A Population Approach to Enzyme Characterization and Identification: Application to Phenacetin O-Deethylation

Donna J. Belle,[1,5] Barbara J. Ring,[1] Sandra R. B. Allerheiligen,[1] Michael A. Heathman,[2] Lisa M. O'Brien,[2] Vikram Sinha,[2] Lorin K. Roskos,[3] and Steven A. Wrighton[1,4]

Received June 27, 2000; accepted September 12, 2000

Purpose. To determine the enzyme kinetics (EK) and identify the human cytochrome(s) P450 (CYP) involved in the deethylation of phenacetin to acetaminophen using a population-based method.
Methods. A sparse data set was generated from incubations containing human liver microsomes ($n = 19$) with phenacetin. Estimates of the EK parameters were obtained by fitting the concentration-velocity data to Michaelis-Menten models by using nonlinear mixed effects modeling. Relationships between the EK parameters and the CYP activities determined for these liver microsomes were examined.
Results. A two-enzyme kinetic model with a saturated, low K_M enzyme and an unsaturated, high K_M enzyme capable of forming acetaminophen best fit the data. The population estimates of the EK parameters were V_{max1}, 911 pmol/min/mg protein; K_{M1}, 11.3 μM; and $Cl_{int2,}$ 0.4 μl/min/mg. The coefficients of variation for interliver variability in V_{max1} and residual error of the model were 39% and 15%, respectively. When the selective catalytic activities were examined as potential covariates, 7-ethoxyresorufin O-deethylation (CYP1A2) activity was found to be associated with the low K_M enzyme, however, the high K_M enzyme(s) could not be identified.
Conclusions. The population approach characterized the EK parameters and identified the low K_M enzyme responsible for phenacetin O-deethylation as CYP1A2. Population modeling of EK provides valuable information on inter- and intraliver variability in CYP dependent activities.

KEY WORDS: cytochromes P450; NONMEM; sparse sampling; phenacetin O-deethylation; enzyme kinetics.

INTRODUCTION

Nonlinear mixed effects regression models were introduced to study and analyze the sparse data sets acquired from population pharmacokinetic (PK) studies (1,2). These models estimate the typical values of the population's PK parameters, assess the relationships between potential covariates (such as age or disease state) and PK parameters, and determine inter- and intraindividual variability associated with these parameters (1,2). There is an increasing interest in the application of population approaches to preclinical drug development programs, particularly in toxicokinetics (3,4). Furthermore, the principles of population analysis can be related to other fields such as enzyme kinetics (EK).

The cytochromes P450 (CYP) play an important role in the metabolism of drugs. A number of *in vitro* techniques have been developed to characterize and identify the CYP(s) responsible for the metabolism of a drug. These studies serve as the foundation for making predictions concerning the *in vivo* potential for drug-drug interactions and population variability with the use of that drug. Characterization of the apparent EK for a given CYP biotransformation involves using nonlinear least squares regression to fit Michaelis-Menten models of EK. Identification of the enzyme(s) involved in the biotransformation employs several approaches including correlation of metabolite formation rate with CYP activity of a panel of human liver microsomes, metabolism by cDNA-expressed enzymes, and selective inhibition of CYPs in liver microsomes (5).

This *in vitro* approach to enzyme characterization and identification is a multifaceted and labor-intensive process. To streamline the current practice, a "population" approach was examined to perform the EK analyses and to define relationships between the determined EK parameters and the form-selective catalytic activities from a characterized liver bank using a sparse sample paradigm. Thus, the population approach would combine the EK characterization and correlation analyses into one step. Phenacetin O-deethylation (POD) was used as a test biotransformation. The results obtained with the population approach were compared to traditional procedures.

MATERIALS AND METHODS

Phenacetin and NADPH were purchased from Sigma (St. Louis, MO). 7-Ethoxyresorufin and resorufin were obtained from Molecular Probes (Eugene, OR). Acetaminophen was obtained from Kodak (Rochester, NY), and furafylline was obtained from Ultrafine Chemicals (Manchester, England). Microsomes prepared from insect cells expressing CYP1A2 and CYP2C19 were obtained from Gentest Corporation (Woburn, MA).

Human liver samples (designated HLA through HLS) were obtained and microsomes prepared and stored as previously reported (6). Protein content was determined by the Lowry method (7).

POD Assay

POD activity was determined by measuring the conversion of phenacetin to acetaminophen. A typical 0.2 ml incubation mixture contained 0.25 mg/ml of microsomes, 100 mM sodium phosphate buffer (pH 7.4), reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1 mM) and phenacetin. After a 3 min 37°C preincubation, the reaction was initiated with NADPH. After 25 min (linear rate conditions), the reaction was stopped by the addition of 0.2 ml cold methanol. The mixture was centrifuged at 4000 rpm for 10 min and

[1] Drug Disposition or [2]Pharmacokinetics, Eli Lilly and Company, Indianapolis, IN 46285.
[3] Abgenix, Fremont, CA 94555.
[4] To whom correspondence should be addressed. (e-mail wrighton_steven@lilly.com)
[5] Current address: Pharmacokinetics, Schering Plough, Kenilworth, NJ 07033.

ABBREVIATIONS: CV, coefficient of variation; CYP, cytochrome P450; EK, enzyme kinetics; EROD, 7-ethoxyresorufin O-deethylation; MLR, multiple linear regression; MOF, minimal value of the objective function; PK, pharmacokinetics; POD, phenacetin O-deethylation; σ^2, intraliver variance; ω^2, interliver variance.

0724-8741/00/1200-1531$18.00/0

acetaminophen in the supernatant was determined by HPLC with UV detection at 254 nm. Metabolites were separated on a heated (35°C), 250 × 4.6 mm Zorbax-Phenyl column (MacMod Analytical, Inc., Chadds Ford, PA) using a mobile phase consisting of 25 mM sodium phosphate buffer (pH 3.0)/methanol (95:5 or 90:10) at a flow rate of 1 ml/min.

Investigation of POD in Human Liver Microsomes

A sparse data set for POD by microsomes from the characterized liver bank was accomplished by sequentially assigning HLA–HLS to one of three incubation groups. All incubations were performed in duplicate. Group one incubations contained phenacetin concentrations of 5, 30, 80, and 300 μM; group two contained phenacetin concentrations of 10, 40, 100, and 500 μM; group three contained phenacetin concentrations of 20, 60, 200, and 1000 μM.

For traditional analysis of enzyme kinetics, POD activity was determined in three samples; samples with low (HLP), moderate (HLG), or high (HLS) CYP1A2 activity. Phenacetin concentrations from 12.5 to 2000 μM (10 concentrations/liver performed in duplicate) were used in the microsomal incubations. Incubations with human recombinant CYPs were performed using the same conditions as described above, except the mixtures contained 25 pmol CYP1A2, or 50 pmol CYP2C19 and were incubated for up to 30 min. Control incubations were performed with microsomes from cells transfected with the expression vector alone and showed no detectable activity.

Determination of CYP Activities

The selective CYP activities were determined by monitoring 7-ethoxyresorufin O-deethylase (EROD) (5), coumarin 7-hydroxylase (5), S-mephenytoin 4′-hydroxylase (5), bufuralol 1′-hydroxylase (5), midazolam 1′-hydroxylase (6), chlorzoxazone 6-hydroxylase (8), diclofenac 4′-hydroxylase (9), and taxol 6-hydroxylase (10). CYP2B6 levels were determined as previously reported (11).

Traditional Analyses

For correlation analyses, microsomes from livers HLA–HLS were incubated with 40 or 1000 μM phenacetin with or without 10 μM furafylline, a mechanism-based inhibitor of CYP1A2 (12). During the inhibition studies, microsomes in buffer were preincubated with or without furafylline at 37°C for 15 min prior to the initiation of the reaction with phenacetin. Univariate or stepwise multiple linear regression (MLR) analysis (JMP, version 3.21, SAS Institute Inc., Cary, NC) was performed to compare POD activity with the CYP-selective activities or protein levels from the microsomal bank. The activities for CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 were log-transformed to normalize their distribution prior to correlation analysis. Stepwise MLR analyses were performed in a forward mode with a probability to enter of 0.15.

Eadie-Hofstee plots were constructed to assess whether one or two enzymes contributed to POD (Excel, Microsoft Corp., Redmond, WA). Apparent Michaelis-Menten parameters were determined from nonlinear, least-squares regression analysis (WINNONLIN version 1.5, Scientific Consulting Inc., Lexington, KY) using the equation for a single enzyme model (13) for the cDNA expressed CYPs:

$$v = (V_{max1} \times [S])/(K_{M1} + [S]) \quad (1)$$

or with the human liver microsomes, using the equations for a two enzyme model (13):

$$v = (V_{max1} \times [S])/(K_{M1} + [S]) + (V_{max2} \times [S])/(K_{M2} + [S]) \quad (2)$$

or

$$v = (V_{max1} \times [S])/(K_{M1} + [S]) + Cl_{int2} \times [S] \quad (3)$$

where v is the velocity of POD; S is the initial concentration of phenacetin in the incubation; K_{M1} and K_{M2} represent the high and low affinity enzymes, respectively; V_{max1} and V_{max2} represent the maximum enzyme velocities for the high and low affinity enzymes; and Cl_{int2} is equal to V_{max2}/K_{M2} for an unsaturated, low affinity enzyme where $K_{M2} > S$.

Population Analyses

The population analyses were conducted utilizing NONMEM (version V, level 1.1, NONMEM Project Group, University of California, San Francisco, CA). The base model was determined (i.e., no covariates) by fitting Michaelis-Menten models of EK [Eqs. (1)–(3)] to sparse concentration-velocity data. First-order conditional estimation was implemented (14). Interliver variability was estimated according to additive, proportional, and exponential error models (14). Residual variability between the observed response (i.e., velocity) and those predicted by the model(s) was also estimated using NONMEM. Intraliver variability was attributable to errors in the POD incubation and/or analytical methods and was estimated using additive, proportional, and exponential error models (14).

In the covariate structural model, the vector population parameters are denoted with θ. All available data (concentration-velocity) were fitted simultaneously to obtain the best estimates of θ parameters, intraliver variances (ω^2) for each random effect, and residual error variance (σ^2) that characterizes the EK statistical model. The iterative extended-least-squares fitting routine continues until a minimal value of the objective function (MOF) is reached. The best fit model in the series was designated as the base model.

CYP activities were introduced as covariates into the base model by testing each covariate on each EK parameter. Those covariates that exhibited a significant relationship with the EK parameters were added to the base model in combination so that a full model contained all possible covariates. To confirm the relationship between the EK parameters and covariates, the process was reversed, with covariates being removed individually from the model. Covariates retained in the final model were those which when removed from the model resulted in a significant increase in MOF (ΔMOF ≧10 points for 1 degree of freedom, $p < 0.01$).

The goodness-of-fit of each NONMEM analysis was also assessed by examining the scatterplot of predicted versus measured POD velocities, the scatterplot of predicted POD velocities versus weighted residuals, the percent standard errors of the mean parameter estimates (%SEM). Empirical Bayesian estimates of the model parameters were obtained

for each liver by means of the POSTHOC option of NONMEM.

RESULTS

Traditional Analyses

Analyses of POD EK in the three livers investigated demonstrated biphasic curves with respect to substrate concentration (data not shown). Data from HLG and HLP were fit to Eq. (3), whereas data from HLS were fit to Eq. (2). The K_{M1} values for HLG, HLP, and HLS were 37 ± 6, 67 ± 4, and 13 ± 3 μM, respectively, with corresponding V_{max1} values of 801 ± 70, 269 ± 10, and 1601 ± 110 pmol/min/mg protein. Estimates of Cl_{int2} for HLG and HLP were 0.34 ± 0.01, and 0.24 ± 0.07 μl/min/mg protein, respectively. For HLS, the K_{M2} value was 624 ± 274 μM with a V_{max2} value of 1248 ± 119 pmol/min/mg protein.

POD at concentrations representing the low K_{M1} (40 μM) and high K_{M2} enzyme (1000 μM) were correlated with eight CYP activities and CYP2B6 content in a liver bank (Table 1). Of the CYP parameters examined, only EROD, a probe for CYP1A2, showed a strong correlation ($r = 0.83$, $p < 0.01$) with POD at 40 and 1000 μM phenacetin. To further elucidate the high K_{M2} enzyme(s) involved in POD, furafylline (10 μM) was preincubated in human liver microsomes to eliminate CYP1A2 involvement in this biotransformation (data not shown). In the furafylline inhibited samples, POD activity at 40 μM phenacetin was almost completely abolished. In incubations containing 1000 μM phenacetin- and furafylline-inhibited microsomes, remaining POD activity strongly correlated with S-mephenytoin 4′-hydroxylation ($r = 0.93$, $p < 0.01$), a probe for CYP2C19.

Kinetic analyses were performed in microsome cells expressing human CYP1A2 and CYP2C19. The apparent K_M values obtained with CYP1A2 and CYP2C19 were 10.4 and 818 μM, respectively.

Population Analyses

The database consisted of 150 POD velocity measurements from 19 livers. The final base model for POD incorporated a two enzyme kinetic model with a low K_M site and an unsaturated high K_M site [Eq. (3)], an exponential error model for interliver variability in V_{max1}, as well as a proportional error model for intraliver variability. Interliver variability was estimated for the V_{max1} term of the EK model only. The interliver variability in V_{max1} was calculated by taking the square root of the ω^2 value (i.e., ω^2_{Vmax1}) obtained by NONMEM and expressing it as percent coefficient of variation (% CV) (14). Intraliver variability was estimated by taking the square root of the estimated error variance, σ^2 (15). Interliver variability terms for K_{M1} and Cl_{int2} could not be determined with good precision. Table 2 summarizes the parameter, standard error, and residual variability estimates for the base model. Figures 1A and B show scatterplots of the measured versus predicted concentrations and weighted residuals versus predicted concentrations from the base model. This model was then used as a basis for evaluating covariate effects.

When the CYP activities and CYP2B6 content (Table 1) were tested as potential covariates on the EK parameters of the base model, only the model incorporating EROD activity on V_{max1} resulted in a statistically significant improvement in fit ($\Delta MOF = 117$, $p < 0.01$) (Table 2). Interliver variability decreased from 70% CV in the base model to 39% CV. The estimated intraliver variability improved from 20.3% CV in the base model to 15.4% CV (Table 2). A dramatic improvement in the scatterplots of observed versus predicted POD velocities (Fig. 1C) and weighted residuals versus predicted velocities (Fig. 1D) was noted when comparing the results from the base model to the final model. The scatterplots from the final model showed that the differences between the observed and predicted values were small, further demonstrating a good fit. Little if any bias was evident in the weighted

Table 1. Form-Selective Catalytic Activites[a] and Immunoquantified Levels of CYPs in a Human Liver Bank

Microsomal sample	EROD (1A2)	7-Hydroxy coumarin (2A6)	6-Hydroxy taxol (2C8)	4′-Hydroxy diclofenac (2C9)	4′-Hydroxy S-mephenytoin (2C19)	1′-Hydroxy bufuralol (2D6)	6-Hydroxy chlorzoxazone (2E1)	1′-Hydroxy midazolam (3A)	CYP2B6 levels
HLA	116.1	470.2	156.8	597.0	58.0	17.3	2210.0	989.0	100.0
HLB	41.5	308.3	279.3	337.0	14.0	30.4	1210.0	833.0	81.8
HLC	53.1	116.2	184.7	254.0	65.6	18.6	2440.0	595.0	79.0
HLD	29.7	836.0	495.8	659.0	0.0	41.1	2790.0	439.0	230.1
HLE	35.4	484.3	456.4	517.0	158.4	97.3	1890.0	4257.0	508.9
HLF	41.5	545.7	141.3	305.0	0.0	28.0	1470.0	2382.0	28.5
HLG	37.2	412.7	114.0	373.0	44.0	44.0	920.0	1632.0	183.2
HLH	40.6	323.7	4.7	546.0	49.2	24.9	1630.0	1143.0	39.2
HLI	43.2	1338.0	183.5	501.0	46.8	38.2	3400.0	5836.0	387.0
HLJ	69.5	387.7	110.3	308.0	87.6	34.3	920.0	1365.0	91.4
HLK	36.4	1031.0	76.3	532.0	34.8	10.7	880.0	1020.0	60.2
HLL	28.7	79.7	52.2	150.0	0.0	15.0	1030.0	681.0	23.8
HLM	21.3	406.0	94.1	591.0	20.8	69.5	1650.0	909.0	116.6
HLN	45.6	428.2	0.0	463.0	99.2	13.9	1280.0	1244.0	78.2
HLO	42.0	1468.0	0.0	298.0	26.0	91.8	1300.0	7897.0	2543.6
HLP	25.2	728.0	0.0	292.0	13.6	44.3	3800.0	3157.0	1512.3
HLQ	31.8	509.8	0.0	677.0	13.2	50.4	1010.0	734.0	148.5
HLR	21.3	137.7	0.0	413.0	31.6	12.5	1080.0	722.0	127.0
HLS	114.4	1594.0	0.0	441.0	125.6	36.7	1890.0	2209.0	287.4

[a] All activities are reported as pmol/min/mg protein. EROD = 7-ethoxyresorufin O-deethylation.

Table 2. Phenacetin O-Deethylation Population Model Parameter Estimates

Parameter	Base model		Final model	
	Estimate	% SEM	Estimate	% SEM
θ_1^a			19.8	9.8
V_{max1} (pmol/min/mg protein)	791	15.0	911.4	
K_{M1} (μM)	6.9	38.5	11.3	18.3
Cl_{in12} (μl/min/mg protein)	0.56	25.3	0.41	21.6
Interliver variability on V_{max1} (% CV)	70.1	33.1	39	31.2
Intraliver variability (% CV)	20.3	26.6	15.4	29.0
MOF	1732		1615	

[a] Structural model: $V_{max} = \theta_1$ *ethoxyresorufin O-deethylation; where θ_1 is the coefficient for ethoxyresorufin O-deethylation activities in V_{max1}.

Abbreviations used: CV(%) = coefficient of variation, SEM = standard error of the mean, MOF = minimum objective function, V_{max1} = maximal velocity of phenacetin O-deethylation of the low K_M enzyme, K_{M1} = phenacetin concentration at 1/2 V_{max1}, and Cl_{int2} is equivalent to V_{max2}/K_{M2}.

residuals (Fig. 1D). The final population model predicted V_{max1} for HLG, HLP, and HLS were 745, 215, and 2079 pmol/min/mg protein, respectively. These values compare well to the V_{max1} values obtained for HLG, HLP, and HLS by traditional methods. An excellent fit was observed between the individual predicted velocities and the observed velocities for each of the 19 livers (Fig. 2).

DISCUSSION

The objectives of this study were to use a population approach to characterize and identify the CYPs that catalyze POD using a sparse data set generated in a bank of 19 microsomal samples and to compare the results to the traditional approach. A two enzyme model with a low K_{M1} enzyme and an unsaturated high K_{M2} enzyme site best fit the sparse concentration-velocity data set. In the final population model, V_{max1} was related to EROD activity (CYP1A2). The addition of EROD into the model explained approximately 30% of the interliver variability. The final model predicted V_{max1} values for the 19 livers ranged from 214.9 to 2315.4 pmol/min/mg protein (data not shown). The typical values for K_{M1} and Cl_{int2} were 11.3 μM and 0.41 μl/min/mg protein, respectively. Because of the relatively small data set, model-predicted estimates for K_{M1} and Cl_{int2} for each liver could not be determined because of the inability to estimate interliver variability for these parameters with adequate precision.

Validation of the population model was obtained from traditional analyses and the literature. The analyses of POD in the three human livers investigated demonstrated biphasic EK suggesting the involvement of at least two CYPs. Involvement of multiple CYPs in POD as demonstrated by both the population and traditional studies is consistent with published in vitro kinetic studies (15,16). Estimates for V_{max1} in HLG, HLP, and HLS for POD from both the population and tradi-

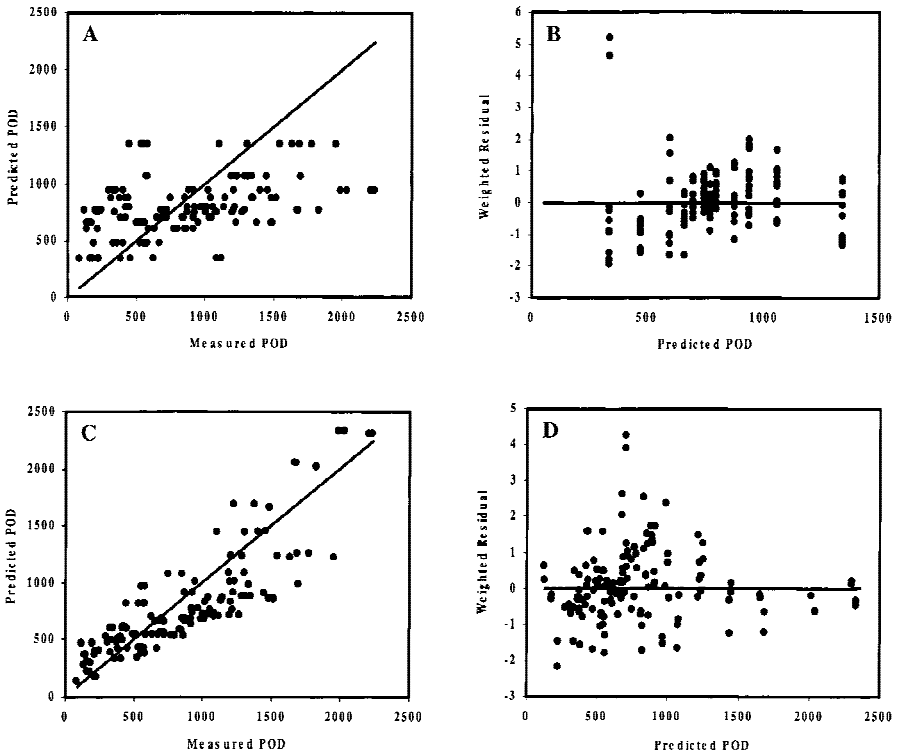

Fig. 1. Scatterplots of (A) base model predicted versus measured phenacetin O-deethylation velocity measurements, (B) base model weighted residuals versus predicted velocity measurements, (C) final model predicted versus measured phenacetin O-deethylation velocity measurements, and (D) final model weighted residuals versus predicted velocity measurements.

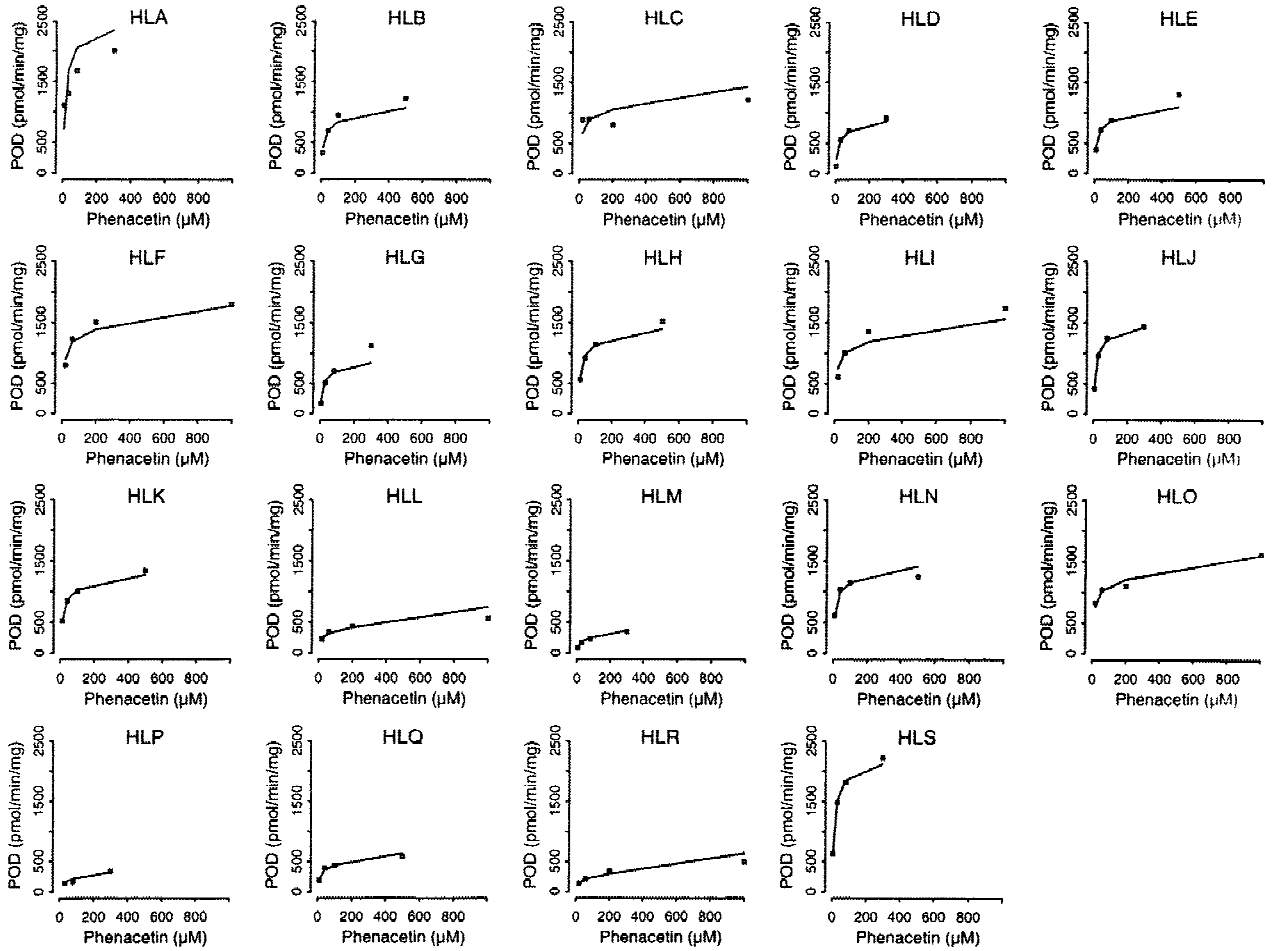

Fig. 2. The individual predicted velocity-phenacetin concentration profiles (solid line) for each liver. The mean measured velocities (open square) for each liver are indicated.

tional approaches demonstrate that the two methods are in good agreement. The population estimate of 11.3 μM for K_{M1} was slightly lower than the estimates obtained from the traditional analysis, which ranged from 13 to 67 μM. However, a K_{M1} value of 11.3 μM is well within in the reported literature values, which span from 6 to 68 μM (15,17).

Incorporation of EROD into the model for V_{max1} significantly improved the objective function. EROD is a known marker reaction for CYP1A2 activity (5). Because EROD was a significant covariate with V_{max1}, this indicates that CYP1A2 is the low K_M enzyme responsible for POD. This finding was corroborated with the traditional analyses and the literature (16,17). As additional confirmation of this conclusion, recombinant CYP1A2 had a K_M value for POD similar to the low K_M enzyme in liver microsomes.

Both population and traditional EK analyses indicated that low and high K_M enzymes contributed to POD. To elucidate the high K_M enzyme, furafylline was preincubated with the microsomes. In incubations pretreated with furafylline and containing 1000 μM phenacetin, POD activity correlated with S-mephenytoin 4-hydroxylase activity. Supplementary evidence for the involvement of CYP2C19 was provided using microsomes derived from cells expressing CYP2C19. The K_M value obtained in recombinant CYP2C19 was similar to the high K_{M2} POD component in HLS. Together, these observations indicate that CYP1A2 is the low K_M enzyme and CYP2C19 is the high K_M enzyme responsible for POD in liver microsomes.

One limitation of the population model was the inability to estimate interliver variability in K_{M1} and Cl_{int2} with subsequent predictions of these parameters in each liver. This probably stems from the fact that the sparse data set was not large enough to estimate these EK parameters, their respective interliver variabilities, and the intraliver variability term. Another shortcoming in the study design was that the data set was biased to ensure identification of the low K_{M1} enzyme. In one-third of the population (Group 1), the highest phenacetin concentration examined was 300 μM. Perhaps there were not enough data points at high substrate concentrations ($S > K_{M2}$) to adequately characterize that section of the concentration-velocity curve. The K_M term is dependent upon factors that influence substrate binding (18). Because all microsomal samples were incubated under the same experimental conditions, the K_M of the enzyme(s) should remain relatively constant between the livers and therefore a composite K_M may be sufficient. However, given this information, $Cl_{int2,}$ which reflects V_{max2}/K_{M2}, should be directly related toV_{max2}. Hence, in the future, it would be beneficial to estimate Cl_{int2} for each liver, although this may require a different experimental design or a larger data set. Simulation studies are

planned to determine an optimal study size and design for complete EK analyses. If a larger data set is necessary, then the population method may not be practical. The advantages of the population approach is that it requires roughly half the number of incubations (140–150 incubations) as the traditional analyses, but provides additional information such as EK parameter estimates for each liver examined and interliver variability for the parameter.

In conclusion, similar EK parameters for POD were obtained with both the traditional and population approach. Both approaches identified CYP1A2 as the low K_{M1} CYP responsible for POD. Traditional studies indicated that CYP2C19 is the enzyme responsible for the high K_{M2} component, whereas the population method failed to identify the high K_{M2} component. A major advantage of the population approach is the ability to estimate the typical EK parameter values for the population and predict the EK parameters for each liver. In addition, the population approach provided information on inter- and intraliver variability for a given biotransformation. In the future, this information may be used to better understand interindividual variability in drug metabolism and better predict drug-drug interactions with new drug entities.

REFERENCES

1. L. B. Sheiner, B. Rosenberg, and V. V. Marathe. Estimation of population characteristics of pharmacokinetic parameters from routine clinical data. *J. Pharmacokinet. Biopharm.* **5**:445–479 (1977).
2. C. C. Peck, D. Z. D'Argenio, and J. H. Rodman. Analysis of pharmacokinetic data for individualized drug dosage regimens. In W. E. Evans, J. J. Schentag, and W. J. Jusko (eds.), *Applied Pharmacokinetics: Principles of Therapeutic Drug Monitoring,* Applied Therapeutic, Inc., Vancouver, 1992, pp. 1–31.
3. A. M. Dahlem, S. R. Allerheiligen, and M. J. Vodicnik. Concomitant toxicokinetics: techniques for and interpretation of exposure data obtained during the conduct of toxicology studies. *Toxicol. Pathol.* **23**:170–178 (1995).
4. S. M. Pai, S. H. Fettner, G. Hajian, M. N. Cayen, and V. K. Batra. Characterization of AUCs from sparsely sampled populations in toxicology studies. *Pharm. Res.* **13**:1283–1290 (1996).
5. S. A. Wrighton, M. Vandenbranden, J. C. Stevens, L. A. Shipley, B. J. Ring, A. E. Rettie, and J. R. Cashman. *In vitro* methods for assessing human hepatic drug metabolism: their use in drug development. *Drug Metab. Rev.* **25**:453–484 (1993).
6. B. J. Ring, S. N. Binkley, L. Roskos, and S. A. Wrighton. Effect of fluoxetine, norfluoxetine, sertraline and desmethyl sertraline on human CYP3A catalyzed 1′-hydroxy midazolam formation in vitro. *J. Pharmacol. Exp. Ther.* **275**:1131–1135 (1995).
7. O. H. Lowry, N. H. Rosenbrough, A. L. Farr, and R. J. Randall. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
8. S. V. Chittur and T. S. Tracy. Rapid and sensitive high-performance liquid chromatographic assay for 6-hydroxychlorzoxazone and chlorzoxazone in liver microsomes. *J. Chromatogr. B Biomed. Sci. Appl.* **693**:479–483 (1997).
9. T. Leemann, C. Transon, and P. Dayer. Cytochrome P450TB (CYP2C): a major mononoxygenase catalyzing diclofenac 4′-hydroxylation in human liver. *Life Sci.* **52**:29–34 (1993).
10. J. W. Harris, A. Rahman, B.-R. Kim, F. P. Guengerich, and J. M. Collins. Metabolism of taxol by human hepatic microsomes and liver slices: participation of cytochrome P450 3A4 and an unknown P450 enzyme. *Cancer Res.* **54**:4026–4035.
11. S. Ekins, M. Vandenbranden, B. J. Ring, J. S. Gillespie, T. J. Yang, H. V. Gelboin, and S. A. Wrighton. Further characterization of the expression in liver and catalytic activity of CYP2B6. *J. Pharmacol. Exp. Ther.* **286**:1253–1259 (1998).
12. K. L. Kunze and W. F. Trager. Isoform-selective mechanism-based inhibition of human cytochrome P450 1A2 by furafylline. *Chem. Res. Toxicol.* **6**:649–656 (1993).
13. I. H. Segel. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems,* John Wiley and Sons, New York, 1993.
14. A. J. Boeckman, S. L. Beal, and L. B. Sheiner (eds). NONMEM Users Guides. NONMEM project group, University of California, San Francisco, CA, 1992.
15. A. R. Boobis, G. C. Kahn, C. Whyte, M. J. Brodie, and D. S. Davies. Biphasic O-deethylation of phenacetin and 7-ethoxycoumarin by human and rat liver microsomal fractions. *Biochem. Pharmacol.* **30**:2451–2456 (1981).
16. W. Tassaneeyakul, D. J. Birkett, M. E. Veronese, M. E. McManus, R. H. Tukey, L. C. Quattrochi, H. V. Gelboin, and J. O. Miners. Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2. *J. Pharmacol. Exp. Ther.* **265**: 401–407 (1993).
17. L. L. von Moltke, D. J. Greenblatt, S. X. Duan, J. Schmider, L. Kudchadker, S. M. Fogelman, J. S. Harmatz, and R. I. Shader. Phenacetin O-deethylation by human liver microsomes in vitro: inhibition by chemical probes, SSRI antidepressants, nefazodone and venlafaxine. *Psychopharmacology* **128**:398–407 (1996).
18. R. A. Copeland. *Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis,* Wiley-VCH, New York 1996.