ORIGINAL RESEARCH ARTICLE

Clin Pharmacokinet 2003; 42 (15): 1393-1409
0312-5963/03/0015-1393/$30.00/0

Nonparametric Expectation Maximisation (NPEM) Population Pharmacokinetic Analysis of Caffeine Disposition from Sparse Data in Adult Caucasians

Systemic Caffeine Clearance as a Biomarker for Cytochrome P450 1A2 Activity

Dimiter Terziivanov,[1] *Kristina Bozhinova*,[1] *Velislava Dimitrova*[1] and *Ivanka Atanasova*[2]

1 Clinic for Therapeutics and Clinical Pharmacology, University Hospital 'St. I. Rilsky', Sofia, Bulgaria
2 Department of Drug Policy, Ministry of Health, Sofia, Bulgaria

Abstract

Objective: To explore the ability of the nonparametric expectation maximisation (NPEM) method of population pharmacokinetic modelling to deal with sparse data in estimating systemic caffeine clearance for monitoring and evaluation of cytochrome P450 (CYP) 1A2 activity.

Design and participants: Nonblind, single-dose clinical investigation in 34 non-related adult Bulgarian Caucasians (18 women and 16 men, aged between 18 and 62 years) with normal and reduced renal function.

Methods: Each participant received oral caffeine 3 mg/kg. Two blood samples per individual were taken according to the protocol for measuring caffeine plasma concentrations. A total of 67 measured concentrations were used to obtain NPEM estimates of caffeine clearance. Paraxanthine/caffeine plasma ratios were calculated and correlated with clearance estimates. Graphical methods and tests for normality were applied and parametric and nonparametric statistical tests were used for comparison.

Results: NPEM median estimates of caffeine absorption and elimination rate constants, k_a = 4.54 h^{-1} and k_{el} = 0.139 h^{-1}, as well as of fractional volume of distribution and plasma clearance, V_{S1} = 0.58 L/kg and CL_{S1} = 0.057 L/h/kg, agreed well with reported values from more 'data rich' studies. Significant correlations were observed between paraxanthine/caffeine ratios at 3, 8 and 10 hours and clearance (Spearman rank correlation coefficients, r_s, >0.74, $p \leq 0.04$). Sex or renal function caused no significant differences in clearance. Heavy smokers and drinkers showed 2-fold higher CYP1A2 activity. Normality tests and graphical methods of analysing caffeine clearance supported a non-Gaussian and multicomponent distribution of CYP1A2 activity.

Conclusions: Collectively, the results show that the NPEM method is suitable and relevant for large-scale epidemiological studies of population phenotyping for cancer susceptibility and for abnormal liver function by monitoring CYP1A2 activity based on sparse caffeine data.

The human *CYP1* gene family is composed of two structural genes, *CYP1A1* and *CYP1A2*, which encode the cytochrome P450 (CYP) 1A1 and 1A2 proteins.[1] The *CYP1A1* and *CYP1A2* genes differ in their tissue-specific expression. In humans, the CYP1A2 enzyme metabolises a broad spectrum of polycyclic aromatic hydrocarbons including potential environmental procarcinogens[2-5] and drugs (recently reviewed by Carrillo and Benitez[6]). CYP1A2 is expressed in the liver[7] and accounts for nearly 15% of the total hepatic CYP content.[8] Therefore, monitoring of CYP1A2 activity may supply useful information concerning progression of hepatic disorders,[9-11] as well as the outcome of liver transplantation.[12] Since the total involvement of CYP1A2 in caffeine metabolism accounts for more than 95% of caffeine plasma or blood clearance,[13,14] caffeine clearance is widely used as a biomarker[15] for monitoring CYP1A2 activity in human populations of interest.

There are different approaches for parametric assessment of caffeine systemic clearance directly from plasma or saliva data,[16-21] or indirectly, by regression of paraxanthine/caffeine plasma (saliva) ratios to caffeine clearance.[18-22] The parametric clearance methods have had their shortcomings recently reviewed by Denaro et al.[9] None of these methods, however, was a true population modelling method, though the Bayesian method they used is similar to a standard two-stage population modelling approach. Conventional parametric drug clearance measurements have needed 'data rich' clinical protocols for each participant in order to obtain sufficient accuracy and precision in the estimation of clearance.[6] Moreover, the parametric methods, which presume a normal distribution of parameter values in the populations, have been used to analyse what is really a non-Gaussian population distribution of CYP1A2 activity, which in a series of studies has been reported to follow a multimodal (bi- or trimodal) distribution.[22-27]

In the last 20 years, both parametric and nonparametric methods have been developed for evaluating population parameter distributions from a given data set.[28] Nonparametric methods, like the nonparametric maximum likelihood (NPML) method of Mallet [29] and the nonparametric expectation maximisation (NPEM) method of Schumitzky,[30] do not make any parametric assumptions about the shape of the estimated parameter distribution, i.e. the distribution of population parameters is not *a priori* related to any particular form of parametric distribution. As outlined recently by Mallet and colleagues,[31] multimodal distributions are described with a higher resolution by the NPML method than by using parametric methods.

In recently published works, several authors[28,32-34] have demonstrated the reliable abilities of the NPEM method, in spite of a rather limited number of patients, to reveal unexpected subpopulations of patients whose drug clearance clearly deviated from a central tendency. Therefore, it was of clinical and research interest to explore the capability of the NPEM method to detect unsuspected subpopulations during a population pharmacokinetic analysis based on sparse data of caffeine.

Participants and Methods

Selection of Volunteers, Study Design and Blood Sampling

Inclusion criteria were non-related volunteers of both sexes, aged between 18 and 65 years. Exclusion criteria were any metabolic, mental or systemic gastrointestinal disorders, age above 65 years, or any intake of methylxanthines, grapefruit juice, cruciferous vegetables (cabbage, turnips, broccoli, radishes, horseradish, alyssum), spinach or alcohol 72

hours prior to the study day, participation in clinical trials with blood withdrawals within the last 3 months, and pregnancy. None of the volunteers had clinical signs or laboratory evidence of hepatic dysfunction. The women participating in the study did not take any oral contraceptives, and they entered the study between two menses. Three of the volunteers (nos. 10, 23 and 24; table I) claimed to be regular consumers of coffee (more than three cups of coffee per day). Heavy smokers were considered those who smoked more than ten cigarettes per day. Heavy consumers of alcohol were considered to be those drinking more than the equivalent of 50mL of distilled alcohol per day.

The study was carried out on 34 adult Bulgarian Caucasians with both normal and reduced renal function as assessed by measuring their endogenous creatinine clearance (table I).[36] Of the 34 participants, 18 were women and 16 were men, aged between 18 and 62 years (mean age 34.9 years, median age 34.5 years), weighing between 45 and 114kg (mean 72.3kg, median 71kg) and height between 148 and 189cm (mean 169.7cm, median 167.5cm). Written informed consent for participation in the study was signed by all of the participants and permission from the local Ethics Committee was granted.

The study was designed as a nonblind, single-dose clinical investigation. The volunteers were hospitalised for at least 24 hours prior to the investigation and put under standard conditions with respect to diet and physical activity. The day before the investigation any drug treatment was discontinued so that during the study period all volunteers were free of any medication. After a 12-hour overnight fast, on the morning of the study day each participant was given an oral caffeine dose of 3 mg/kg bodyweight (as *Coffeinum purum*; Knoll) in a capsule with 100mL of water, and remained in bed for 2 hours, when a standard breakfast was served.

Table I. Caffeine median population model parameters, as estimated by the nonparametric expectation maximisation (NPEM) method. Values are medians ± dispersion factor[a]

Parameter and unit	Estimate
k_a (h^{-1})	4.54 ± 4.01
V_{S1} (L/kg)	0.58 ± 0.29
CL_{S1} (L/h/kg)	0.057 ± 0.066
k_{el} (h^{-1})	0.139 ± 0.089

a The dispersion factor is the average of the dispersion factor for the central 50% of the density and for the central 95% and represents an ad hoc single measure similar to the SD, but suited best for the shape actually found for each parameter distribution.[35]

CL_{S1} = fractional systemic caffeine clearance (slope of plasma clearance to bodyweight for one-compartment disposition model); **k_a** = absorption rate constant; **k_{el}** = elimination rate constant; **V_{S1}** = fractional volume of distribution (slope of volume of distribution to bodyweight for one-compartment disposition model).

For the determination of caffeine and paraxanthine plasma concentrations, blood samples were collected from each participant immediately before drug intake (time zero) and at two different time points over a 24-hour interval (table I) in such a way that at least one blood sample belonged to the anticipated elimination phase of caffeine.[37,38] According to the study protocol, eight subjects were sampled at 0.5 and 3 hours after caffeine administration, six subjects had samples obtained at 1 and 7 hours, another six subjects at 2 and 6 hours, seven subjects at 4 and 8 hours, six subjects at 10 and 24 hours, and one subject at 4 hours after drug intake. Blood samples (5mL) were collected into heparinised tubes, centrifuged at 1240*g* for 10 minutes, and the plasma was transferred to labelled tubes. All samples were stored at –20°C until assay.

Assay Method for Caffeine and for its Primary Metabolites

Reagents and Chemicals

Caffeine (p.a. grade), theobromine (p.a. grade), paraxanthine (~98%) and theophylline (p.a. grade) were purchased from Sigma Aldrich Chemie GmbH (Deisenhofen, Germany).

Methanol (gradient grade), tetrahydrofuran (for spectroscopy), acetic acid (p.a. grade), chloroform (for chromatography), isopropanol (p.a. grade), ammonium sulphate (p.a. grade) and disodium tetraborate (p.a. grade) were obtained from Merck (Darmstadt, Germany).

Instruments and Instrumental Conditions

Analyses were performed on a high performance liquid chromatography (HPLC) system consisting of an automated gradient controller (Millipore Corp., Waters Assoc., Milford, MA, USA), two Waters model 501 pumps, a manual Waters model U6K injector, a photodiode detector (Waters model 911) set at 274nm and a computer (Nec Power Mate SX/16) with a printer-plotter (Waters 5200). The column was reversed phase 10µm LiChrosphere 100 RP-18, 250 ×4mm (Merck) with a precolumn of 10µm LiChrosphere 100 RP-18, 40 × 4 mm (Merck). The mobile phase consisted of 0.01 mol/L acetic acid/methanol/tetrahydrofuran (92 : 6 : 2, v/v/v). The eluent was filtered through a Millipore filter (pore size 0.22µm) before it was used. A flow rate of 1.5 mL/min and a pressure of 1500 psi were used.

Standard Solutions and Sample Preparation

All standard stock solutions (100 mg/L) were prepared by dissolving 10mg of caffeine or of the primary metabolites in 35mL of disodium tetraborate, pH 7.0, and made up to 100mL with distilled water. A modification of the method of Grant et al.[39] was used.[40] Plasma samples (500µL) were transferred to glass tubes and 6mL of chloroform/isopropanol (95 : 5, v/v) was added. The tubes were vortex-mixed vigorously for 1 minute. After centrifugation for 10 minutes at 4500 rev/min, the organic phase was removed, and 5mL of the organic layer was transferred to a centrifuge tube and evaporated at 40°C under a stream of air. The residue was dissolved in HPLC mobile phase solvent, and 100µL was injected into the HPLC system.

Validation of the Analytical Method and Determination of the Assay Error Pattern

Very low variation was observed in the retention times for the study compounds within and between days over a 6-month period. Mean values for caffeine and paraxanthine only, in minutes with their SDs, were as follows: within-day, caffeine 11.7 (0.12) and paraxanthine 6.49 (0.05) [n = 10 analyses per working day]; between-day, caffeine 11.4 (0.65) and paraxanthine 6.32 (0.32) [n = 8 analyses]. Mean percentage recoveries from plasma, with percentage coefficients of variation (CV%), were: within-day, caffeine 87.53% (5.83%) and paraxanthine 74.46% (2.43%) [n = 10 analyses]; between-day, caffeine 88.57% (9.86%) and paraxanthine 76.67% (2.85%) [n = 3 analyses]. At a concentration of 0.24 mg/L the accuracy of the method (closeness of determined value to the true value) was 100% for caffeine and 87.5% for paraxanthine; at 2.4 mg/L it was 93.3% for caffeine and 97.9% for paraxanthine; and at 9.6 mg/L it was 97.2% for caffeine and 96.1% for paraxanthine [n = 4 measurements]. The within-day CV% for each of the compounds was below 15% for low, 7% for medium and 8% for high drug concentrations. Between-day CV% was below 20% for all samples. The calibration curves (from 0.024 to 9.6 mg/L) showed a high degree of linearity ($r = 0.99$, $p < 0.05$). The lower limit of quantification (LOQ) was 0.024 mg/L for both caffeine and paraxanthine when defined as the lowest concentration of analyte measured with a CV% of ≤20%. To determine the assay error pattern over the entire working range of caffeine concentrations from 0.024 to 18 mg/L, the assay SDs were fitted to a third-order polynomial equation. The results were:

$$SD = 0.002060 + 0.200717\ C - 0.039061\ C^2 + 0.002854\ C^3$$

where C is the measured caffeine plasma concentration. The equation reflects a CV% of 12% for the average, equal to 2.92 mg/L, of all 67 measured caffeine concentrations. This equation was introduced into the NPEM2 program. The dispersion factor gamma, representing the intra-individual variability found by prior analysis with the IT2B iterative two-stage Bayesian population modelling program,[41] was 4.30, showing that about 25% of overall intra-individual SD was due to the assay error itself, and that about 75% was due to other environmental causes such as errors in preparing and administering the doses, errors in recording when the doses were actually given, errors in recording when the serum samples were actually drawn, misspecification of the structural pharmacokinetic model, and any changes in a patient's parameter values that may have taken place during the period of data collection.

Population Pharmacokinetic and Statistical Analyses

Actual population modelling consisted of developing a population pharmacokinetic model using the NPEM2 algorithm based on sampled individual caffeine concentrations. The study was divided into two steps. Step 1 involved NPEM2 analysis of a total of 67 sparse, randomly distributed, caffeine concentration-time points (full data set) over a 24-hour period. This generated estimates of caffeine clearance that were used for testing the normality of the distribution of the systemic drug clearance. Step 2 of the study consisted of analysing the time distribution of the relationship between paraxanthine/caffeine plasma ratios and caffeine clearance as assessed by the NPEM2 estimator.

Development of a Nonparametric Population Pharmacokinetic Model of Caffeine Based on Sparse Data Sets.

Individual patient clinical data files were entered into the USC*PACK clinical (PASTRX) files (version 10.6). A total of 67 caffeine concentrations (two per volunteer except for participant no. 10, with one drug concentration; table I) were available for population analysis with the NPEM2 estimator. One- and two-compartment disposition models (1-COM and 2-COM) with first-order input were compared. The appropriateness of both models was tested based on criteria for evaluating the goodness of fit (coefficients of correlation and determination, r and r^2) and on measures of predictive performance as suggested by Sheiner and Beal.[42] The bias was evaluated as the mean prediction error, ME, the mean difference between observed and predicted drug concentrations. The precision was calculated as root mean squared prediction error, RMSE, and is equal to the square root of the mean squared prediction error. This is used to convert the measure of precision back into concentration units. Both measures of predictive performance were derived from the NPEM2 analysis. The comparison did not reveal any substantial difference between models in fitting population plasma caffeine concentrations ($r^2_{1\text{-COM}}$ = 0.95 and $r^2_{2\text{-COM}}$ = 0.93; $r_{1\text{-COM}}$ = 0.98 and $r_{2\text{-COM}}$ = 0.97; $p < 0.0001$ for both models), as well as in their predictive ability ($ME_{1\text{-COM}}$ = –0.11 mg/L and $ME_{2\text{-COM}}$ = –0.26 mg/L; $RMSE_{1\text{-COM}}$ = 0.64 mg/L and $RMSE_{2\text{-COM}}$ = 0.79 mg/L; log-likelihoods = –195.59 for 1-COM and –523.49 for 2-COM). Based on these data, on the law of parsimony, and on its greater likelihood, further population modelling was performed using the one-compartment disposition model with first order input (1-COM). Two types of parameterisation were compared using the full data set with means and medians as single measures of central tendency.

The first type of parameterisation was carried out with the slope of volume of distribution to bodyweight, V_{S1} (L/kg), the slope of caffeine plasma clearance to bodyweight, CL_{S1} (L/h/kg), and the absorption rate constant, k_a (h^{-1}). The second type of parameterisation included the elimination rate constant, k_{el} (h^{-1}), instead of CL_{S1} (table II). With both types of parameterisation, caffeine bioavailability was assumed to be 100%.[37] The respective log-likelihoods were –195.59 and –178.92. The values for skewness and kurtosis for the distribution of CL_{S1} were 1.165 and 3.585, and for that of k_{el} were 0.701 and 2.674, respectively. The parameterisation with k_{el} increased both the skewness and kurtosis for the distribution of V_{S1} as compared with the parameterisation with CL_{S1}, from 1.788 and 7.969 to 2.122 and 10.360, respectively. Furthermore, the inclusion of CL_{S1} in the parameterisation resulted in decreased population parameter CV% values: the CV% values of V_{S1} and k_a decreased from 56% to 51% and from 114% to 106%, respectively. Moreover, there was no significant difference between the mean values of V_{S1} after either type of parameterisation (0.595 ± 0.304 vs 0.530 ± 0.297 L/kg, p = 0.374). In order to estimate individual caffeine clearances directly from drug plasma concentrations, the caffeine population pharmacokinetic model was subsequently parameterised with CL_{S1} instead of k_{el}. The predictive performance of the population model was measured as described above.

Analysis of the Relationship between Paraxanthine/Caffeine Ratio and Systemic Caffeine Clearance.

The paraxanthine/caffeine plasma molar ratios[22] were calculated for all of the sampling times (from 0.5 to 24 hours) and each subset of patients sampled at that particular timepoint was analysed for correlation with the corresponding patient's plasma clearances (CL_{S1}). Similar correlation analysis was performed with caffeine plasma concentrations instead of paraxanthine/caffeine ratios. Correlation analyses were evaluated by means of nonparametric Spearman rank correlation coefficients (r_s). The correlations were accepted as statistically significant at $p < 0.05$ and the indicated p values in the text were two-tailed.

Statistical Analyses

Statistical assessments and graphics were performed by means of STATISTICA version 4.3, GraphPad PRISM version 2.01 and Microcal Origin 4.1. The distributions of individual CL_{S1} estimates, untransformed and logarithmically transformed (to the base 10), were tested for normality by the following statistical tests: Kolmogorov-Smirnov, Lilliefors, Shapiro-Wilk's W test and χ^2 test. The null hypothesis for a Gaussian distribution was rejected at $p < 0.05$. Furthermore, skewness and kurtosis were used as additional ad hoc criteria for testing normality. The estimates of skewness and kurtosis of the distributions of untransformed and log-transformed clearance data, as computed by STATISTICA version 4.3, were checked for statistical significance (symbolism according to Afifi and Azen[43]) and the following two null hypotheses were verified: H_0: skewness = 0 and H_0: kurtosis = 3. Asymptotically, each of the statistics z_0 = skewness/(standard error of skewness) and z_0 = (kurtosis – 3)/(standard error of kurtosis) is distributed according to a standardised normal distribution N(0,1), and p-values could be read from statistical tables of this distribution elsewhere.[43]

Additionally, frequency distributions and cumulative frequencies of untransformed and log-transformed estimates of caffeine plasma clearance were tested for normality by probit analysis. Taking into account the outcomes of normality tests, showing non-Gaussian distribution of systemic caffeine clearance (see also the Results section), the results were presented as medians along with the 25th and 75th percentiles in parentheses (25th percentile;

Table II. Individual values of fractional systemic caffeine clearance as estimated by the nonparametric expectation maximisation (NPEM) method, ordered by increasing magnitude

Subject	CL_{S1} (L/h/kg)
34	0.00253
1	0.00758
14	0.00782
18	0.00909
15	0.01250
23	0.01253
31	0.01750
13	0.04250
17	0.04250
21	0.04250
22	0.04750
26	0.04750
7	0.04752
6	0.04760
10	0.04825
2	0.04829
11	0.05192
12	0.05704
5	0.07675
19	0.07779
9	0.08749
33	0.08857
8	0.08869
28	0.10250
3	0.12706
20	0.12755
24	0.12755
32	0.14228
4	0.14278
29	0.14440
25	0.21259
30	0.24775
16	0.27206
27	0.27250
Mean ± SD	0.08620 ± 0.07468
Geometric mean	0.05437
25th percentile	0.0425
Median	0.05448
75th percentile	0.12755

CL_{S1} = fractional systemic caffeine clearance (slope of plasma clearance to bodyweight for one-compartment disposition model).

75th percentile). The individual values of the structural pharmacokinetic parameter estimates, resulting from NPEM2 analysis, were the medians from each subject's Bayesian posterior joint density function, if not stated otherwise.

The two-tailed Mann Whitney U (the rank sum) test for evaluation of differences between medians was used as a nonparametric test at level of significance $\alpha = 0.05$.

Results

Evaluation of Systemic Caffeine Clearance by NPEM2 Estimator Based on Sparse Data Observed Over 24 Hours

The medians of the structural pharmacokinetic parameters are listed in table II. Visual inspection of figure 1a and figure 1b, describing the approximate marginal density of the distribution of CL_{S1} and joint density of V_{S1} and CL_{S1}, respectively, suggested an apparently multimodal distribution of systemic caffeine clearance. Figure 1a displays the discrete distribution of clearance in our panel of individuals. The total probability of the clearances below 0.02 L/h/kg was about 21%, and for the clearances from 0.04 to 0.15 L/h/kg it was 66%. A well-segregated subgroup, located at clearance values > 0.2 L/h/kg with a total probability of about 13%, was clearly seen. The highest probability, greater than 17%, was of a clearance about 0.050 L/h/kg.

Moreover, as shown in table III, when clearance values were arranged in increasing order, three clusters or subgroups of subjects were seen: subpopulation 1 (n = 7) had clearance values up to 0.02 L/h/kg, subpopulation 2 (n = 23) formed an intermediate group with clearances from 0.04 to 0.15 L/h/kg, and subpopulation 3 (n = 4) had clearance estimates over 0.21 L/h/kg.

There was no tendency for slower clearance in the individuals who were sampled at the later time points. For instance, subjects 13, 14, 15, 18, 23 and 31 were sampled at the earlier times (0.5 and 3 hours) but their clearance values were about 6–13 times lower than those of subjects 20, 30 and 32 who were sampled at the latest time points (10 and 24 hours) after administration (see tables I and II). The general question of a correlation between the times at which the serum samples were obtained and the resulting values of clearance was examined. No significant correlation was found. The relationship between clearance and sampling time was found to be: clearance = 74.2 + 1.99 × time ($r = 0.1699$, $r^2 = 0.0289$, $p > 0.10$).

Figure 2 shows the predictions made using each subject's individual nonparametric Bayesian posterior median parameter values to predict measured caffeine concentration in each volunteer. There was highly significant correlation between individual measured and population model predicted drug concentrations. The predictive performance of the population model was characterised by low bias and good precision: ME = –0.11 mg/L and RMSE = 0.64 mg/L. The population model slightly underestimated measured drug concentrations.

The participants with estimates of caffeine clearance between 0.12 and 0.15 L/h/kg (subjects 3, 20, 24, 32, 4 and 29; table III) were not smokers or heavy consumers of alcohol, except for subjects 32 and 20, who were as reported to smoke 5 and 17 cigarettes per day, respectively (see also table I). In contrast, all subjects (except one, no. 16) with highest systemic clearance, ranging between 0.210 and 0.275 L/h/kg, were heavy smokers and heavy drinkers of alcohol (table I). The participant with the lowest NPEM2 estimate of systemic caffeine clearance (subject 34, 0.00253 L/h/kg) had been treated with ciprofloxacin 500mg twice daily for 5 days).

Statistical analysis for comparing the effect of smoking on CYP1A2 activity in heavy smokers (≥10 cigarettes per day) and nonsmokers was carried out after excluding the medicated volunteer (subject 34). The Mann-Whitney test was used to compare 21 nonsmokers (including subject 8, who claimed to smoke three or four cigarettes weekly; table I) and eight heavy smokers. The heavy smokers had a 2-fold higher median clearance of 0.115 (0.01253; 0.2725) versus 0.05192 (0.00758; 0.2721) L/h/kg and geometric mean clearance of 0.09848 versus 0.04882 L/h/kg. The inclusion of light smokers (one to six cigarettes daily, subjects 2, 6, 7 and 32; table I)

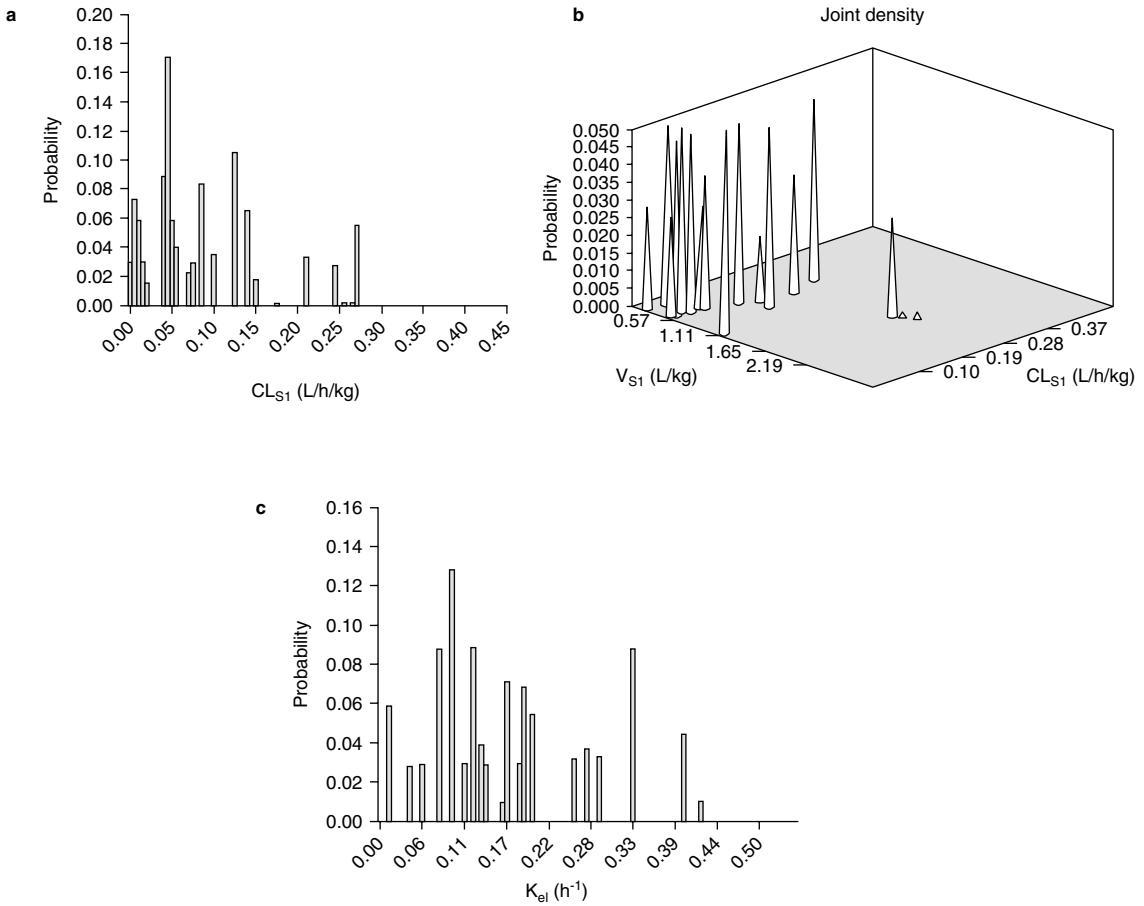

Fig. 1. (**a**) Discrete marginal density for slope of clearance to bodyweight, (CL_{S1}), as found with NPEM2 estimator (n = 34). The abscissa represents CL_{S1} estimates and the ordinate represents the probability of having the particular value of CL_{S1}. The mean and SD were 0.08749 ± 0.07282 L/h/kg. The estimates of the 25th, 50th (median) and 75th percentiles were 0.04249, 0.05696 and 0.12773 L/h/kg, respectively The value of the mode was 0.0475 L/h/kg. The skewness and kurtosis were 1.1646 and 3.5849, respectively The distribution was non-Gaussian and was skewed to the right with a mean value higher than the median. There are apparently three clusters of subjects. At least one small outlying one is clearly segregated. (**b**) Three-dimensional plot of the population joint probability density function found with NPEM2 estimator using the slope of volume of distribution to bodyweight, (V_{S1}), and CL_{S1} parameterisation. V_{S1} and CL_{S1} were uncorrelated. Apparently three clusters are perceived; all subjects have almost the same values for V_{S1} but clearly differ in their values for CL_{S1} (n = 34). (**c**) Discrete marginal density of elimination rate constant, (k_{el}). The mean and SD were 0.1695 ± 0.1026 h^{-1} (n = 34). The estimates of the 25th, 50th (median) and 75th percentiles were 0.0954, 0.1391 and 0.2031 h^{-1}, respectively The value of the mode was 0.0962 h^{-1}. The skewness and kurtosis were 0.7011 and 2.6742, respectively The distribution was non-Gaussian and skewed to the right with a mean value being closer to the 75th percentile and higher than the median. The small outlying cluster of subjects is seen and sharply segregated. V_{S1} and k_{el} parameterisation.

in the analysis lowered both the median clearance to 0.09554 (0.01253; 0.2725) L/h/kg and the geometric mean clearance to 0.08476 L/h/kg. This observation is in line with the results of Tantcheva-Poór et al.[44]

When testing caffeine clearance distribution for normality, three out of the four tests rejected the null hypothesis (Lilliefors, distance = 0.181, $p < 0.01$; Shapiro-Wilk's $W = 0.85$, $p < 0.0002$; $\chi^2 = 6.069$, $p = 0.048$) whereas the Kolmogorov-Smirnov statistic did not (distance = 0.181, $p < 0.20$). This agrees with the statement of Afifi and Azen[43] stressing the fact that when parameters of the distribution of a varia-

ble are estimated upon a population sample, p-values of the Kolmogorov-Smirnov statistic are not correct. After logarithmic transformation of caffeine systemic clearance estimates, all but two tests (Shapiro-Wilk's W = 0.923, $p < 0.024$; Lilliefors, distance = 0.208, $p < 0.01$) agreed with the null hypothesis for normality of the underlying distribution. Recently, the Shapiro-Wilk's W test[45] has become the preferred test of normality because of its good power properties; if the W statistic is significant, than the hypothesis that the respective data are sampled from a Gaussian population should be rejected. Whereas the distribution of untransformed systemic caffeine clearance was skewed to the right (skewness = 1.244 ± 0.403), that of log-transformed CL_{S1} was skewed to the left (skewness = –0.856 ± 0.403). Computation of Z statistics for skewness and kurtosis pointed out that the H_0 hypothesis had to be rejected for both distributions ($p_{skewness} = 0.001$ and 0.017, respectively, and $p_{kurtosis} = 0.005$ and 0.0007, respectively, one-sided), thus supporting a non-Gaussian shape of the two distributions.

The distribution tests showed that in participants with normal renal function (n = 27, see table I) the caffeine clearance distribution strongly deviated from Gaussian ($\chi^2 = 5.79$, $p = 0.016$; Lilliefors, $p < 0.01$ and Shapiro-Wilk, $p = 0.0001$). Because of the small number of subjects with reduced creatinine clearance (n = 7, table I), normality tests in that smaller group failed to distinguish any significant departure from normality. The Mann Whitney test revealed no significant between-group differences of CL_{S1} medians for subjects with normal versus reduced renal function. The median CL_{S1} for the group of subjects with decreased creatinine clearance (subjects 28–34 in table I) was 0.1025 (0.0025; 0.2477) L/h/kg (n = 7) compared with 0.0483 (0.0076; 0.2725) L/h/kg (n = 27) for the group of participants with normal renal function ($U = 73$, $p = 0.371$). Furthermore, the paraxanthine/caffeine ratios for the corresponding sampling times of these subjects were of the same extent as those of participants with normal renal function (data not shown). Moreover, the between-subject variability was comparable for both groups, with CV% of 90% and 80%, respectively, for the normal and the reduced renal function groups regardless of the 4-fold difference in sample size. The values of geometric means for the two groups were very close at 0.05362 and 0.05728 L/h/kg. The mean values (SD) for age (39.7 ± 14.1 years), bodyweight (67 ± 14.3kg) and height (170.3 ± 8.9cm) for the renal failure group did not differ significantly from those of the normal creatinine clearance group: 33.7 ± 12.7 years, 73.6 ± 18.3kg and 169.5 ± 9.9cm ($p > 0.05$).

The Shapiro-Wilk test revealed that systemic caffeine clearance was not normally distributed either in the female or in the male group ($p < 0.0129$ and $p < 0.0126$, respectively). There were no significant differences between median caffeine plasma clearance for females and males: 0.05265 (n = 18) versus 0.06485 L/h/kg (n = 16) [$U = 137$, $p = 0.823$], as well as between their mean (SD) ages: 34.6 ± 12.2 and 35.3 ± 14.2 years, respectively ($p = 0.869$). Clearance variability was higher between males than between females (CV% = 92% and 82%, respectively). The lack of significant difference between the medians of caffeine clearance persisted after both male and female heavy smokers and heavy drinkers were excluded from comparison, so that 13 females and 12 males entered the Mann-Whitney U-test. The median values for females and males were 0.057 and 0.0479 L/h/kg, respectively ($U = 54.5$, $p = 0.2108$) but variability within females and males became comparable (CV% = 76.52% and 77.14%, respectively) due to the substantial reduction in the number of male subjects, since of the four subjects with the highest estimates of caffeine clearance, three (subjects 25, 30 and 27) were males (see table III and table I).

Probit analysis of cumulative frequencies of untransformed and log-transformed individual estimates of caffeine clearance and elimination rate constant, k_{el}, showed a clear deviation of the underlying distribution from Gaussian with three breakpoints (figure 3). Testing the probit plots for departure from linearity demonstrated that it was highly significant ($p < 0.0001$) for untransformed as well as for log-transformed data ($p = 0.032$ and $p = 0.0006$, respectively). Both the NPEM2 analysis and

Table III. Clinical data

Subject	Sex	Age (y)	Height (cm)	Weight (kg)	CL_{CR} (mL/min/ 1.73m^2)	Reported smoking habits[a]	Reported alcohol consumption[b]	Adverse events	Comedication	Sampling times (h) [caffeine plasma conc., mg/L]
1	M	58	170	83	82	No	0.5 L/week	None	Vitamins	4; 8 [2.23; 3.32]
2	M	41	179	96.5	126	5–6	~0.2 L/day	None	No	4; 8 [4.10; 2.45]
3	F	40	167	67	103	No	0.5L beer/10days	None	No	4; 8 [2.69; 1.14]
4	F	41	160	50	132	No	0.05 L/2 weeks	None	No	1; 7 [3.01; 1.28]
5	F	18	172	72	103	No	No	None	No	1; 7 [4.89; 2.21]
6	F	47	148	55.9	119	1–2	0.15 L/week	None	No	4; 8 [4.39; 3.19]
7	M	18	187	78.5	92	2–3	1 L/week	None	Propranolol	1; 7 [5.85; 2.78]
8	M	22	180	74	106	3–4/week	No	None	No	4; 8 [2.76; 1.55]
9	M	39	176	95.2	79	No	1L wine/month	None	No	1; 7 [3.90; 1.89]
10	F	25	164	55	143	No	No	None	No	4 [3.95]
11	M	40	176	83	111	No	0.05–0.1 L/day	None	No	4; 8 [5.26; 2.78]
12	F	44	156	49.5	102	No	No	None	No	1; 7 [7.89; 2.66]
13	F	41	160	62	133	No	No	None	No	0.5; 3 [1.38; 6.48]
14	M	45	176	90	101	No	0.05 L/day	None	No	0.5; 3 [0.27; 3.38]
15	M	19	189	80	99	No	No	None	Atenolol	0.5; 3 [0.10; 2.78]
16	F	27	167	57.5	138	No	No	None	No	0.5; 3 [3.88; 1.48]
17	F	60	160	114	125	No	No	None	No	2; 6 [9.36; 4.63]
18	F	24	165	66	131	No	No	None	No	0.5; 3 [0.28; 5.44]
19	M	18	163	77	176	No	No	None	No	10; 24 [1.51; 0.19]
20	F	23	175	98	197	17	No	None	No	10; 24 [0.86; 0.04]
21	F	26	158	45	128	15	No	None	No	10; 24 [1.8; 0.07]
22	F	28	161	78	93	No	0.02 L/day	None	Enalapril	10; 24 [2.05; 0.70]
23	F	24	167	65	152	10	No	None	No	0.5; 3 [0.09; 3.60]
24	F	23	168	50	107	No	No	None	No	0.5; 3 [1.53; 2.66]
25	M	51	172	70	108	20	0.1 L/day	None	No	2; 6 [2.26; 0.78]
26	M	43	181	107	122	No	No	None	Atenolol, diclofenac	2; 6 [4.81; 4.27]
27	M	24	180	69	117	20	0.05 L/day	None	No	2; 6 [2.06; 0.40]

Continued next page

Table III. Contd

Subject	Sex	Age (y)	Height (cm)	Weight (kg)	CL_{CR} (mL/min/1.73m²)	Reported smoking habits[a]	Reported alcohol consumption[b]	Adverse events	Comedication	Sampling times (h) [caffeine plasma conc., mg/L]
28	M	25	176	91.4	44	20	0.15 L/month	None	No	1; 7 [6.57; 1.27]
29	F	37	174	75	52	No	No	None	Enalapril, prednisone	4; 8 [2.07; 0.80]
30	M	62	167	63	59	20	0.3 L/day	None	Enalapril	10; 24 [0.42; 0.06]
31	M	32	164	56.5	29	No	No	None	Famotidine	0.5; 3 [0.05; 2.36]
32	M	28	186	72	22	5	No	None	No	10; 24 [0.68; 0.08]
33	F	38	160	64	41	10–15	No	None	Enalapril	2; 6 [3.32; 2.17]
34	F	56	165	47	11	10	No	None	Ciprofloxacin, diclofenac	2; 6 [16.09; 14.09]

a Cigarettes/day unless otherwise reported.

b Distilled alcohol unless otherwise reported.

CL_{CR} = creatinine clearance; **F** = female; **M** = male.

the probit plots suggested that the distributions of CL_{S1} and k_{el} in the study population were not unimodal. Probit analysis of the cumulative frequencies of untransformed and log-transformed estimates of CL_{S1} and k_{el} in the population of subjects with normal renal function also showed deviation from normality ($p = 0.012$ and $p < 0.0001$, respectively).

Time Distribution of the Relationship Between Paraxanthine/Caffeine Ratios and Systemic Caffeine Clearance

When plotting individual paraxanthine/caffeine ratios at different sampling times versus individual values of CL_{S1} for corresponding subjects, highly significant positive correlations were observed for 3, 8 and 10 hours after caffeine administration, r_s being greater than 0.7 at $p \leq 0.04$. Plotting the medians of the nonparametric Bayesian posterior distributions of clearance for each individual subject, as assessed by the NPEM2 estimator, versus corresponding caffeine concentrations at sampling times 2, 6, 7, 8 and 10 hours revealed even higher significant negative correlations ($r_s > -0.94$, $p < 0.005$).

Discussion

After the demonstration of caffeine *N*-demethylation activity of human CYP1A2 by Butler et al.,[46] caffeine has been largely accepted as a safe and reliable probe for *in vivo* phenotyping for hepatic CYP1A2 activity,[19,47] as the enzyme is expressed essentially only in the liver.[46,48] The activity of the enzyme varies 40-fold among individuals.[7,23,46] Interindividual differences in enzyme expression may contribute to cancers caused by procarcinogens and also can increase the risk of unexpected drug interactions.[49,50]

The population model parameter values for k_a, V_{S1}, CL_{S1} and k_{el} obtained by the NPEM2 estimator (see table II) were very similar to those reported by other authors from 'data rich' pharmacokinetic protocols[6,18,38,51] (for an overview of published data concerning caffeine pharmacokinetics, see also table II in Rostami-Hodjegan et al.[26]). With the population estimates for k_a and k_{el} (table II), the estimated

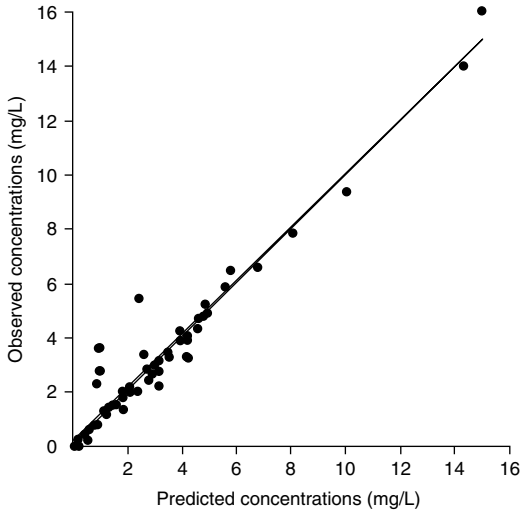

Fig. 2. Scatter plot of observed versus individual model-predicted caffeine plasma concentrations (n = 67) using the individual maximum *a priori* (MAP) Bayesian posterior parameter values of k_a, V_{S1} and CL_{S1}, based on the population median parameter values and their SDs as the MAP Bayesian priors, in the population ($r = 0.98$, $p < 0.0001$). **CL_{S1}** = fractional systemic caffeine clearance (slope of plasma clearance to bodyweight); **k_a** = absorption rate constant; **V_{S1}** = fractional volume of distribution (slope of volume of distribution to bodyweight).

time at which caffeine peak plasma concentration should be reached, t_{max}, equalled 48 min and was identical to the value of 47 ± 5 minutes experimentally observed by Bonati et al.[37] in healthy volunteers. The population estimate of V_{S1} was also close to the value for the volume of distribution (0.56 L/kg) reported by Bonati et al.[37] after a dose of caffeine of 5 mg/kg, and was almost identical to the values published recently by Carillo et al.[6] (0.55 and 0.59 L/kg for nonsmoker and smoker males, respectively, and 0.53 and 0.61 L/kg for nonsmoker and smoker females, respectively). The population estimate of CL_{S1} (table II) was very close to the value of 0.068 L/h/kg reported by Denaro et al.[20] for oral caffeine clearance on low doses (4.2 mg/kg/day). Those results were all obtained with 'data rich' clinical scenarios. The lack of effect of reduced creatinine clearance on caffeine plasma clearance was consistent with the data of Ullrich et al.[52]

In the present study, the population distribution of systemic caffeine clearance was found to be skewed, with a large between-subject variability. Since it is well established that age does not account for between-subject variability of CYP1A2 activity,[44,53] the study was not designed to investigate the contribution of this covariate. The influence of sex on CYP1A2 activity is still under investigation. Some authors claim activity to be lower in females,[25,53] but others find no sex difference in CYP1A2 activity.[51,54] Since our study was not designed to discern any sex-related differences, the luteal and follicular phases of the menstrual cycle were not investigated. We found no significant differences in CYP1A2 activity between females and males. These findings correspond to those of Kashuba et al.[55] and Zaigler et al.[56]

The observed 2-fold higher geometric mean and arithmetic mean (0.1383 L/h/kg) of caffeine plasma clearance in heavy smokers as compared with nonsmokers (0.0732 L/h/kg) reflects increased CYP1A2 activity in human liver due to the enzyme-inducing effect of smoking.[23,57] Moreover, the three heavy smokers with the highest clearance estimates were also heavy drinkers, and a combined effect of both inducers[44,57] was likely. All participants with smoking habits were self-designated smokers, and smoking status was not confirmed by a cotinine test, as the investigation was not designed to evaluate the effect of smoking on CYP1A2 activity. There was a 36-fold range of CYP1A2 activity, as monitored by caffeine clearance, in nonsmokers (0.00758–0.2721 L/h/kg) and a 22-fold range in heavy smokers as well as in all smokers (heavy and light smokers, 0.01253–0.2725 L/h/kg). These findings agree with these of Butler et al.,[23,46] and show the enhancing effect of smoking on CYP1A2 activity. However, the effect of other environmental factors on such activity should not be overlooked. Of the four subjects with the highest CYP1A2 activity (table III), one (no. 16) had never smoked or consumed alcohol.

The lowest value of caffeine clearance was found in subject 34, who was treated with ciprofloxacin. This is in agreement with the data of Harder et al.[58] and Healy et al.[59] showing ciprofloxacin to be an effective inhibitor of caffeine metabolism in humans at dosages exceeding 250mg twice daily. Regarding

the six volunteers (excluding subject 34) with the lowest clearance values (table III), a genetic deficiency of CYP1A2 might be suspected, but it is too rare to show up in the size of the population investigated here.[54]

About 87% of the studied population showed low or moderate clearances (up to 0.150 L/h/kg; see figure 1a), which coincides with the observation of Kadlubar et al.[60] concerning the proportion of slow metabolisers of caffeine (77%), as measured by paraxanthine/caffeine urine ratio, in a population of comparable size (n = 30). The remaining 13% might be regarded as rapid metabolisers of caffeine, and/or with metabolism accelerated by smoking (clearances >0.200 L/h/kg).

The tests for normality of the population distribution of caffeine clearance, taken together with the probit plot analysis of untransformed and log-transformed data, indicated that the distribution in our population was neither normal nor log-normal. Recently, Vesell and Gaylor[61] pointed out that histograms and probit plots, frequently used as initial screens to detect genetic variations of drug metabolism, have their inherent limitations and uncertainty in identifying the presence of bimodality in frequency distributions. On the other hand, Jackson et al.[62,63] stressed the need to test the strength of the hypothesis that two or more populations are present. Actually, experimental and clinical data often represent a mixture of several normal distributions (clusters of subpopulations). The identification of subpopulations by setting up such a multicomponent distribution using common graphical methods is only possible for bimodality if more than two SDs are present between the two means.[62,64] Bimodality could be detected reliably (>90%) when the separation was as great as four to six SDs.[62] Moreover, further parametric assumptions should be made concerning the effect of the size ratio of components and the effect of relative width, i.e. the effect of varying the ratio of the two individual SDs of the two component distributions.[62] Since the relative proportions may vary between studied populations, describing such a distribution of clusters optimally is not possible with either a normal or lognormal distribution.[28]

The NPEM method of Schumitzky[30] makes no assumptions about the shape of the joint probability distribution. It computes the entire joint density or distribution of points, each of which contains a set of parameter values, and each of which has a certain probability.[28] A useful feature of the NPEM method is its ability to discover unsuspected subpopulations.[28,33]

The statistical comparison of NPEM2 estimates of CL_{S1} between the three subpopulations (table III) indicated highly significant differences between their means: 0.00993 ± 0.00477, 0.08072 ± 0.03765 and 0.2512 ± 0.02824 L/h/kg ($p < 0.0001$). Testing the variances revealed highly significant differences between subpopulations 1 and 2 ($F_{22,6} = 62.37$, $p < 0.0001$) and subpopulations 1 and 3 ($F_{6,3} = 35.09$, $p = 0.0003$). The variances of subpopulations 2 and 3 were not significantly different ($F_{23,3} = 1.78$, $p = 0.355$). As predicted by theory, since the means of subpopulations 2 and 3 were more than six SDs apart from the mean of subpopulation 1, this distribution was resolved with probit plots (see figure 3). Moreover, in accordance with the predictions of Endrenyi and Patel[64] concerning the effect of relative widths, i.e. of SDs, a small but narrow component distribution (subpopulation 1) is indicated by a sharp inflection region on the probit plots (figure 3).

Individual estimates of V_{S1} and k_{el} were not intercorrelated in the set of 34 subjects (data not shown). There was no significant difference between the mean values of V_{S1} for the subpopulation with NPEM2 clearance estimates up to 0.020 L/h/kg (n = 7) and with clearance estimates higher than 0.040 L/h/kg (n = 27) [0.65 ± 0.33 vs 0.59 ± 0.30 L/kg, respectively, $p = 0.652$]. In contrast, the mean value of the elimination rate constant, k_{el}, was more than 3-fold higher for the subpopulation with caffeine clearance values > 0.040 L/h/kg (n = 27) [0.052 ± 0.029 vs 0.197 ± 0.096 h^{-1}, respectively, $p = 0.0005$]. The F-test revealed significantly different variances of the parameter ($F_{26,6} = 10.27$, $p = 0.004$) and supports the inference about the different distributions of k_{el} in these subpopulations. This implies

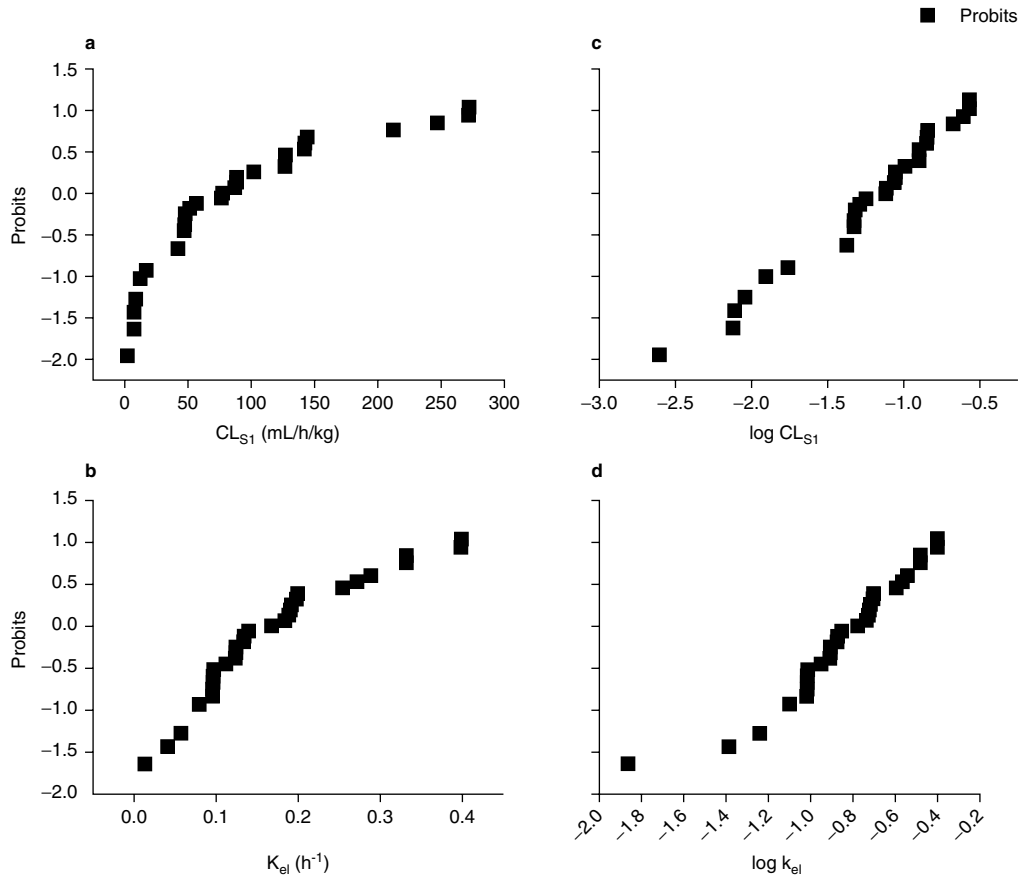

Fig. 3. Probit transformations of systemic caffeine clearance (CL_{S1}) and elimination rate constant (k_{el}) data. The ordinates of the figures are cumulative frequency distributions in probit units. (**a**) Probit plot of untransformed CL_{S1} values as estimated with the nonparametric expectation maximisation (NPEM) method (abscissa); (**b**) probit plot of untransformed k_{el} values as estimated with the NPEM method (abscissa); (**c**) probit plot of logarithmically transformed (to the base 10) CL_{S1} values (abscissa); (**d**) probit plot of logarithmically transformed (to the base 10) k_{el} values (abscissa).

that the reason for the observed substantially higher clearances was the significantly higher elimination rate constant of caffeine in these participants, not an increased volume of distribution. This inference was further supported by the discrete marginal density of k_{el}, where a cluster of unsuspected individuals with extremely high values was seen (figure 1c). Moreover, there were no significant differences between mean age (36.86 ± 16.09 vs 34.41 ± 12.35 years, p = 0.663), mean height (170.9 ± 9.01 vs 169.4 ± 9.83cm, p = 0.719) and mean bodyweight (69.64 ± 15.39 vs 72.94 ± 18.23kg, p = 0.664) among the two subpopulations. There was also no significant difference between the values of body mass index (23.71 ± 4.18 vs 25.32 ± 5.74 kg/m^2, p = 0.495) suggesting that the higher clearance values could not be attributed, at least in this study, to that covariate (Tantcheva-Poór et al.[44]), most probably because of the small number of individuals.

As outlined by Eaton et al.,[4] a polymodal distribution of CYP1A2 has been reported from studies where smokers and nonsmokers have been analysed separately. The same authors claim that the analysis of a mixed population of smokers and nonsmokers results in the masking of a polymodal distribution of CYP1A2 activity. The results of the present study,

however, demonstrate a distribution of CYP1A2 activity that appears to be multimodal, even in this mixed population of smokers and nonsmokers. The study was not designed to evaluate the contribution of within-subject variability (unaccounted-for intra-individual variation) of CYP1A2 activity, since the investigations of Kalow and Tang[54] have confirmed its rather low level (17.2%). However, if taking into consideration the value of gamma (4.30) the fraction of unaccounted-for intra-individual variability is reduced to about 13%. Nevertheless, one has to bear in mind that within-subject variability might be a latent source of bias in population model parameter evaluation.[65]

Comparing ME and RMSE estimates with the average measured caffeine concentration of 2.92 ± 2.94 mg/L reveals rather high predictive accuracy and precision of the population model. The results of correlation analyses between caffeine clearance estimates and paraxanthine/caffeine molar ratios at different sampling points, as well as with caffeine plasma concentrations, firmly agree with the observations of others,[44,66,67] and support the high reliability of caffeine clearance values obtained with the NPEM method.

Conclusion

The results of this investigation show that NPEM2 analysis is a reliable and sufficiently sensitive method for clinical testing of liver function, even in the presence of reduced renal function, using systemic clearance of caffeine as a biomarker. Additionally, taken together with already published data,[33,34] these results reveal the NPEM method as suitable and relevant for large-scale epidemiological studies with regard to phenotyping the cancer susceptibility of high-risk populations by monitoring their CYP1A2 activity based on random sparse data. The method can even deal with mixed populations (smokers and nonsmokers) in discerning multicomponent distributions with departure from normality, which the parametric methods have failed to do.

Based on this population model and on the sampling strategy employed, it should now be quite feasible in the near future to obtain nonparametric Bayesian posterior individual parameter estimates for individual patients in order to screen them either for abnormal liver function or for risk of various types of cancer associated with unusual values of caffeine clearance. For this purpose, this population model can be incorporated into software such as the USC*PACK programs, especially the newer nonparametric MM-USC*PACK programs.

Acknowledgements

The authors are deeply indebted to Roger W. Jelliffe, MD, for generously making available to them the USC*PACK collection of PC programs, v.10.6. They also wish to express their sincere gratitude to him for working with them on the revised version of the manuscript. The authors have provided no information on sources of funding or on conflicts of interest directly relevant to the content of this study.

References

1. Nebert DW, Nelson DR, Coon MJ, et al. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. DNA 1991; 10: 1-4
2. Ioannides C, Parke DV. The cytochrome P450 1 gene family of microsomal hemoproteins and their role in the metabolic activation of chemicals. Drug Metab Rev 1990; 22: 1-85
3. Shimada T, Iwasaki M, Martin MV, et al. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by umu gene response in Salmonella typhimurium TA 1535/pSK1002. Cancer Res 1989; 49: 3218-28
4. Eaton DL, Gallagher EP, Bammler TK, et al. Role of cytochrome P4501A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. Pharmacogenetics 1995; 5: 259-74
5. Gonzalez FJ, Gelboin HV. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. Drug Metab Rev 1994; 26: 165-83
6. Carrillo JA, Benitez J. Clinically significant pharmacokinetic interactions between dietary caffeine and medications. Clin Pharmacokinet 2000; 39: 127-53
7. Schweikl H, Taylor JA, Kitareewan S, et al. Expression of CYP1A1 and CYP1A2 genes in human liver. Pharmacogenetics 1993; 3: 239-49
8. Shimada T, Yamazaki H, Mimura M, et al. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 1994; 270: 414-23
9. Denaro CP, Jacob III P, Benowitz NL. Evaluation of pharmacokinetic methods used to estimate caffeine clearance and comparison with a Bayesian forecasting method. Ther Drug Monit 1998; 20: 78-87
10. Bechtel YC, Haffen E, Lelouët H, et al. Relationship between the severity of alcoholic liver cirrhosis and the metabolism of caffeine in 226 patients. Int J Clin Pharmacol Ther 2000; 38: 467-75

11. Lelouët H, Bechtel YC, Paintaud G, et al. Caffeine metabolism in a group of 67 patients with primary biliary cirrhosis. Int J Clin Pharmacol Ther 2001; 39: 25-32
12. Bechtel YC, Lelouët H, Hrusovsky S, et al. Caffeine metabolism before and after liver transplantation. Int J Clin Pharmacol Ther 2001; 39: 53-60
13. Lelo A, Miners JO, Robson RA, et al. Quantitative assessment of caffeine partial clearances in man. Br J Clin Pharmacol 1986; 22: 183-6
14. Gu L, Gonzalez FJ, Kalow W, et al. Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. Pharmacogenetics 1992; 2: 73-7
15. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001; 69: 89-95
16. Jost G, Wahllander A, Von Mandach U, et al. Overnight salivary caffeine clearance: a liver function test suitable for routine use. Hepatology 1987; 7: 338-44
17. Nagel RA, Dirix LY, Hayllar KM, et al. Use of quantitative liver function tests: caffeine clearance and galactose elimination capacity: after orthotopic liver transplantation. J Hepatol 1990; 10: 149-57
18. Tanaka E, Ishikawa A, Yamamoto Y, et al. A simple useful method for the determination of hepatic function in patients with liver cirrhosis using caffeine and its three major dimethylmetabolites. Int J Clin Pharmacol Ther Toxicol 1992; 30: 336-41
19. Tang BK, Zhou Y, Kadar D, et al. Caffeine as a probe for CYP1A2 activity: potential influence of renal factors on urinary phenotypic trait measurements. Pharmacogenetics 1994; 4: 117-24
20. Denaro C, Brown CR, Wilson M, et al. Dose-dependency of caffeine metabolism with repeated dosing. Clin Pharmacol Ther 1990; 48: 277-85
21. Carrillo JA, Christensen M, Ramos SI, et al. Evaluation of caffeine as an in vivo probe for CYP1A2 using measurements in plasma, saliva, and urine. Ther Drug Monit 2000; 22: 409-17
22. Fuhr U, Rost KL. Simple and reliable CYP1A2 phenotyping by the paraxanthine/caffeine ratio in plasma and in saliva. Pharmacogenetics 1994; 4: 109-16
23. Butler MA, Lang NP, Young JF, et al. Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. Pharmacogenetics 1992; 2: 116-27
24. Lang NP, Butler MA, Massengill J, et al. Rapid metabolic phenotypes for acetyltransferase and cytochrome P4501A2 and putative exposure to food-borne heterocyclic amines increase risk for colorectal cancer or polyps. Cancer Epidemiol Biomark Prevent 1994; 3: 675-82
25. Nakajima M, Yokoi T, Mizutani M, et al. Phenotyping of CYP1A2 in Japanese population by analysis of caffeine urinary metabolites: absence of mutation prescribing the phenotype in the CYP1A2 gene. Cancer Epidemiol Biomarkers Prev 1994; 3: 415-21
26. Rostami-Hodjegam A, Nurminen S, Jackson PR, et al. Caffeine urinary metabolite ratios as markers of enzyme activity: a theoretical assessment. Pharmacogenetics 1996; 6: 121-49
27. Schrenk D, Brockmeier D, Mörike K, et al. A distribution study of CYP1A2 phenotypes among smokers and non-smokers in a cohort of healthy Caucasian volunteers. Eur J Clin Pharmacol 1998; 53: 361-7
28. Jelliffe RW, Schumitzky A, Bayard D, et al. Model-based, goal-oriented, individualised drug therapy. Clin Pharmacokinet 1998; 34: 57-77
29. Mallet A. A maximum likelihood estimation method for random coefficient regression models. Biometrika 1986; 73: 645-56
30. Schumitzky A. Nonparametric EM algorithms for estimating prior distributions. Appl Math Comput 1991; 45: 143-57
31. Jerling M, Merlé Y, Mentré F, et al. Population pharmacokinetics of nortriptyline during monotherapy and during concomitant treatment with drugs that inhibit CYP2D6: an evaluation with the nonparametric maximum likelihood method. Br J Clin Pharmacol 1994; 38: 453-62
32. Preston SL, Drusano GL. Nonparametric expectation maximization population modeling of ganciclovir. J Clin Pharmacol 1996; 36: 301-10
33. Terziivanov D, Atanasova I, Dimitrova V. Population pharmacokinetics of ciprofloxacin in patients with liver impairments analyzed by NPEM2 algorithm: a retrospective study. Int J Clin Pharmacol Ther 1998; 36: 376-82
34. Terziivanov D, Atanasova I, Dimitrova V, et al. Pharmacokinetic variability of nimodipine disposition after single and multiple oral dosing to hypertensive renal failure patients: parametric and nonparametric population analysis. Int J Clin Pharmacol Ther 1999; 37: 404-12
35. Jelliffe RW, Schumitzky A, Van Guilder M. User manual for version 10.6 of the USC*PACK collection of PC programs. Los Angeles (CA): Laboratory of Applied Pharmacokinetics, University of Southern California. School of Medicine, 1995
36. Jeliffe RW, Jeliffe SM. A computer program for estimation of creatinine clearance from unstable serum creatinine levels, age, sex and weight. Math Biosci 1972; 14: 17-24
37. Bonati M, Latini R, Galetti F, et al. Caffeine disposition after oral doses. Clin Pharmacol Ther 1982; 32: 98-106
38. Blanchard J, Sawers SJA. The absolute bioavailability of caffeine in man. Eur J Clin Pharmacol 1983; 24: 93-7
39. Grant DM, Tang BK, Kalow W. Variability in caffeine metabolism. Clin Pharmacol Ther 1983; 33: 591-602
40. Bojinova C, Terziivanov D. Measurement of drug concentrations in biological fluids for the purposes of population pharmacokinetic analysis: I. validation of HPLC assay for simultaneous determination of caffeine and its primary metabolites in blood and urine [in Bulgarian]. Anal Lab 1997; 6: 142-8
41. Ette EI. Stability and performance of a population pharmacokinetic model. J Clin Pharmacol 1997; 37: 486-95
42. Sheiner LB, Beal SL. Some suggestions for measuring predictive performance. J Pharmacokinet Biopharm 1981; 9: 503-12
43. Afifi AA, Azen SP. Statistical analysis: a computer oriented approach [in Russian]. Moscow: Mir, 1982
44. Tantcheva-Poór I, Zaigler M, Rietbrock S, et al. Estimation of cytochrome P-450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine test. Pharmacogenetics 1999; 9: 131-44
45. Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). Biometrika 1965; 52: 591-611
46. Butler MA, Iwasaki M, Guengerich FP, et al. Human cytochrome $P450_{PA}$ (P450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. Proc Natl Acad Sci U S A 1989; 86: 7696-700
47. Kalow W, Tang BK. The use of caffeine for enzyme assays: a critical appraisal. Clin Pharmacol Ther 1993; 53: 503-14

48. Guengerich FP. Human cytochrome P450 enzymes. In: Ortiz de Montellano PR, editor. Cytochrome P450. 2nd ed. New York: Plenum, 1995: 473-535
49. Kadlubar FF. Biochemical individuality and its implications for drug and carcinogen metabolism. Recent insights from acetyltransferase and cytochrome P4501A2 phenotyping and genotyping in humans. Drug Metab Dispos 1994; 26: 37-46
50. Guengerich FP, Parikh A, Turesky RJ, et al. Inter-individual differences in the metabolism of environmental toxicants: cytochrome P450 1A2 as a prototype. Mutat Res 1999; 428: 115-24
51. Campbell ME, Spielberg SP, Kalow W. A urinary metabolite ratio that reflects systemic caffeine clearance. Clin Pharmacol Ther 1987; 42: 157-65
52. Ullrich D, Compagnone D, Münch B, et al. Urinary caffeine metabolites in man: age-dependent changes and pattern in various clinical situations. Eur J Clin Pharmacol 1992; 43: 167-72
53. Relling MV, Lin J-S, Ayers G, et al. Racial and gender differences in N-acetyltransferase, xanthine oxidase, and CYP1A2* activities. Clin Pharmacol Ther 1992; 52: 643-58
54. Kalow W, Tang BK. Use of caffeine metabolite ratios to explore CYP1A2 and xanthine oxidase activities. Clin Pharmacol Ther 1991; 50: 508-19
55. Kashuba ADM, Bertino JS, Kearns GL, et al. Quantitation of three-month intraindividual variability and influence of sex and menstrual cycle phase on CYP1A2, N-acetyltransferase-2, and xanthine oxidase activity determined with caffeine phenotyping. Clin Pharmacol Ther 1998; 63: 540-51
56. Zaigler M, Rietbrock S, Szymanski J, et al. Variation of CYP1A2-dependent caffeine metabolism during menstrual cycle in healthy women. Int J Clin Pharmacol Ther 2000; 38: 235-44
57. Kalow W, Tang BK. Caffeine as a metabolic probe: exploration of the enzyme-inducing effect of cigarette smoking. Clin Pharmacol Ther 1991; 49: 44-8
58. Harder S, Staib AH, Beer C, et al. 4-Quinolones inhibit biotransformation of caffeine. Eur J Clin Pharmacol 1988; 35: 651-6
59. Healy PD, Polk RE, Kanawati L, et al. Interaction between oral ciprofloxacin and caffeine in normal volunteers. Antimicrob Agents Chemother 1989; 33: 474-8
60. Kadlubar FF, Talaska G, Butler MA, et al. Determination of carcinogenic arylamine N-oxidation phenotype in humans by analysis of caffeine urinary metabolites. In: Mendelsohn ML, Albertini RJ, editors. Mutation and the environment. Part B: metabolism, testing methods, and chromosomes. New York: Wily-Liss, 1990: 107-14
61. Vesell ES, Gaylor DW. Limitations of probit plots in pharmacogenetics: requirement of genetic analyses to test hypotheses based on graphical methods. Pharmacogenetics 1995; 5: 18-23
62. Jackson PR, Tucker GT, Woods HF. Testing for bimodality in frequency distributions of data suggesting polymorphisms of drug metabolism: histograms and probit plots. Br J Clin Pharmacol 1989; 28: 647-53
63. Jackson PR, Tucker GT, Woods HF. Testing for bimodality in frequency distributions of data suggesting polymorphisms of drug metabolism: hypothesis testing. Br J Clin Pharmacol 1989; 28: 655-62
64. Endrenyi L, Patel M. A new, sensitive graphical method for detecting deviations from the normal distribution of drug response: the NTV plot. Br J Clin Pharmacol 1991; 32: 159-66
65. Karlsson MO, Sheiner LB. The importance of modeling interoccasion variability in population pharmacokinetic analyses. J Pharmacokinet Biopharm 1993; 21: 735-50
66. Schmider J, Brockmöller J, Arold G, et al. Simultaneous assessment of CYP3A4 and CYP1A2 activity in vivo with alprazolam and caffeine. Pharmacogenetics 1999; 9: 725-34
67. Spigset O, Hägg S, Söderström E, et al. The paraxanthine:caffeine ratio in serum or in saliva as a measure of CYP1A2 activity: when should the sample be obtained? Pharmacogenetics 1999; 9: 409-12

Correspondence and offprints: Professor *Dimiter Terziivanov,* Clinic for Therapeutics and Clinical Pharmacology, University Hospital 'St. I. Rilsky', 15 D. Nestorov Street, Sofia, 1431, Bulgaria.
E-mail: terziiv@yahoo.com