
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<tr>
<td>17X/137X</td>
<td>5.97</td>
<td>0.38</td>
<td>6.3</td>
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<td></td>
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</table>

Ten aliquots extracted and analyzed on the same day.

* Present that coeluted with the internal standard and several caffeine metabolites; these peaks were likely related to her ingestion of the drug salicylazosulfapyridine (Azulfidine). Of the 19 subjects, only 2 exhibited no detectable 17X or 137X in T0 urines for all 3 weeks. One of these subjects (man 2) had an excellent phenotype precision (4.2% CV), while the other (man 5) had an unexpectedly large 17X peak during week 1 only, which led to a poor phenotype precision (58.2% CV). The other 17 subjects had 17X or 137X peaks present in 1 or more weeks in their T0 urines. The T0 urines in which 17X or 137X peaks were present at 5–12% or above 12% full-scale height on the chromatogram are marked.

In the 9 women studied, we observed 17X/137X values ranging from 2.19 to 14.04, with a precision ranging from 3 to 71.5% CV. The 10 men displayed molar ratios ranging from 2.7 to 11.24, with a precision ranging from 4.2 to 60.4% CV. The T0 urine from week 2 of man 9 was inadvertently spilled, hence this sample was not considered.

Fig. 1. Actual chromatograms obtained from injections of standard solution and extracted urine samples from two subjects (detection at 280 nm; 0.02 absorbance unit at full scale [AUFS]). Arrows, times of peaks of interest. a, 50-μl injection of sextet of known standards; b, 20-μl injection of extracted T0 urine sample from man A; c, 20-μl injection of extracted T4-5 urine sample from man A; d, 5-μl injection of extracted T0 urine sample from man B; e, 5-μl injection of extracted T4-5 urine sample from man B; IS, internal standard.

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Table 2  Molar ratio of 17X/137X as a measure of CYP1A2 phenotype

<table>
<thead>
<tr>
<th>Subject</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Mean</th>
<th>SD</th>
<th>% CV</th>
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<tbody>
<tr>
<td>Woman 1</td>
<td>3.62a</td>
<td>3.77a</td>
<td>2.79b</td>
<td>3.39</td>
<td>0.53</td>
<td>15.6</td>
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<tr>
<td>Woman 2</td>
<td>7.82b</td>
<td>4.23b</td>
<td>5.20b</td>
<td>5.75</td>
<td>1.86</td>
<td>32.3</td>
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<tr>
<td>Woman 3</td>
<td>3.75b</td>
<td>2.95b</td>
<td>4.58b</td>
<td>3.76</td>
<td>0.82</td>
<td>21.8</td>
</tr>
<tr>
<td>Woman 5</td>
<td>3.00b</td>
<td>2.93b</td>
<td>3.10b</td>
<td>3.01</td>
<td>0.09</td>
<td>3.0</td>
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<td>Woman 6</td>
<td>6.84b</td>
<td>14.04a</td>
<td>2.87</td>
<td>7.92</td>
<td>5.66</td>
<td>71.5</td>
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<td>Woman 7</td>
<td>3.41b</td>
<td>4.98b</td>
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<td>4.04</td>
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<td>Woman 8</td>
<td>4.63a</td>
<td>4.99a</td>
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<td>4.91</td>
<td>1.56</td>
<td>31.8</td>
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<td>Woman 9</td>
<td>2.41a</td>
<td>2.19</td>
<td>2.53a</td>
<td>2.38</td>
<td>0.17</td>
<td>7.1</td>
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<td>Woman 10</td>
<td>4.48b</td>
<td>4.93a</td>
<td>6.03a</td>
<td>5.15</td>
<td>0.80</td>
<td>15.5</td>
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<tr>
<td>Man 1</td>
<td>6.68</td>
<td>5.01b</td>
<td>6.61</td>
<td>6.10</td>
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<tr>
<td>Man 2</td>
<td>8.22</td>
<td>7.95</td>
<td>7.56</td>
<td>7.91</td>
<td>0.33</td>
<td>4.2</td>
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<tr>
<td>Man 3</td>
<td>3.98a</td>
<td>5.75a</td>
<td>6.29</td>
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<td>3.90</td>
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<td>11.24</td>
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<td>4.36</td>
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<td>Man 6</td>
<td>5.76b</td>
<td>7.91b</td>
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<td>Man 7</td>
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<td>3.15a</td>
<td>2.70a</td>
<td>3.24</td>
<td>0.58</td>
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<td>Man 8</td>
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<td>4.00a</td>
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* T0 urines with 17X or 137X peaks present at 5-12% full-scale height of chromatogram.

Discussion
Optimization of Chromatographic Conditions and Showing Representative Chromatograms. We modified chromatographic procedures reported previously by several other groups in order to obtain near-baseline separation of this ratio to 17X/137X for 5 women (women 1–3, 6, and 9) but not for the other four women studied (women 5, 7, 8, and 10). We observed (17U + 17X)/137X values ranging from 4.15 to 24.32, and within-subject variation ranging from 4.1 to 63.9% CV.
analytes from endogenous UV-absorbing material in urine. This modified procedure has resulted in an improved separation of analytes of interest, simplified gradient conditions, and a reduction in total time per cycle.

We found that chromatograms of urine from healthy subjects exhibited greater amounts of endogenous peaks relative to the size of the caffeine metabolite peaks, when compared to those published previously (4, 7-9). This difference might have resulted from the use of lower detector sensitivities by other groups or from the administration of a higher dose of caffeine to the subjects (7, 8). For example, in our experiments coffee was selected as a source of caffeine (containing approximately 200 mg 137X) to enhance subject compliance, whereas other groups have given known amounts of caffeine in powder or tablets to ensure ingestion of a known amount of caffeine (4, 7, 8). We also observed that salicylazosulfapyridine treatment probably interferes with the detection and integration of some caffeine metabolites.

In our modified protocol, the sample injection volumes were reduced when analyte peaks of interest were off-scale (falling outside the range of the standard curve). In this regard, the injection volumes were reduced to 5 μl to obtain the results shown in Figs. 1, d and e; 2b; 3b; and 4b.

We derived standard curves to quantitate the amounts of caffeine and caffeine metabolites in sample injections in pmol amounts (e.g., pmol injected in 20 μl volume). We then calculated actual molar ratios from pmol quantities. Since we were measuring the molar ratios of peaks of interest, we did not need to correct for percentage recovery of the internal standard. Rather, we used the recovery of the internal standard as a quality control to be sure that the extraction/chromatography procedure was consistent (our mean recovery of internal standard ± SD was 86.9% ± 6.3%).

Metabolic Molar Ratios and Within-Subject Variation in CYP1A2 (Using 17X/137X) and NAT2 Phenotypes. Most of our subjects who were caffeine free for 36 h before being tested demonstrated low between-day variation in their phenotypes (<25% CV). Our observed range of the 17X/137X molar ratio (2.19–14.04) is comparable to ranges reported previously. In 30 randomly selected individuals studied on one occasion, Kadlubar et al. (2) observed molar ratios of 0.36–10.62, and in 97 subjects, Lang et al. (3) observed a molar ratio range from 0.3 to 10.

Our observed range of the NAT2 molar ratio (0.27–2.77) also is comparable to previously described values.
Kadlubar et al. (2) observed a range from <0.1 to 3.33, Lang et al. (3) from approximately 0.002 to 5.0 in 95 individuals, Butler et al. (4) from approximately 0.2 to 3.0 in 81 nonsmokers, Grant et al. (8) from approximately 0.03 to 1.0 in 68 nonsmokers, and Bechtel et al. (11) from approximately 0.0 to 2.0 in 245 unrelated individuals.

Comparing our within-subject NAT2 variation observations (3.3–34.7% CV) to others described previously, Butler et al. (4) observed a range from 8.9 to 21.1% CV for 8 subjects studied once a week for 5 weeks and from 9.9 to 33.4% CV for 5 different subjects studied once daily for 5 days. Because no 17X/137X variability studies are described, no comparisons can be made.

**Excretion of Caffeine and Its Metabolites Over Time.** A 36-h caffeine-free period was used in our study initially. A longer period would be more desirable, but some subjects who consumed coffee chronically complained of lethargy and headaches during the 36-h abstinence. In these circumstances, the headaches were personally treated with medications other than acetaminophen (Tylenol and others). However, even with a 36-h caffeine-free period, some subjects still excreted small amounts of caffeine metabolites in their T0 urines. While these small amounts of metabolites in T0 urines could cause different molar ratios to be observed, we don’t believe that they would have a major impact on molar ratio values because caffeine-related peaks 4–5 h after a new dose of caffeine would be much larger than the peaks remaining 36 h after a previous dose for most subjects. We concluded that it is preferable for participants to refrain from ingesting caffeine-containing products for at least 48 h before testing due to the different rates of metabolite formation, retention, and excretion in our study volunteers. We also concluded that it is necessary to collect a T0 urine from each volunteer on each study day in order to evaluate any contributions of caffeine metabolites to the results.

By following the patterns of excretion of caffeine and its metabolites for 48 h after caffeine ingestion, we have affirmed that the 4–5-h period is a convenient time for the collection of urine samples for which molar ratios could be determined, since all metabolites of interest were relatively stable.

**Different Molar Ratios Used for Phenotyping CYP1A2.** CYP1A2 activity was also phenotyped in 9 women, studied once a week for 3 weeks, using the molar ratio (17U + 17X)/137X. Butler et al. (4) and Nakajima et al. (10) have reported that the (17U + 17X)/137X ratio better reflects the actual activity of CYP1A2 when compared to other proposed molar ratios used to phenotype CYP1A2. In our study, the determination of this molar ratio was easily accomplished and showed essentially the same within-subject variation as the simpler 17X/137X ratio.

We observed within-subject variation in the (17U + 17X)/137X molar ratio of 4.1 to 63.9% CV, similar to previous reports. In four nonsmoking subjects studied once weekly for 5 weeks, Butler et al. (4) observed a range of 23–48% CV, and in 4 different nonsmokers studied daily for 5 days, 12.9–36% CV. Nakajima et al. (10), describes within-subject variations ranging from 0.7 to 84.5% in 8 Japanese nonsmokers studied twice over 11 months.

When using (17U + 17X)/137X to phenotype CYP1A2, Butler et al. (4) observed a trimodal distribution of data creating “breakpoints” at molar ratio values of approximately 4 and 12, separating subjects into poor, intermediate, and extensive metabolizer phenotypes. Nakajima et al. (10) observed a bimodal distribution of data with a breakpoint at 5 in nonsmokers and at 6 in smokers, when using the same molar ratio, denoting poor and extensive classifications. Using this ratio, women 6, 8, and 10 changed from extensive to intermediate metabolizers within the 3-week test period with respect to the findings of Butler et al. (4). Women 5 and 6 changed from extensive to poor metabolizers within the 3-week test period when regarding the observations of Nakajima et al. (10).

We also observed greater within-subject variation for CYP1A2 when compared to NAT2. The molar ratio of AFMU/1X may have less variation over time because both analytes (which are products of 17X) increase and decrease “in parallel,” whereas 17X increases as 137X decreases. Within-subject variation over longer times (>3 weeks) should be tested, especially if patients begin or start smok-
other groups, are preliminary and tentative because:

(a) variations in CYP1A2 and NAT2 phenotypes, and those from our molar ratio and percentage CV values compared well to those observed by other groups, despite our molar ratio and percentage CV values coming closer to those observed by other groups. Further, even if only qualitative classification into extensive and poor metabolizers is used, our method may be more accurate than previous methods.

(b) enzyme activity in liver biopsy samples, for example. Even though our molar ratio and percentage CV values corresponded to those observed by other groups, comparison between our determinations of within-subject variation in CYP1A2 and NAT2 phenotypes, and those from other groups, are preliminary and tentative because: (a) other groups describe various times that study subjects were caffeine-free; (b) control urines collected before caffeine dosing were reported in only two previous papers (7, 8). Also, because our study was not conducted in a completely controlled environment (such as a metabolic ward), such variables as diet, exercise, sleep, alcohol consumption, intercurrent illnesses, and exposure to other possible enzyme inducers (such as charbroiled meat) could not be totally controlled. These factors may have contributed to higher within-subject variation. Additionally, the relatively small group of subjects (i.e., 9 women and 10 men) may not reflect expected between-subject variation associated with differences in race, sex, and geographic location (3, 4).

In summary, we found that most subjects demonstrated moderate within-subject variation from week to week in their molar ratios when using caffeine to phenotype CYP1A2 and NAT2, and that similar findings have been reported previously. This degree of variation is usually acceptable provided that subjects have been caffeine-free for at least 36 h, and preferably 48 h, prior to ingesting the test dose of caffeine. While more difficult, it may also be preferable to study subjects twice rather than once if a specific molar ratio (rather than qualitative classification) is important. If only qualitative classification into extensive or poor metabolizer is important, then studying a subject on only one occasion usually provides an accurate classification of that individual.

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