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Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites

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The wide variations in urinary bladder and colo-rectal cancer incidence in humans have been attributed in part to metabolic factors associated with exposure to carcinogenic aromatic and heterocyclic amines. Cytochrome P-4501A2 (CYP1A2), which catalyses *N*-oxidation, and acetyltransferase (NAT2) which catalyses *N*- and *O*-acetylation, both appear to be polymorphically distributed in human populations; and slow and rapid NAT2 phenotypes have been implicated as risk factors for these cancers. Caffeine has also been shown to undergo 3-demethylation by CYP1A2, and it is further acetylated to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) by the polymorphic NAT2. In this report, we describe a metabolic phenotyping procedure that can be used to determine concomitantly the hepatic CYP1A2 and NAT2 phenotypes. For the NAT2 phenotype, we confirm the valid use of the urinary molar ratio of AFMU/1-methylxanthine, even in alkaline urines. For the CYP1A2 phenotype, the urinary molar ratio of [1,7-dimethylxanthine + 1,7-dimethyluric acid]/caffeine, taken at 4–5 h after caffeine ingestion, was identified from pharmacokinetic analyses of 12 subjects as being better correlated ($r = 0.73$; $p = 0.007$) with the rate constant for caffeine 3-demethylation than other previously suggested ratios. This procedure was then used to determine the CYP1A2 phenotype in subjects from Arkansas ($n = 101$), Italy ($n = 95$), and China ($n = 78$). Statistical and probit analyses of nonsmokers indicated that the CYP1A2 activity was not normally distributed and appeared trimodal. This trimodality allowed arbitrary designation of slow, intermediate, and rapid phenotypes, which ranged from 12–13% slow, 51–67% intermediate, and 20–37% rapid, in the different populations. A reproducibility study of 13 subjects over a 5 day or 5 week period showed that, with one exception, intraindividual variability did not alter this CYP1A2 phenotypic classification. Induction of CYP1A2 by cigarette smoking was also confirmed by the increased caffeine metabolite ratios observed in the Arkansas and Italian smokers (blonde tobacco). However, Italian smokers of black tobacco and Chinese smokers did not appear to be induced. Furthermore, probit analyses of Arkansas and Italian blonde tobacco smokers could not discriminate between phenotypes, apparently as a consequence of enzyme induction.

Introduction

Two drug-metabolizing enzymes in humans, cytochrome P-4501A2 (CYP1A2) and *N*-acetyltransferase (NAT2), are generally regarded as the principal catalysts for the major metabolic reactions involved in both caffeine biotransformation and in the activation and detoxication of carcinogenic arylamines and heterocyclic amines (Kadlubar *et al.*, 1992). For this reason, caffeine, a relatively innocuous drug, has potential for use in characterizing the proficiency of individuals to metabolize this class of chemical

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carcinogens and could serve to assess individual differences in susceptibility to aromatic amine-induced cancers.

The wide variations in the incidence of colo-rectal and urinary bladder cancer observed in human populations (Tomatis, 1990; Boring *et al.*, 1991) are consistent with environmental and genetic factors that define the NAT2 and CYP1A2 phenotypes and that may predispose individuals to these cancers. A number of studies have already shown that individuals with the slow acetylator phenotype, inherited as an autosomal co-dominant trait, appear to be more susceptible to the development of urinary bladder cancer, particularly in subgroups occupationally exposed to carcinogenic arylamines (Hein, 1988); while individuals with the rapid acetylator phenotype appear to be predisposed to the development of colo-rectal cancer (Lang *et al.*, 1986; Ilett *et al.*, 1987).

The basis for these phenotypic differences in susceptibility can be rationalized from the apparent metabolic pathways for aromatic amines in humans. It is well established that human CYP1A2 catalyses the metabolic activation of several primary arylamines and heterocyclic amines through *N*-oxidation (Guengerich & Shimada, 1991). NAT2, on the other hand, catalyses both the *N*-acetylation of arylamines, a detoxication step for arylamines such as 4-aminobiphenyl and 2-naphthylamine, and the *O*-acetylation of the *N*-hydroxy metabolites to reactive *N*-acetoxy esters that bind to DNA and are regarded as ultimate carcinogens (Hein, 1988). For arylamines known to be human bladder carcinogens, such as 4-aminobiphenyl, *N*-oxidation by CYP1A2 and *N*-acetylation by NAT2 represent competing activation and detoxication reactions in the liver that control the levels of the *N*-hydroxy metabolite that can enter the circulation and be transported to the urinary bladder where reabsorption into the bladder epithelium and arylamine-DNA adduct formation can occur (Kadlubar *et al.*, 1991). In contrast, the heterocyclic amines, which have been implicated in human colorectal cancer (Wohlleb *et al.*, 1990; Schiffman & Felton, 1990), are poor substrates for *N*-acetylation in the liver. However, their *N*-hydroxy metabolites are readily conjugated by glucuronidation (Kaderlik *et al.*, 1991) and transported through the bile to the colon lumen where hydrolysis and reabsorption can occur. Subsequently, NAT2, which is present at high levels in human colon, can catalyse the *O*-acetylation of the *N*-hydroxy metabolite to form the ultimate carcinogenic derivative (Turesky *et al.*, 1991).

Although the contribution of the CYP1A2 phenotype to cancer susceptibility seems likely, this hypothesis has not yet been fully investigated. Clearly, there is need for the identification of a relatively safe drug substrate for CYP1A2 and the development of a method to provide a reliable indicator of CYP1A2 activity *in vivo*. Since CYP1A2 was shown to be responsible for the 3-demethylation of caffeine (Butler *et al.*, 1989b), the initial major step in the biotransformation of caffeine in humans, we proposed that CYP1A2 activity in humans could be characterized by measuring urinary caffeine metabolite ratios reflecting CYP1A2 activity (Butler *et al.*, 1989b). In this report, we describe a caffeine metabolite ratio determined in urine obtained at a 4–5 h interval after coffee consumption that accurately reflects the rate constant for the CYP1A2-catalysed 3-demethylation of caffeine *in vivo*. Furthermore, we describe a modification of a previously published method using caffeine for acetylation phenotyping (Grant *et al.*, 1984) that now allows for the simultaneous determination of both NAT2 and CYP1A2 phenotypes. We also present results for several human populations that suggest the existence of slow, intermediate, and rapid CYP1A2 phenotypes and their inducibility by cigarette smoking.

Materials and methods

Chemicals

1,3,7-Trimethylxanthine (137X, caffeine), 3,7-dimethylxanthine (37X, theobromine), 1,7-dimethylxanthine (17X, paraxanthine), 1,3-dimethylxanthine (13X, theophylline), 1-methylxanthine (1X), 3-methylxanthine (3X), 7-methylxanthine (7X), 3,7-dimethyluric acid (37U), 1,3-dimethyluric acid (13U), 1-methyluric acid (1U), 3-methyluric acid (3U), 7-methyluric acid (7U), β-hydroxyethyltheophylline (BHET), and 4-acetamidophenol (internal standard, IS) were purchased from Sigma Chemical Company (St Louis, MO). 5-Acetylamino-6-formylamino-3-methyluracil (AFMU) was obtained from Drs W. Kalow and B. K. Tang, University of Toronto, Toronto, Canada. 1,3,7-Trimethyluric acid (137U) and 1,7-dimethyluric acid (17U) were purchased from Fluka Chemical Corporation (Ronkonkoma, NY). 5-Acetylamino-6-amino-3-methyluracil (AAMU) was synthesized from AFMU by a 10 min incubation at room temperature in aqueous solution at pH 10.0 (Tang *et al.*, 1983).

Pharmacokinetic study

Healthy, non-medicated males ($n = 2$) and females ($n = 10$) between the ages of 24 and 56, who did not have a sensitivity to caffeine or a history of abnormal liver or kidney function, were selected for participation. These subjects were recruited from Pulaski County, Arkansas, USA. All subjects had serum

values for creatinine of $<1.8\,mg\,dl^{-1}$, for bilirubin of $<1.5\,mg\,dl^{-1}$, for alkaline phosphatase of $<100\,IU\,l^{-1}$, and for aspartate aminotransferase of $<40\,IU\,l^{-1}$. Subjects refrained from consumption of methylxanthine-containing foods and beverages for 72 h prior to the study and until 48 h after caffeine dosing. After overnight fasting, the subjects received 3 mg caffeine per kg body weight, which was added to a 180 ml cup of coffee prepared from 1.8 g decaffeinated instant coffee. Venous blood samples (< 5 ml) were collected from a forearm vein prior to and at 0.25, 0.5, 1, 2, 4, 8, 24, 32, and 48 h after the caffeine dose. A urine sample was obtained just prior to the caffeine dose, and all urine formed in the 0–3, 3–4, 4–5, 5–6, 6–24, and 24–48 h intervals was collected. These samples were immediately frozen on dry ice and then stored at –20° C until analyses. Data from the plasma concentration-time curves of 137X, 13X, 17X, and 37X and from urinary accumulation of AAMU, AFMU, 1U, 3U, 7U, 1X, 3X, 7X, 13X, 17X, 37X, 13U, 17U, 37U, 137U, and 137X over the 48 h period were analysed using a computerized pharmacokinetic model system (SimuSolve Modeling and Simulation Software, Dow Chemical Co., Midland, MI) that determined rate constants for the metabolism of 137X to 13X, 17X, 37X, and 137U, for the metabolism of 13X to 1X, 3X, and 13U, for the metabolism of 17X to 1X, 7X, 17U, AAMU, and AFMU, for the metabolism of 37X to 3X, 7X, and 37U, and for the metabolism of 1X to 1U, 3X to 3U, and 7X to 7U. The proficiency of caffeine 3-demethylation by hepatic CYP1A2 was judged by the ratio of rate constants, $k_{137X\rightarrow 17X}/k_{137X\rightarrow urine}$, because these represent the initial competing pathways for elimination of caffeine from the blood and they allow a direct comparison to urinary metabolite ratios. The pharmacokinetic model, defined by a series of differential equations that comprise known caffeine metabolic pathways (Bonati *et al.*, 1982), will be described fully in a separate publication.

Study groups and dosing regimen

Subjects were recruited from Arkansas, Italy, and the Peoples' Republic of China. After providing written informed consent, the volunteers from Arkansas and Italy filled out a questionnaire on smoking status, diet, medication use, and illness. Only volunteers who reported no history of liver and kidney abnormalities or who did not limit their caffeine consumption due to a known sensitivity to caffeine were selected for participation. Subjects from the Peoples' Republic of China provided oral consent but information on liver and kidney function was not available. Subjects refrained from consuming methylxanthine-containing foods and beverages from midnight before the test until 5 h after consuming the dose cup of coffee (*ca.* 114 mg caffeine), with the exception of subjects from China, who did not necessarily restrict tea consumption. This was judged to be acceptable since the level of caffeine in tea (< 50 mg per cup; Gilman *et al.*, 1990), if consumed prior to phenotyping, would be insufficient to alter caffeine metabolite ratios at 4–5 h after coffee dosing (Bonati *et al.*, 1982). At 9 a.m. or earlier on the morning of the test, the subjects in each of the study groups received a 9-ounce cup of coffee prepared from 3.6 g instant coffee, voided 4 h after consuming the coffee, and provided a 1 h urine specimen at the end of the 5th hour following dosing. The urine specimens were then frozen on dry ice and stored at −20° C.

Subject descriptions

One-hundred and eight healthy subjects between the ages of 23 and 70, who were not taking any medications previously reported to affect caffeine metabolism, were recruited from Arkansas, USA. This group was composed of 40 males, 68 females, 27 smokers, 81 nonsmokers, 18 Blacks, and 90 Caucasians. One-hundred healthy male Caucasian volunteers were selected in Turin, Italy. These subjects were between the ages of 45 and 64 years. Thirty-one of the subjects were smokers of blonde tobacco cigarettes and sixteen smoked black tobacco cigarettes. Seventy-eight male subjects were selected from the Peoples' Republic of China. All of these subjects had been employed in benzidine production for at least 1 year between 1945 and 1977, and 39 of the subjects had a history of urinary bladder cancer. Forty-eight of the subjects were smokers.

Intraindividual variability studies

Thirteen healthy Caucasian subjects from Pulaski County, Arkansas and Bethesda, Maryland participated in the reproducibility portion of the study. Four males and four females were administered caffeine once weekly for 5 weeks. Of these, two of each sex smoked 8–30 cigarettes daily. These subjects also abstained from methylxanthine-containing foods and beverages after midnight on the night before the study and until 5 h after caffeine administration. The subjects were then administered a 9-ounce cup of coffee prepared from 3.6 g instant coffee at 8:30 a.m. once weekly for 5 weeks, on the same day of the week.

Five Caucasian subjects (three female, and two male, one of whom smoked 6–8 cigarettes per day) were administered caffeine once daily for 5 days. These subjects were given 100 mg caffeine as a tablet on

day 1 of the study after refraining from methylxanthines for 48 h, and on day 2 after refraining for 24 h, and a caffeinated beverage of their choice (a cola soft drink, coffee, or tea) on days 3 to 5, with no dietary restrictions. Each subject consumed their caffeine-containing beverage before 9 a.m. each morning of the study. The subjects voided 4 h after consuming the beverage, and provided a 1 h urine specimen at the end of the 5th hour following dosing.

The urine specimens were then frozen on dry ice and stored at $-20°$ C. These subjects were not taking any medications previously reported to affect caffeine metabolism, other than oral contraceptives.

Analytical procedures

Urine samples for analysis were quickly thawed by placing them in cold, running water. The samples were thoroughly mixed and then adjusted to pH 3.5 with 1 M HCl. Caffeine and its metabolites were extracted from the urine samples using a modification of a procedure described by Grant *et al.* (1984). A 200 µl aliquot of urine was added to a 15 ml centrifuge tube, followed by a saturating amount (*ca.* 120 mg) of ammonium sulfate. After mixing for 2 min, 200 µl of the internal standard (120 mg l^{-1} 4-acetamido-phenol in chloroform) and 6 ml of chloroform:isopropanol was added. Urine from all subjects was extracted using chloroform:isopropanol (3:1) with the exception of urine from subjects from Arkansas, which was extracted with chloroform:isopropanol (19:1). The tube was mixed for 1 min and was centrifuged for 5 min at 3000 rpm. A 5.5 ml aliquot of the organic phase was removed and evaporated to dryness under a stream of nitrogen. For analyses of caffeine and metabolites in blood, Bakerbond Octadecyl (C18) disposable extraction columns (J. T. Baker, Inc., Phillipsburg, NY) were used to extract 137X, 37X, 17X, and 13X from heparinized plasma (400 µl) containing 0.07 µg ml^{-1} BHET as internal standard. The columns were eluted with methanol and the eluates were evaporated to dryness under nitrogen. The residues from urine or plasma extracts were dissolved in 0.14 to 0.5 ml of 0.05% acetic acid and filtered through a 0.45 µm filter. The extracts (25 to 100 µl) were analysed by HPLC using a program that separated caffeine and all of its known metabolites from each other and from other plasma and urinary constituents as described below.

Instrumentation

HPLC analyses of reconstituted extracts of urine and plasma for caffeine and its metabolites were conducted on a Waters Associates instrument consisting of a Model 6000A solvent delivery system, WISP 712 Automated Sample Injector, and a Model 680 Automated Gradient Controller (Waters Chromatography Division, Millipore Corporation, Milford, MA), which was equipped with a Beckman/Altex 5 µm Ultrasphere ODS (4.6 mm × 25 cm) column (Rainin Instrument Co., Woburn, MA), a Waters Guard-PAK Precolumn Module containing a µBondapak C18 insert (Waters Chromatography Division, Millipore Corporation, Milford, MA) and a computerized Hewlett-Packard Model 1040M Diode-Array Detection System (Hewlett-Packard Co., Atlanta, GA) set at 280 nm. The solvents used for elution were 0.045% acetic acid containing 9% methanol (A) and 100% methanol (B). Typical conditions for elution were: 0% B (0–3.5 min), 0–2% B (3.5–3.8 min), 2% B (3.8–15 min), 2-16% B (15–20 min), and 16% B (20–35 min). Linear gradients were used for all solvent changes. The flow rate was 1.1 ml min^{-1}. Retention times (min) for a representative column were as follows: AAMU, 3.1; AFMU, 4.3; 3U, 5.2; 7U, 7.1; 7X, 7.9; 1U, 8.7; 3X, 9.5; 37U, 10.3; 1X, 11.2; IS, 13.0; 13U, 14.8; 37X, 15.4; 17U, 22.9; 17X, 23.5; 13X, 24.8; BHET, 25.5 min; 137U, 26.5, and 137X, 29.7. When new columns were installed, it was often necessary to make minor modifications in the elution program in order to obtain the best resolution of metabolites, for example, changing the final conditions to 15% B. Some columns were found to be incapable of resolving caffeine from endogenous components in the urine extracts and could not be used. Caffeine and its metabolites in urine and plasma were identified by absorption spectra and retention time compared to standards. The concentration (µM) of caffeine and its metabolites in these samples was determined after calculating the nmol present in the extract injected by comparison of their integrated areas with areas from a calibration curve prepared from known standards. The limit of detection was 0.10–0.18 nmol injected.

Data analysis

Median values were compared using the Mann-Whitney U-test; and the data were examined for normality of distribution by the use of probit plots, tests for skewness and kurtosis, the Wilk-Shapiro test of normality ($n < 50$) (Shapiro & Wilk, 1965), and the Chi-Squared goodness of fit test ($n > 50$), all using RS/1 statistical programs (BBN Software Products Corporation, Cambridge, MA). Probit transformations of the data were conducted by plotting the ratios against their corresponding percentage area under the normal probability curve on probability paper (Finney, 1964). The ratios were ranked from the lowest to highest value, and the percent area under the probability curve calculated for each data point (x). The

percentage area under the normal probability curve is $100i/(n+1)$, where i = rank of the data point, and n = sample size. Using probability paper, a plot of x, or log x, versus the percentage area under the normal probability curve produces a straight line for data that are normally, or log normally, distributed. Curved or broken lines indicate that x is not normally distributed. The probit plots and associated linear regression lines were plotted using Sigma Plot software (Jandel Corp., Corte Madera, CA).

Results

Method for separation and identification of caffeine and its metabolites in urine and plasma

Determination of caffeine and its metabolites was achieved by extraction with chloroform:isopropanol and HPLC analysis as illustrated in Fig. 1. AFMU, 1X, 17U, 17X, and 137X, as well as 7X, 1U, 3X, 13U, 37X, 13X, and 137U are identified in this extract, which was prepared from urine collected during the 4–5 h interval following ingestion of a 9-ounce cup of coffee prepared from 3.6 g instant coffee. Chloroform: isopropanol (3:1) afforded good recovery of all caffeine metabolites (80–98%), while a 19:1 solvent ratio provided excellent recovery (>90%) of AFMU, 1X, 17U, 17X, and 137X, the metabolites selected for CYP1A2 and NAT2 phenotyping. The use of chloroform:isopropanol (19:1) also decreased the extraction of uric acid and other water-soluble endogenous components present in urine that elute near many of the caffeine metabolites. Because of the presence of these endogenous components, computerized photodiode-array detection, rather than conventional UV detection, was essential for the positive identification of caffeine and its metabolites. The spectral information provided by the photodiode-array detection system allowed a comparison of the absorption spectra of the

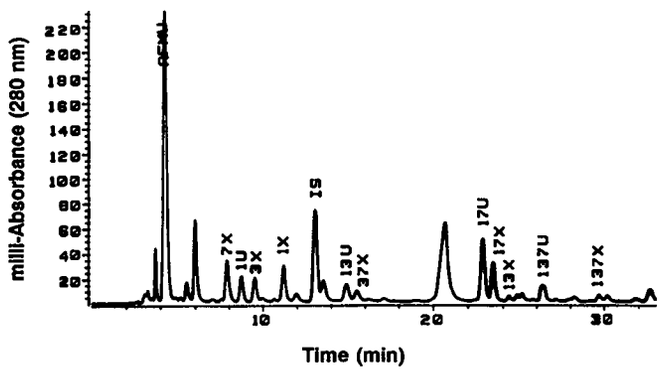

Fig. 1. HPLC chromatogram of a chloroform:isopropanol (19:1) extract of urine obtained at 4–5 h interval after ingestion of a cup of coffee.

Table 1. Correlation of the rate constant ($k_{137X \to 17X}/k_{137X \to urine}$) for caffeine 3-demethylation with urinary metabolite ratios determined in the 4–5 h interval after caffeine consumption[a]

Urinary metabolite ratio	*r*	*p*
[17X + 17U]/137X[b]	0.73	0.007
17X/137X	0.54	0.07
[AFMU + 1X + 1U]/17U	0.33	0.30
[AAMU + 1X + 1U]/17U	0.34	0.29

[a]The term, $k_{137X \to 17X}/k_{137X \to urine}$, represents the ratio of rate constants for the hepatic conversion of caffeine to paraxanthine ($k_{137X \to 17X}$) and the excretion of caffeine into urine ($k_{137X \to urine}$), and is thus a measure of the proficiency of hepatic 3-demethylation of caffeine catalysed by CYP1A2. The correlation coefficient (r) was determined by least-squares linear regression analysis. p is the significance probability.

[b]Correlational analysis of ratios that included additional urinary metabolites derived from 137X did not significantly increase the r or p values obtained.

putative metabolites with known standards and provided information on peak homogeneity. Furthermore, automation of peak identification and integration by the software was necessary for time-efficient analyses of large numbers of samples.

Correlation of rate constants for caffeine 3-demethylation with caffeine urinary metabolite ratios

Rate constants for the proficiency of hepatic caffeine 3-demethylation ($k_{137X \to 17X}/k_{137X \to urine}$) were obtained from the pharmacokinetic study conducted on 12 subjects where levels of caffeine and its 15 metabolites in urine and blood were measured over a 48 h period after 3 mg kg^{-1} caffeine dose. The results (Table 1) indicated that the urinary molar ratio of [17X + 17U]/137X obtained from the 4–5 h interval after caffeine consumption accurately reflected caffeine 3-demethylation activity *in vivo* ($r^2 = 0.53$; $r = 0.73$). By comparison, the previously proposed ratios 17X/137X (Kadlubar *et al.*, 1990), [AFMU + 1X + 1U]/17U (Campbell *et al.*, 1987b) and [AAMU + 1X + 1U]/17U (Kalow & Tang, 1991) were not as well correlated.

CYP1A2 and NAT2 phenotypes of Arkansas subjects

The caffeine phenotyping procedure for the concomitant determination of CYP1A2 and NAT2 phenotypes was then applied to 108 healthy subjects from Arkansas. Seven subjects could not be phenotyped for CYP1A2 because of endogenous components in

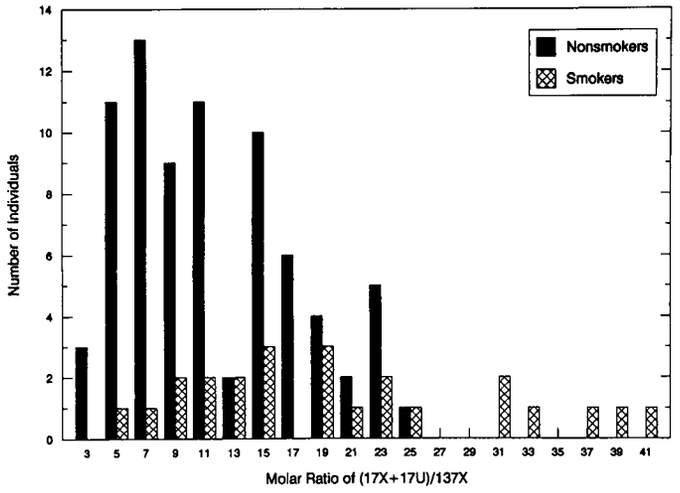

Fig. 2. Frequency distribution of the urinary molar ratio of [17X+ +17U]/137X in Arkansas nonsmokers and smokers.

the urine that interfered with caffeine quantitation. Results of the CYP1A2 phenotyping procedure are presented as a frequency distribution in Fig. 2. The [17X + 17U]/137X ratio in the 77 nonsmokers was not normally distributed, as indicated by the Chi-Squared goodness of fit test of normality ($\chi^2 = 10.5$, $p = 0.033$). Although the frequency distribution in the 24 smokers also did not appear normal, statistical tests (Wilk-Shapiro; $W = 0.936$; $p > 0.1$) could not confirm this difference, probably due to the limited sample size. However, the distribution of ratios was skewed (0.63, 0.59) and showed negative kurtosis (−0.63, −0.53) in both smokers and nonsmokers, respectively. The median [17X + 17U]/137X ratio was significantly higher ($p = 0.0002$) in smokers (17.3) than in nonsmokers (9.3).

Probit transformation of [17X + 17U]/137X urinary ratios, determined in the 77 nonsmokers, resulted in nonlinear probit plots (Fig. 3a) and indicate further that this group did not have a normal distribution of [17X + 17U]/137X ratios. Using linear regression analysis, the data suggested an apparent trimodal distribution with breakpoints at a [17X + 17U]/137X ratio near 4 and 12, suggesting the existence of slow, intermediate and rapid phenotypes. In contrast, probit plots of the 24 smokers could not clearly discriminate between phenotypes.

For comparison, probit transformations, which have long been used to determine the distribution of slow and rapid NAT2 phenotypes (Grant *et al.*, 1983), clearly shows the expected bimodal distribution of NAT2 phenotypes in this population with a breakpoint at 0.6 for both smokers and nonsmokers (Fig. 3b). Interestingly, there appeared to be a greater

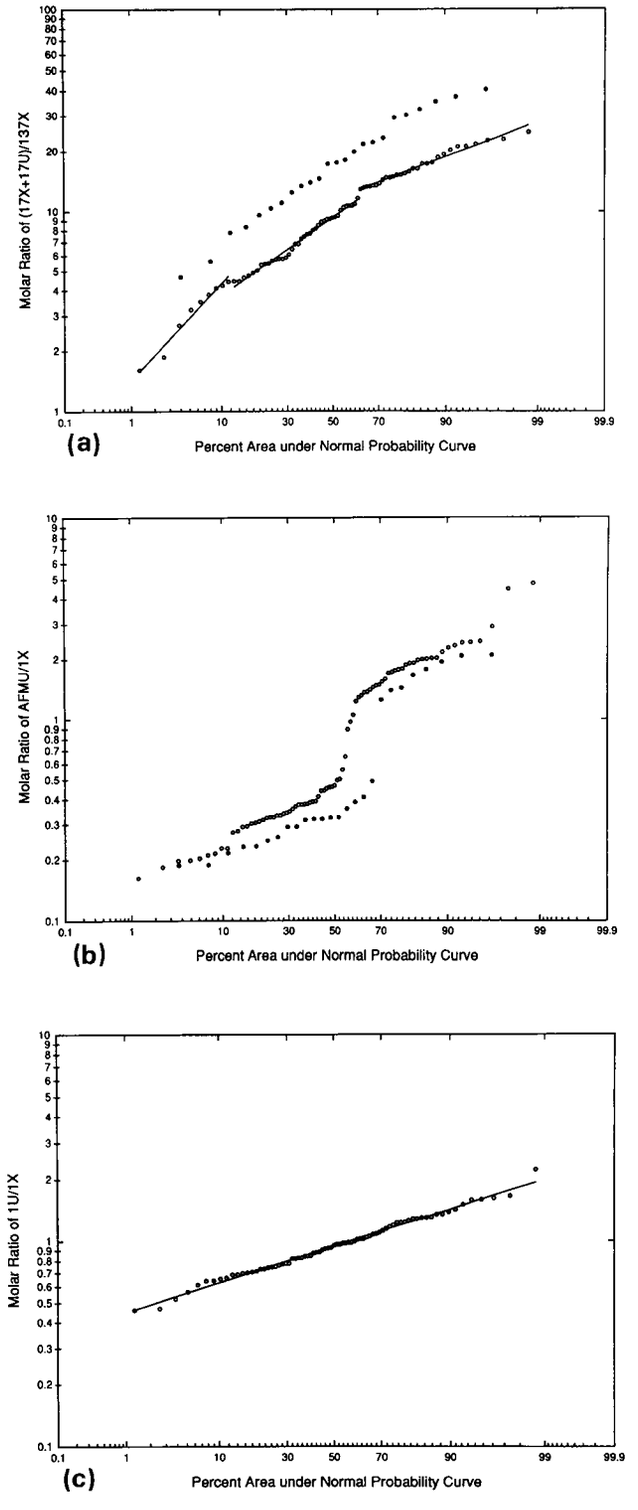

Fig. 3. Probit analyses of Arkansas nonsmokers and smokers for the urinary molar of: [17X + 17U]/137X (a); AFMU/1X (b); and 1U/1X (c). The ordinate denotes values obtained from analysis of caffeine urinary metabolites in different individuals. The abscissa represents the percentage area under the probability curve calculated for each data point as described in *Materials and methods*. Nonsmokers (open circles); smokers (closed circles).

proportion of slow NAT2 individuals among the smokers, but this difference was not statistically significant ($p = 0.25$) using a two-tailed Fisher's Exact Test. Similarly, probit transformations of the urinary molar ratio of 1U/1X, which reflects xanthine oxidase activity and is known to be normally distributed (Grant *et al.*, 1983), clearly showed a linear, unimodal pattern (Fig. 3c).

Intraindividual variability in CYP1A2 activity

Since CYP1A2 enzyme activity in humans may be a consequence of both heritable and environmental factors, the intraindividual variability in the urinary [17X + 17U]/137X ratio was examined in eight subjects (four smokers and four nonsmokers) once a week for 5 weeks and in five subjects (one smoker and four nonsmokers) daily for 5 days. These subjects maintained their normal smoking status and their regular diets of choice throughout the study. Weekly coefficients of variation (calculated as SD/mean × 100%) ranged from 23.0 to 48.0% in the weekly subjects who did not smoke, and from 13.5 to 27.4% in the smokers. Daily coefficients of variation were 12.9 to 36.0% in the four nonsmokers, and 34.7% in the smoker. For the eight nonsmokers who could be classified phenotypically (*vide supra*), one individual consistently had urinary ratios of [17X + 17U]/137X at the breakpoint between slow and intermediate metabolizers and could not be classified. Of the remaining individuals, no phenotypic misclassification occurred out of 35 analyses.

Intraindividual variability in NAT2 activity

Since the AFMU/1X ratio used to characterize the NAT2 phenotype was concomitantly determined in our procedure, intraindividual variability in NAT2 status was compared in the eight subjects tested over the 5 week period. Coefficients of variation ranged from 8.9 to 14.7% in the four subjects characterized as slow acetylators (AFMU/1X < 0.6) and from 10.0 to 21.1% in the four rapid acetylators (AFMU/1X > 0.6); while urine pH varied from 5.0 to 7.2. Each of the eight subjects remained in the same phenotypic category throughout the 5 week sampling period. For the five subjects administered caffeine-containing beverages daily for 5 days, the coefficient of variation in the AFMU/1X ratio ranged from 9.9 to 33.4%. Again, the NAT2 phenotype was the same for each of the five individuals over the 5 day period.

AFMU stability in vivo

Since AFMU spontaneously deformylates to AAMU under *in vitro* conditions in which the urine is adjusted to pH 10 (Tang *et al.*, 1983), it has been suggested that AFMU may spontaneously deformylate to AAMU while in the bladder whenever the urine is neutral or basic (Tang *et al.*, 1991). However, the AFMU/1X ratios were independent of normal urine pH variations in the eight subjects over the 5 week period, indicating that AFMU does not deformylate in the bladder to a significant extent that would affect phenotype classification under normal conditions. In order to determine directly if alkaline urine in the bladder during the 4–5 h interval would promote the deformylation of AFMU and result in NAT2 misclassification, 1 week after the 5 week reproducibility study, one subject consumed one teaspoon of sodium bicarbonate in a glass of water at 1, 2, and 3 h after taking caffeine. Although the urine collected at 3–4, 4–5, and 5–6 h was elevated to pH 8.0, the urinary AFMU/1X ratio (2.40) measured at the 4–5 h interval was not different from ratios (2.45 ± 0.27) determined in weeks 1–5 where the urine pH ranged from 5.9 to 7.2. Furthermore, the AFMU/1X molar ratio (2.34), determined after the pH 8.0 urine specimen was stored at −20° C for 11 days prior to adjustment to pH 3.5 for analysis, indicates that AFMU did not decompose during storage at pH 8.0 to an extent that would change phenotypic characterization.

CYP1A2 phenotypes of Italian subjects

The phenotyping procedure used to determine the urinary [17X + 17U]/137X and AFMU/1X molar ratios was then applied to 47 smokers and 50 nonsmokers from an Italian population. The use of black tobacco in Italy allowed a further comparison between this tobacco type and the more common blonde tobacco. Since the NAT2 phenotypes of this population have been published earlier (Bartsch *et al.*, 1990), only the results of the CYP1A2 phenotyping are presented here. The [17X + 17U]/137X molar ratios were successfully determined in 92 of the urine samples, with five samples from nonsmokers containing an unknown interfering substance that coeluted with caffeine that made quantitation impossible.

As observed for the Arkansas nonsmokers, the frequency distribution (not shown) of the urinary [17X + 17U]/137X ratio in the 45 Italian nonsmokers was not normal, as indicated by the Wilk-Shapiro test ($W = 0.785$, $p < 0.01$). Moreover, the frequency distribution for the 31 smokers of blonde tobacco ($W = 0.617$; $p < 0.01$) and 16 smokers of black tobacco ($W = 0.438$; $p < 0.01$) also did not appear normal. In addition, the distribution of ratios was skewed (1.65, 3.80, 3.77) and showed positive kurtosis (2.19, 17.80, 14.70) in nonsmokers, and smokers of blonde and black tobacco, respectively. The median [17X + 17U]/137X ratio in smokers of blonde tobacco

(4.90) was significantly higher ($p = 0.024$) than the median value in nonsmokers (3.02) or in smokers of black tobacco (3.44, $p = < 0.05$). The median ratios in nonsmokers and black tobacco smokers were not significantly different ($p = 0.85$).

Probit transformation of the [17X + 17U]/137X ratios, determined in the 45 nonsmoking subjects, indicate further that the ratios in this group did not have a normal distribution (Fig. 4). Using linear regression analysis, an apparent trimodal distribution is suggested with breakpoints at [17X + 17U]/137X ratios near 2 and 5, which is again consistent with the existence of slow, intermediate and rapid phenotypes. As with the Arkansas nonsmokers, a trimodal distribution in smokers of blonde or black tobacco could not be detected.

CYP1A2 phenotypes of Chinese subjects

The caffeine phenotyping procedure used to describe the CYP1A2 phenotypes in the Arkansas and Italian subjects was also applied to a population of 78 Chinese male subjects. As observed for the Italian population, the Wilk-Shapiro test of normality indicated that the [17X + 17U]/137X ratios did not have a normal distribution in either the 30 nonsmokers ($W = 0.574$, $p < 0.01$) or in the 48 smokers ($W = 0.576$, $p < 0.01$). The distribution of ratios was skewed (3.59, 3.80) and showed positive kurtosis (15.03, 17.37) for nonsmokers and smokers, respectively. In contrast to the Arkansas and Italian blonde tobacco smokers, there were no differences ($p = 0.586$) in the median urinary ratios between the smokers (5.12) and nonsmokers (5.37).

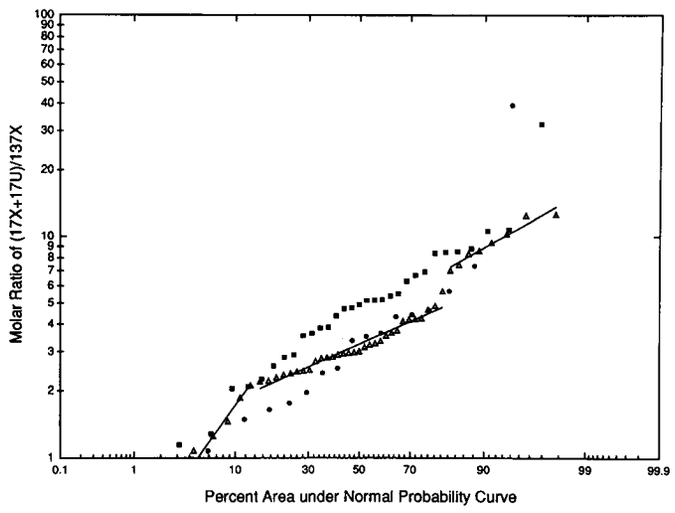

Fig. 4. Probit analyses of the urinary molar ratio of [17X + 17U]/137X in Italian nonsmokers and smokers. The abscissa and ordinate are as described in Fig. 3. Nonsmokers (open triangles); blonde tobacco smokers (closed squares); black tobacco (closed circles).

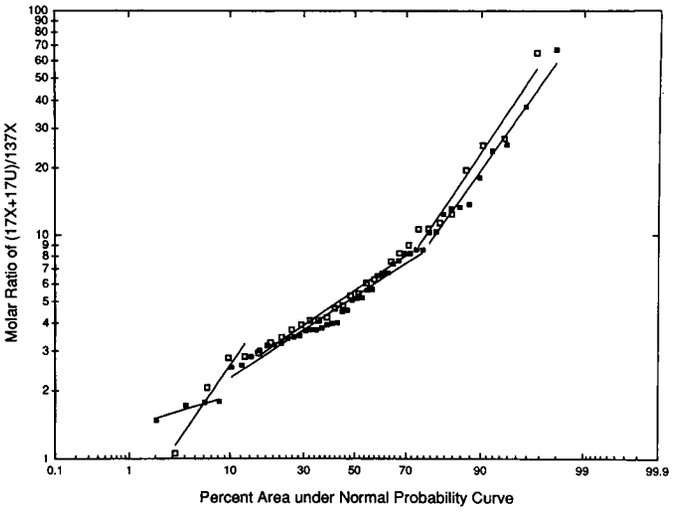

Fig. 5. Probit analyses of the urinary molar ratio of [17X + 17U]/137X in Chinese nonsmokers and smokers. The abscissa and ordinate are as described in Fig. 3. Nonsmokers (open squares); smokers (closed squares).

Probit transformations of the urinary [17X + 17U]/137X ratios in Chinese smokers and nonsmokers, as shown in Fig. 5, also indicated that neither ratio was normally distributed in either the smokers or the nonsmokers. Using linear regression analyses, a breakpoint near 2 and near 10 could be used to divide the smokers and nonsmokers into slow, intermediate, and rapid CYP1A2 phenotypes.

Discussion

Using a simple, noninvasive assay in which caffeine is administered in coffee, urine is collected between 4–5 h after dosing, and caffeine metabolites are analysed by HPLC, we present data that suggest the existence of slow, intermediate, and rapid CYP1A2 phenotypes in each of several human populations and we have confirmed that smoking can induce CYP1A2 activity. Apparent tri-linear probit plots of the urinary molar ratio of [17X + 17U]/137X were observed for nonsmoking populations from Arkansas, Italy, and the Peoples' Republic of China (Fig. 6). In both the Arkansas and Italian blonde tobacco smokers, the median [17X + 17U]/137X ratio was increased and probit plots did not show apparent breakpoints. This is consistent with partial enzyme induction by cigarette smoking, which would be expected to obscure the distinction between phenotypes. The percentage of individuals with the rapid phenotype varied in the different groups, ranging from 20% rapid in the Italian nonsmokers to 37% rapid in the Arkansas

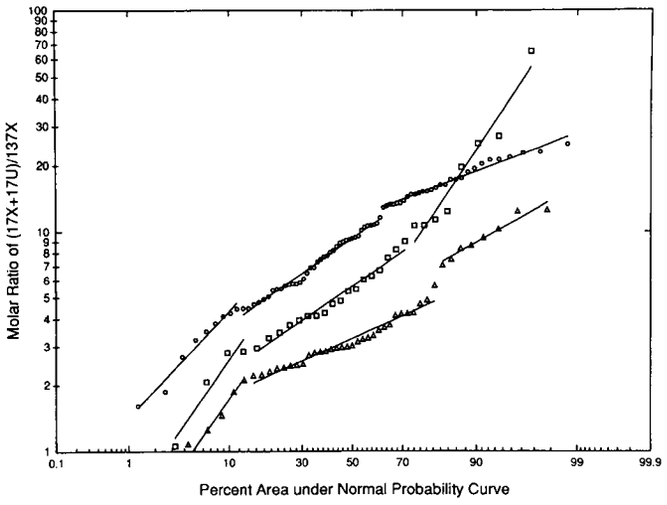

Fig. 6. Probit analyses of the urinary molar ratio of [17X + 17U]/137X in nonsmokers from Arkansas, Italy, and the Peoples' Republic of China. The abscissa and ordinate are as described in Fig. 3. Arkansas (open circles); Italy (open triangles); China (open squares).

nonsmokers. The proportion of individuals with the apparent slow phenotype narrowly ranged from 12–13% in the three populations. This is consistent with previous findings that about 10% of populations are deficient in phenacetin *O*-deethylase activity (Devonshire *et al.*, 1983), which is now known to be exclusively catalysed by CYP1A2 (Distlerath *et al.*, 1985). These phenotypes may be due to a genetic polymorphism, dependent on the gene frequency distribution in the populations. A genetic polymorphism for CYP1A2 has been suggested by a family study in which impaired phenacetin-*O*-deethylation ability was noted in two siblings (Shahidi, 1968). However, additional family studies or population genotyping will be needed to confirm this finding.

In previous reports, the determination of CYP1A2 variability and the effect of inducers on its activity were based on the results of either *in vitro* assays utilizing human liver microsomes or on *in vivo* assays using other caffeine urinary metabolite ratios. The latter include the ratios [AFMU + 1X + 1U]/17U (Vistisen *et al.*, 1991) and [AAMU + 1X + 1U]/17U (Kalow & Tang, 1991) that reflect paraxanthine 7-demethylation activity. In contrast to caffeine 3-demethylation, paraxanthine 7-demethylation has not been demonstrated to be catalysed by CYP1A2 using classical approaches such as inhibition by chemical inhibitors or antibodies specific for CYP1A2, or catalysis by purified CYP1A2. Although there are significant correlations between paraxanthine 7-demethylation and caffeine 3-demethylation ($r = 0.83$) *in vitro* (Campbell *et al.*, 1987a), and the urinary metabolite ratio (AFMU + 1X + 1U)/17U and also caffeine clearance ($r = 0.91$) *in vivo* (Campbell *et al.*, 1987b), such correlations are not definitive proof that a common enzyme is involved. For example, studies with specific inhibitors have shown that, although caffeine 3-demethylation, caffeine 1-demethylation, and caffeine 7-demethylation are highly correlated ($r > 0.92$) in human liver microsomal preparations (Campbell *et al.*, 1987a), CYP1A2 is the principal enzyme responsible for only the 3-demethylation of 137X (Berthou *et al.*, 1991; Fuhr *et al.*, 1990; Butler *et al.*, 1990b). Moreover, when we used the urinary [AFMU + 1X + 1U]/17U ratio in a direct comparison with our [17X + 17U]/137X ratio in the Italian urines, a normal rather than trimodal distribution was observed, as has been reported by Vistisen *et al.* (1991) and by Kalow & Tang (1991).

The apparent lack of a normal distribution of [17X + 17U]/137X ratios *in vivo* is also strongly supported by previous *in vitro* studies with human liver microsomes that show a bimodal distribution for the CYP1A2-dependent *N*-oxidation of 2-acetylaminofluorene (Minchin *et al.*, 1985) and the *O*-deethylation of phenacetin, as well as for levels of immunoreactive CYP1A2 (Sesardic *et al.*, 1988).

Large interindividual variations in the activity of hepatic CYP1A2 have been demonstrated in our study, where the urinary metabolite [17X + 17U]/137X ratio that reflects CYP1A2 activity varied 38-fold in the subjects from Arkansas, 70-fold in the Italian subjects, and 67-fold in the Chinese subjects. This is consistent with the observation that phenacetin metabolism also varied 58-fold in different human subjects (Alvares *et al.*, 1979). Similar interindividual variations in levels of hepatic microsomal CYP1A2 have been shown in several earlier *in vitro* studies. For example, rates of *N*-oxidation of 4-aminobiphenyl and 2-naphthylamine varied >44-fold (Butler *et al.*, 1989a) and >28-fold (Hammons *et al.*, 1985), respectively, in different human liver microsomes. Likewise, the amount of immunoreactive CYP1A2 varied >60-fold in other preparations (Sesardic *et al.*, 1988). Similar results were also reported for the mutagenic activation of the heterocyclic arylamines, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), which is likewise catalysed selectively by human liver microsomal CYP1A2 (Yamazoe *et al.*, 1988). Furthermore, greater than 15-fold differences in CYP1A2 mRNA levels have been observed among 12 human liver samples (Ikeya *et al.*, 1989). In contrast, only a 6.3-fold range was found in 178

subjects using the [AAMU + 1X + 1U]/17U ratio (Kalow & Tang, 1991).

Large interindividual differences in [17X + 17U]/137X ratios may also reflect exposure to enzyme inducers such as cigarette smoke, charbroiled food, and certain cruciferous vegetables known to induce CYP1A2 (Guengerich & Shimada, 1991). Cigarette smoking, for example, has been demonstrated to stimulate phenacetin metabolism (Pantuck *et al.*, 1974) and is associated with increased amounts of immunoreactive CYP1A2 in human liver microsomes (Sesardic *et al.*, 1988) and decreased plasma half-lives of caffeine *in vivo* (Parsons & Neims, 1978). Similarly, the rate of phenacetin *O*-deethylation in humans *in vivo* is increased by consumption of charcoal-broiled meat (Conney *et al.*, 1976) and is also stimulated in humans fed cabbage and brussels sprouts (Pantuck *et al.*, 1979). In our study, smoking increased CYP1A2 activity in all populations with the exception of the Chinese group. One possibility is that these Chinese are a fully induced group, perhaps as a consequence of components in their diet and/or environment, such that smoking has no further inductive effect. Alternatively, this population may be uninducible or the cigarette tobacco used does not contain inducers. This apparent lack of induction was also observed in Italian smokers of black but not blonde tobacco. A remaining possibility is that black tobacco may contain potent *in vivo* inhibitors of CYP1A2, as has been reported in *in vitro* studies with cigarette smoke condensate (Shimada & Guengerich, 1991). Other evidence further suggests that these study populations may differ in the extent of enzyme induction. As shown in Fig. 6, the median (50 percentile) 17X + 17U]/137X ratios vary from 3.02 to 5.37 to 9.27 in Italian, Chinese, and Arkansas nonsmokers, respectively. Thus, the differences in median caffeine 3-demethylation proficiency between the three populations, combined with the observation that smoking does not induce CYP1A2 in the Chinese subjects, suggest that these populations may have different gene frequencies and may also be exposed to dissimilar inducers and/or inhibitors in their diet and environment.

Exposure to dietary and environmental inducers and inhibitors may also contribute to the magnitude of the coefficients of variation in CYP1A2 activity that was observed in the subjects who were phenotyped once weekly for 5 weeks. Coefficients of variation were larger in the individuals who did not smoke, compared with those seen in the smokers. Similar intraindividual variations in phenacetin metabolism have been reported in seven individuals who were administered phenacetin on five separate occasions (Alvares *et al.*, 1979). It has been suggested that environmental factors are responsible for approximately 50% of the variability observed in phenacetin *O*-deethylation (Devonshire *et al.*, 1983). In contrast, intraindividual variability in NAT2 activity was lower than variability in CYP1A2 in the same individuals in our study. NAT2 is generally considered to be a non-inducible enzyme in humans (Hein, 1988), although it has been reported that the rate of acetylation may be affected by dietary components such as alcohol which alter the availability of the co-substrate, acetyl coenzyme A (Olsen & Mørland, 1978).

One of the advantages of the method described herein is that both the CYP1A2 and NAT2 phenotypes can be determined from one urine extract and HPLC profile. Although it was reported that there is complete concordance between NAT2 phenotyping with caffeine using the AFMU/1X molar ratio, and results using a standard sulfamethazine phenotyping procedure (Grant *et al.*, 1984), several investigators have voiced concerns about the instability of AFMU, particularly under alkaline conditions occasionally present in the urinary bladder, and have suggested the use of an alternate method in which AFMU is converted to AAMU prior to analysis (Tang *et al.*, 1987; Kilbane *et al.*, 1990, Tang *et al.*, 1991). AFMU has been shown to convert to AAMU *in vitro* at pH 10 (Tang *et al.*, 1983) and was reported as unstable in buffered solutions (Lorenzo & Reidenberg, 1989). However, our results indicate that AFMU/1X ratios are similar when urine remains in the bladder between 4–5 h at pH 8.0 or at pH <7.0, and when stored at pH 8.0 at $-20°$ C for 11 days before analysis.

The AFMU/1X ratio appears to be a more precise measure of NAT2 activity than that obtained after the conversion of AFMU to AAMU. It has been proposed that AFMU forms from an unstable intermediate, yet to be identified, which arises from the 7-demethylation of 17X (Grant *et al.*, 1983). In rapid acetylators, the unstable intermediate is acetylated by the polymorphic NAT2 and remains in the open-ring form; while in slow acetylators, it closes to form 1X before acetylation can occur (Grant *et al.*, 1983). However, the data also allow the possibility that AAMU is a metabolite of caffeine that forms from another intermediate compound (Callahan *et al.*, 1983), which may be acetylated by the monomorphic *N*-acetyltransferase (NAT1) rather than by decomposition of AFMU. In this regard, in individuals phenotyped with both isoniazid and caffeine (Callahan *et al.*, 1983), the cumulative urinary excretion of AFMU[1] shows a much better

[1]The metabolite referred to as A2 by Callahan *et al.* (1983) and Branfman *et al.* (1983) has been deduced to be AFMU, based on comparisons of its stability in methanol, chromatographic properties, and decomposition to AAMU as described by Tang *et al.* (1983).

correlation ($r = 0.97$) with acetylisoniazid/isoniazid ratios than the cumulative urinary excretion of AAMU ($r = 0.23$) or AAMU + AFMU ($r = 0.72$).

The characterization of metabolism of drugs that are relatively safe and acceptable to the human population has long been proposed as a possible indicator to determine how individuals metabolize carcinogens that are activated and/or detoxified by the same enzymes (Conney & Levin, 1974). Determination of urinary caffeine metabolite ratios in humans (Grant *et al.*, 1983; Campbell *et al.*, 1987a) and the caffeine breath test (Wietholtz *et al.*, 1981; Kotake *et al.*, 1982) have been proposed as methods to characterize polycyclic aromatic hydrocarbon inducible enzymes in humans. The determination of CYP1A2 phenotypes by the urinary metabolite ratio [17X + 17U]/137X, along with the identification of the NAT2 phenotypes by the AFMU/1X molar ratio, using a simple method of analysis of caffeine urinary metabolites in urine from the 4–5 h interval after an individual has consumed caffeine in a cup of coffee now provides a method for assessing the role of both CYP1A2 and *N*-acetyltransferase in individual susceptibility to arylamine-induced cancers.

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