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# Utility of Intersystem Extrapolation Factors in Early Reaction Phenotyping and the Quantitative Extrapolation of Human Liver Microsomal Intrinsic Clearance Using Recombinant Cytochromes P450

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**ABSTRACT:**

**Reaction phenotyping using recombinant human cytochromes P450 (P450) has great utility in early discovery. However, to fully realize the advantages of using recombinant expressed P450s, the extrapolation of data from recombinant systems to human liver microsomes (HLM) is required. In this study, intersystem extrapolation factors (ISEFs) were established for CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 using 11 probe substrates, based on substrate depletion and/or metabolite formation kinetics. The ISEF values for CYP2C9, CYP2D6, and CYP3A4 determined using multiple substrates were similar across substrates. When enzyme kinetics of metabolite formation for CYP1A2, 2C9, 2D6, and 3A4 were used, the ISEFs determined were generally within 2-fold of that determined on the basis of substrate depletion. Validation of ISEFs was conducted using 10 marketed drugs by comparing the extrapolated data with published data. The major isoforms responsible for the metabolism were identified, and the contribution of the predominant P450s was similar to that of previously reported data. In addition, phenotyping data from internal compounds, extrapolated using the rhP450-ISEF method, were comparable to those obtained using an HLM-based inhibition assay approach. Moreover, the intrinsic clearance ($CL_{int}$) calculated from extrapolated rhP450 data correlated well with measured HLM $CL_{int}$. The ISEF method established in our laboratory provides a convenient tool in early reaction phenotyping for situations in which the HLM-based inhibition approach is limited by low turnover and/or unavailable metabolite formation. Furthermore, this method allows for quantitative extrapolation of HLM intrinsic clearance from rhP450 phenotyping data simultaneously to obtaining the participating metabolizing enzymes.**

## Introduction

Identification of drug-metabolizing enzymes is critical in assessing potential drug interactions and human pharmacokinetic variation. Quantitative in vitro reaction phenotyping data can be obtained through studies using chemical inhibitors in human liver microsomes or expressed cytochrome P450 (P450) isoforms. Recombinant human P450s (rhP450s) have been used increasingly in early discovery because of their availability and simplicity; however, translation of this type of data directly to a contribution to metabolism in human liver microsomes can be problematic. The disconnect can be attributed to intrinsic differences between rhP450 and HLM systems and include variability in intrinsic activity or turnover number (activity per unit amount of P450 enzyme) among different rhP450 expression systems and HLM, differences in the expression level of accessory proteins between recombinant systems and HLM (primarily cytochrome P450 reductase and cytochrome $b_5$), and, most important, the abundance of the respective P450 isoforms (Crespi, 1995; Crespi and Miller, 1999; Venkatakrishnan et al., 2000). Proper consideration and correction of these factors is necessary when reaction phenotyping data generated from rhP450 systems are scaled to HLM.

The use of intersystem extrapolation factors (ISEFs) integrates the variables of intrinsic activity and accessory protein expression between two systems (Proctor et al., 2004). When the ratio of metabolic rates in rhP450 and HLM are calculated for ISEF, the turnover numbers in HLM are expressed as per picomole of P450 by including the hepatic P450 abundance. This approach allows the prediction of metabolism due to the variability of P450 abundance in HLM and rhP450 systems. ISEF values may vary widely depending on the rhP450 expression system, HLM preparation, probe substrate selected, and/or assay conditions used in each laboratory (Proctor et al.,

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**ABBREVIATIONS:** P450, cytochrome P450; rhP450, recombinant human cytochrome P450; HLM, human liver microsomes; ISEF, intersystem extrapolation factors; $CL_{int}$, intrinsic clearance; CLISEF, ISEF defined with respect to intrinsic clearance $CL_{int}$; VISEF, ISEF defined with respect to intrinsic clearance $V_{max}$; RMSE, root mean square error; MRS, mean residual sum; RAF, relative activity factor.

TABLE 1

*Selected probe substrate for each P450 and corresponding concentrations used in ISEF determination*

| P450 | Probe Substrate | Isoform-Specific Metabolite | Substrate Concentration | HLM Conc. | rhP450 Conc.[a] |
|---|---|---|---|---|---|
| | | | *μM* | *mg/ml* | *pmol/ml* |
| 1A2 | Phenacetin[b] | *O*-Deethylation | 0.5 | 0.5 | 76 |
| 2C8 | Paclitaxel | 6α-Hydroxylation | 0.5 | 0.5 | 185 |
| 2C9 | Diclofenac[b] | 4-Hydroxylation | 5 | 0.25 | 89 |
| | *S*-Warfarin | 7-Hydroxylation | 0.5 | 0.5 | 179 |
| | Tolbutamide | Hydroxylation | 1 | 0.5 | 179 |
| 2C19 | *S*-Mephenytoin | 4-Hydroxylation | 1 | 1.0 | 213 |
| 2D6 | Bufuralol[b] | 1-Hydroxylation | 0.5 | 0.25 | 27 |
| | Dextromethorphan | *O*-Demethylation | 0.5 | 0.25 | 27 |
| 3A4 | Midazolam[b] | 1′-Hydroxylation | 1 | 0.25 | 32 |
| | Testosterone | 6β-Hydroxylation | 1 | 0.25 | 32 |
| | Nifedipine | Hydroxylation | 1 | 0.25 | 32 |

[a] Total protein concentration in rhP450 and HLM incubations was constant.
[b] Enzyme kinetics ($K_m$ and $V_{max}$) were determined for CYP1A2, 2C9, 2D6, and 3A4 using these substrates.

2004). In previous studies, ISEFs were defined with respect to either the $V_{max}$ of metabolite formation of a probe substrate or intrinsic clearance ($CL_{int}$) calculated from metabolite formation enzyme kinetics: $CL_{int} = V_{max}/K_m$ (Proctor et al., 2004). In addition, the extrapolation of rhP450 data measured as either metabolite formation or substrate depletion using extrapolation factors calculated from kinetic data of metabolite formation has been used for reaction phenotyping (Crespi, 1995; Nakajima et al., 1999; Venkatakrishnan et al., 2000) and $CL_{int}$ prediction (Emoto et al., 2006). However, extrapolation of substrate depletion data of test compounds obtained in rhP450 using extrapolation factors (such as ISEF) determined on the basis of substrate depletion of probe substrate has not been previously reported and could be a more practical approach in drug discovery.

In the present study, we determined ISEF for the six most prominent P450s responsible for drug metabolism, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. The ISEF values were determined using multiple substrates and three different methods, substrate depletion ($CL_{int}$) and metabolite formation enzyme kinetics ($V_{max}$ and $CL_{int} = V_{max}/K_m$). Using rhP450-ISEF extrapolation, we estimated the relative contribution of the major P450 isoforms to the overall metabolism for 10 marketed drugs and 10 internal compounds. In addition, we explored a novel approach in which the intrinsic clearance in rhP450s is extrapolated using ISEF determined on the basis of substrate depletion to an intrinsic clearance in human liver microsomes. The overall extrapolation accuracy for P450 contribution and $CL_{int}$ prediction between ISEFs determined using different approaches were compared.

## Materials and Methods

**Chemicals and Biological Reagents.** Probe substrates and associated metabolites for CYP1A2 CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Table 1) were purchased from BD Bioscience (San Jose, CA). NADPH, dimethyl sulfoxide, formic acid, acetic acid, acetonitrile, and methanol were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade commercially available.

Recombinant expressed P450s and control preparations from baculovirus-infected Sf9 insect cells (Supersomes) were purchased from BD Gentest (Woburn, MA). Cytochrome *c* reductase was coexpressed in all preparations, and cytochrome $b_5$ was expressed in cDNA-expressed CYP2C8, 2C9, 2C19, and 3A4. Human liver microsomes, 50-donor pool, were purchased from BD Gentest.

**Incubations in rhP450s and HLM.** The substrate and protein concentrations for the $CL_{int}$. determinations used in ISEF calculation are summarized in Table 1. The probe substrate concentrations were chosen to be less than $K_m$ so that the depletion of substrate is predominantly through a specific P450 pathway. Reaction mixtures containing phosphate buffer (50 mM, pH 7.4), magnesium chloride (5 mM), recombinant Supersomes or HLM, and probe substrates, according to final concentrations listed in Table 1, were prepared. The mixture was preincubated at 37°C for 5 min before transfer to a Biomek 2000 liquid handler (Beckman Coulter, Fullerton, CA) for subsequent processing and incubation. Reactions were initiated by the addition of NADPH (2 mM) and terminated by addition of 1 volume of acetonitrile at predetermined time points (0, 5, 10, and 20 min). After quenching, 1 volume of sample was transferred to an injection plate containing 1 volume of 0.1% acetic acid in water with internal standard (100 ng/ml, 7-hydroxycoumarin). The quenched reaction mixtures were centrifuged, and supernatants were collected and analyzed using liquid chromatography-tandem mass spectrometry.

The $CL_{int}$ values in rhP450 for marketed drugs used in the validation (Table 2) and internal compounds were determined using incubation conditions similar to those mentioned previously. The test compounds (0.1 or 1.0 μM) were incubated with six P450s plus an insect control. All experiments included six probe substrate incubations that were used as positive controls. The $CL_{int}$ was calculated on the basis of the parent compound percentage remaining normalized to insect control. The $CL_{int}$ in each P450 was scaled to $CL_{int}$ in HLM using ISEF and the P450 contributions ($f_m$) were calculated using the equations shown under *Data Analysis*.

TABLE 2

*Selected drugs for ISEF validation*

| Drugs | Major Metabolizing Enzyme | Determination of Metabolizing-Enzyme Contribution | Reference |
|---|---|---|---|
| Metoprolol | CYP2D6, 3A4 | Metabolite formation $V_{max}$ and $K_m$ in HLM | Simcyp |
| Sildenafil | CYP3A4, 2C9 | $CL_{int}$ in rhP450, converted by software | Simcyp |
| Imipramine | CYP2D6, 2C19 | Metabolite formation $V_{max}$ and $K_m$ in HLM | Simcyp |
| Carbamazepine | CYP3A4 | Metabolite formation in HLM with inhibitory antibody | Kerr et al. (1994) |
| Rosiglitazone | CYP2C8, 2C9 | $CL_{int}$ in HLM with chemical inhibitors | Simcyp |
| Mirtazapine | CYP1A2, 2D6, 3A4 | Metabolite formation $V_{max}$ and $K_m$ in rhP450 and extrapolated using RAF and HLM with inhibitors | Störmer et al. (2000a,b) |
| Omeprazole | CYP2C19, 3A4 | Metabolite formation $V_{max}$ and $K_m$ in rhP450 | Simcyp |
| Fluoxetine | CYP2D6, 2C9, 3A4 | Metabolite formation $V_{max}$ and $K_m$ in rhP450 scaled using P450 content and HLM with chemical inhibition | Margolis et al. (2000) |
| Tolterodine | CYP2D6, 3A4 | Metabolite formation $V_{max}$ and $K_m$ in HLM | Simcyp |
| Harmine | CYP1A2, 2C9, 2D6 | Metabolite formation in HLM with monoclonal antibodies | Yu et al. (2003) |

The enzyme kinetics ($K_m$ and $V_{max}$) of selected probe substrates for CYP1A2 (phenacetin), CYP2C9 (diclofenac), CYP2D6 (bufuralol), and CYP3A4 (midazolam) were determined in HLM and recombinant P450s on the basis of the formation of the isoform-specific metabolites (Table 1) at concentrations ranging from 0.1 to 100 $\mu$M using similar incubation procedures. The $V_{max}$ and $CL_{int}$, calculated on the basis of $CL_{int} = V_{max}/K_m$, were used for the ISEF calculation (see *Data Analysis*).

Human liver microsomal inhibition studies were performed using isoform-selective chemical inhibitors or inhibitory antibodies, furafylline (1A2), sulfaphenazole (2C9), quinidine (2D6), ketoconazole (3A4), and antibodies to either CYP2C8 or CYP2C19. Reaction mixtures were prepared containing phosphate buffer (50 mM, pH 7.4), magnesium chloride (5 mM), HLM (0.5 mg/ml), and chemical or inhibitory antibody. The mixture was preincubated at 37°C for 5 min. Reactions were initiated by the addition of NADPH (2 mM) and terminated by addition of acetonitrile at predetermined time points (0, 5, 10, and 20 min). $CL_{int}$ was determined on the basis of substrate depletion, and the percent contribution of each P450 isozyme was calculated on the basis of the changes of $CL_{int}$ in the presence and absence of isoform-specific inhibitors.

**High-Performance Liquid Chromatography-Tandem Mass Spectrometry Analysis.** The high-performance liquid chromatography system consisted of Shimadzu Prominence LC-20AD binary pumps (Shimadzu, Columbia, MD), an HTC PAL autosampler (Leap Technologies, Carrboro, NC), and a BDS Hypersil C18 column (50 × 2.1 mm). The mobile phase contained 0.1% formic acid in water with 5 mM ammonium acetate (A) and 0.1% formic acid in acetonitrile-methanol (1:1) (B). The gradient was as follows: 5% mobile phase B was held for 1.0 min followed by an increase to 90% over 3 min. The total flow was 0.4 ml/min, and the sample injection volume was 10 $\mu$l.

An API 4000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an electrospray ionization source was used for the detection of marker substrates and their metabolites using multiple reaction monitoring transitions at optimized mass spectrometry conditions. The system was operated by Analyst 1.4.1 software (Applied Biosystems/MDS Sciex).

**Data Analysis.** *Calculation of $CL_{int}$.* The percentage of parent drug remaining at each incubation time was calculated. The slope (reaction rate) was obtained from the linear regression of the natural log percent parent remaining versus time plot. The $CL_{int}$ was calculated by normalizing the rate to the amount of P450 (picomoles per milliliter) or HLM protein (milligrams per milliliter).

*Estimation of $K_m$ and $V_{max}$.* Nonlinear curve-fitting was performed using WinNonlin (version 5.2; Pharsight, Mountain View, CA). $K_m$ and $V_{max}$ were estimated using the simple $E_{max}$ model ($E = E_{max} \cdot C/C + EC_{50}$, where $E_{max}$ represents $V_{max}$, $EC_{50}$ represents $K_m$, $C$ represents [$S$] substrate concentration, and $E$ represents the rate of metabolism).

*Calculation of ISEF.* ISEFs were defined with respect to either intrinsic clearance $CL_{int}$ ($= V_{max}/K_m$) (CLISEF) or $V_{max}$ (VISEF) depending on whether or not a similar $K_m$ was assumed (Proctor et al., 2004) (eqs. 1 and 2). In this study, we also determined an ISEF using $CL_{int}$ calculated from substrate depletion, defined as ISEF-$CL_{int}$ (eq. 3).

$$\text{VISEF } (V_{max}) = \frac{V_{max}(\text{HLM})}{V_{max}(\text{rhP450}) \times \text{P450 abundance (HLM)}} \quad (1)$$

$$\text{CLISEF } (CL_{int}) = \frac{V_{max}/K_m(\text{HLM})}{V_{max}/K_m(\text{rhP450}) \times \text{P450 abundance (HLM)}} \quad (2)$$

$$\text{ISEF-}CL_{int} = \frac{CL_{int}(\text{HLM})}{CL_{int}(\text{rhP450}) \times \text{P450 abundance (HLM)}} \quad (3)$$

where $CL_{int}$ (HLM) is intrinsic clearance of marker substrate in HLM, $CL_{int}$ (rhP450) is intrinsic clearance of marker substrate in recombinant P450, and P450 abundance (HLM) is P450 abundance in HLM.

In our studies, the primary ISEF for each P450 was calculated using $CL_{int}$ determined on the basis of substrate depletion measured in rhP450 and HLM at a single substrate concentration ($<K_m$) (eq. 3). For the purpose of confirmation and comparison, the ISEFs were also calculated on the basis of $V_{max}$ (VISEF) and $CL_{int}$ ($= V_{max}/K_m$) determined in enzyme kinetic studies for CYP1A2, CYP2C9, CYP2D6, and CYP3A4 using selected marker substrates and the formation of their isoform selective metabolites (Table 3).

*Extrapolation of rhP450 data.* For each validation drug, the intrinsic clearance mediated through individual P450s in HLM ($CL_{int\ P450_j}$) was scaled on the basis of measured $CL_{int}$ in recombinant P450s and ISEF factor using eq. 4:

$$CL_{int\ P450J} = CL_{int\ (rhP450j)} \times P450_J \text{ abundance} \times ISEF_{(P450J)} \quad (4)$$

where $P450_j$ is the $j$th P450 isoform tested/analyzed, $CL_{int\ P450_j}$ is intrinsic clearance by $P450_j$ in HLM (microliters per minute per milligram of protein), $CL_{int\ (rhP450_j)}$ is intrinsic clearance in $rhP450_j$ (microliters per minute per picomole of P450), $P450_j$ abundance is abundance of $P450_j$ (picomoles of P450 per milligram of protein), and $ISEF_{(P450_j)}$ is ISEF for $P450_j$.

*Calculation of extrapolated human liver microsome intrinsic clearance (HLM $CL_{int}$).* The $CL_{int}$ in HLM was extrapolated from scaled rhP450 data as in eq. 5:

$$CL_{int\ HLM} = \sum_{j=1}^{n} CL_{int\ P450_J} \quad (5)$$

where $CL_{int\ P450_j}$ is extrapolated intrinsic clearance by $P450_j$ in HLM (microliters per minute per milligram of protein).

*Calculation of contribution of each P450.* The involvement and contribution of each P450 in HLM was calculated as in eq. 6:

$$\%\text{ contribution, } P450_j = CL_{int\ P450_j} \Big/ \sum_{j=1}^{n} (CL_{int\ P450_j}) \quad (6)$$

**Statistical Analysis.** The correlation between measured and ISEF-extrapolated $CL_{int}$ and the variability of extrapolated data using ISEF determined from different approaches were evaluated. The correlation coefficient ($R$), root mean square error (RMSE), and mean residual sum (MRS) were calculated using a data analysis tool (Microsoft Excel 2003) based on eqs. 7 and 8. The

TABLE 3

*Summary of ISEF determined based on substrate depletion in rhP450 and HLM*

| P450 | Probe Substrate | $CL_{int}$-HLM[a] | $CL_{int}$-rhP450[a] | P450 Abundance[b] | ISEF-$CL_{int}$ |
|---|---|---|---|---|---|
| | | *μl per min/mg* | *μl per min/pmol* | *pmol/mg protein* | |
| 1A2 | Phenacetin | 28.6 ± 4.63 | 3.30 ± 0.11 | 52 | 0.17 |
| 2C8 | Paclitaxel | 22.7 ± 4.64 | 0.671 ± 0.07 | 24 | 1.41 |
| 2C9 | Diclofenac | 298 ± 12.8 | 7.58 ± 0.96 | 73 | 0.54 |
| | *S*-Warfarin | 8.73 ± 2.11 | 0.115 ± 0.01 | | 1.04 |
| | Tolbutamide | 9.12 ± 1.43 | 0.122 ± 0.01 | | 1.02 |
| 2C19 | *S*-Mephenytoin | 4.68 ± 1.59 | 1.35 ± 0.02 | 14 | 0.25 |
| 2D6 | Bufuralol | 41.6 ± 2.72 | 33.8 ± 1.1 | 8 | 0.15 |
| | Dextromethorphan | 61.8 ± 8.35 | 37.5 ± 1.34 | | 0.21 |
| 3A4 | Midazolam | 410 ± 21.4 | 13.8 ± 1.32 | 142 | 0.21 |
| | Testosterone | 76.6 ± 6.86 | 3.62 ± 0.28 | | 0.15 |
| | Nifedipine | 210 ± 14.3 | 14.3 ± 0.51 | | 0.11 |

[a] $CL_{int}$ data are means ± S.D ($n$ = 6 or 7 from two or three assays, triplicate measurements per assay).
[b] P450 abundance reported in Simcyp based on Rowland-Yeo et al. (2003).

RMSE was calculated to compare variables between different ISEF extrapolated data and directly measured data. A smaller RMSE indicates less variability between the two methods. The MRS was used to measure the bias of different methods. A negative value indicates underestimation and a positive value indicates overestimation.

$$\text{RMSE} = \sqrt{\frac{\sum_{1}^{n}(ISEF\ extrapolated - measured\ in\ HLM)^2}{n}} \quad (7)$$

$$\text{MRS} = \frac{\sum_{1}^{n}(ISEF\ extrapolated - measured\ in\ HLM)}{n} \quad (8)$$

The overall accuracies of $CL_{int}$ and P450 contribution extrapolation methods were determined using eq. 9 (Obach, 1999):

$$\text{Average fold error} = 10^{\left|\frac{\sum \log\left(\frac{ISEF\ extrapolated}{measured\ in\ HLM}\right)}{n}\right|} \quad (9)$$

## Results

**ISEF Determined on the Basis of Substrate Depletion ($CL_{int}$).** Intrinsic clearance of each marker substrate in HLM and rhP450s was determined in at least two separate assays with triplicate incubations in each assay. ISEFs calculated on the basis of measured $CL_{int}$ (eq. 3) in rhP450 and HLM for each P450 are presented in Table 3. The ISEF values determined on the basis of multiple substrates for CYP3A4, CYP2D6, and CYP2C9 were within 2-fold for all the substrates tested. The statistical mean for each ISEF was used for the extrapolation of the rhP450 data in subsequent studies.

**ISEF Determined on the Basis of Enzyme Kinetics.** For CYP1A2, CYP2C9, CYP2D6, and CYP3A4, the $K_m$ and $V_{max}$ were also determined on the basis of metabolite formation of probe substrates. The calculated ISEFs using $V_{max}$ and $CL_{int}$ ($= V_{max}/K_m$) are shown in Table 4 and compared with the ISEFs obtained using the substrate depletion method (ISEF-$CL_{int}$). The ISEFs determined from each method (metabolite formation and substrate depletion) were similar (within 2-fold), with the exception of the VISEF for CYP2D6, which was slightly greater than 2-fold compared with the ISEF-$CL_{int}$.

**Validation of ISEF.** Ten compounds were selected for the validation of in-house generated ISEFs (Table 2). These compounds were chosen on the basis of available literature data and multiple P450s contributing to their metabolism. The $CL_{int}$ determined in each recombinant P450 isoform was extrapolated to HLM $CL_{int}$ using ISEF (eq. 4). The sum of extrapolated HLM $CL_{int}$ values by each P450 represents the total HLM $CL_{int}$ and was used for the calculation of percent contribution by each isoform (eqs. 5 and 6). The contribution of individual P450 isoforms to the metabolism was determined and compared with reported data (Fig. 1). The total HLM $CL_{int}$ based on extrapolated data (using ISEF-$CL_{int}$) was also compared with the measured HLM $CL_{int}$ data to ensure that no major P450 pathways were missed (all within 2-fold), and a good correlation ($R^2 = 0.928$) was observed (Fig. 2).

In general, the P450 profile obtained using the rhP450-ISEF extrapolation approach is in good agreement with reported data. For all drugs tested, the major P450 isoforms responsible for the HLM metabolism were identified, and the contributions of predominant P450s to total metabolism are similar to previously reported data. In consideration of the relatively high variability of phenotyping data reported across different laboratories, data generated from these studies using the rhP450-ISEF approach fall in the range that are well accepted.

**rhP450-ISEF Extrapolated Data for Internal Compounds.** Data from a retrospective analysis of more than a dozen proprietary compounds were used as a test set to gain confidence in the use of ISEF generated in-house. For 10 internal compounds, full phenotyping data previously obtained from an HLM assay using chemical inhibition were compared with the data extrapolated using the rhP450-ISEF method and are presented in Table 5. The percent contributions of each P450 extrapolated from the rhP450 assay and measured from HLM chemical inhibition assay were compared. The data obtained from both methods are comparable regarding the major contributing isoform and their contribution to the total $CL_{int}$ in HLM. For 20 internal compounds, the $CL_{int}$ by each P450 in HLM was obtained using rhP450-ISEF (ISEF-$CL_{int}$) extrapolation. The sum of $CL_{int}$ by each P450 in HLM, representing extrapolated intrinsic $CL_{int}$ in HLM, was compared with measured HLM $CL_{int}$. A good correlation ($R^2 = 0.962$) was observed, and all extrapolated data were within 2-fold of the corresponding HLM $CL_{int}$ values (Fig. 2).

## Discussion

The accurate prediction of P450 isoform contributions to a specific transformation in the metabolism of a drug can be challenging, especially in early drug discovery, because often the detailed metabolic pathway and complete structural information of metabolites are unavailable. In practice, the relative contribution of major isoforms has been determined using changes in substrate depletion in the presence and absence of selective P450 inhibitors. This approach can be problematic for compounds that have a low $CL_{int}$. Often, the use of rhP450 isoforms can overcome this challenge by providing a system with greater metabolic activity compared with HLM. However, the scaling of data generated from recombinant P450 incubations introduces other confounding factors. The use of ISEFs has been proposed as a method to extrapolate these types of data and use of ISEFs established in an individual laboratory is necessary (Proctor et al., 2004).

TABLE 4

*ISEF determined based on metabolite formation and comparison with ISEF determined using substrate depletion*

| P450 | Probe Substrate | HLM | | rhP450 | | ISEF (Metabolite Formation) | | ISEF (Substrate Depletion): ISEF-$CL_{int}$[c] |
|---|---|---|---|---|---|---|---|---|
| | | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | VISEF[a] | CLISEF[b] | |
| | | *pmol per min/mg* | *μM* | *pmol per min/pmol P450* | *μM* | | | |
| 1A2 | Phenacetin | 511 | 47 | 52 | 34 | 0.19 | 0.14 | 0.17 |
| 2C9 | Diclofenac | 1609 | 17.7 | 29 | 9.7 | 0.76 | 0.42 | 0.54 |
| 2D6 | Bufuralol | 135 | 11 | 52 | 8.3 | 0.32 | 0.23 | 0.15 |
| 3A4 | Midazolam | 973 | 2.94 | 21 | 2.89 | 0.33 | 0.32 | 0.21 |

[a] VISEF-calculated on the basis of $V_{max}$ (eq. 1).
[b] CLISEF-calculated on the basis of $CL_{int} = V_{max}/K_m$ (eq. 2).
[c] ISEF-$CL_{int}$ calculated on the basis of measured substrate depletion, $CL_{int}$ (eq. 3).

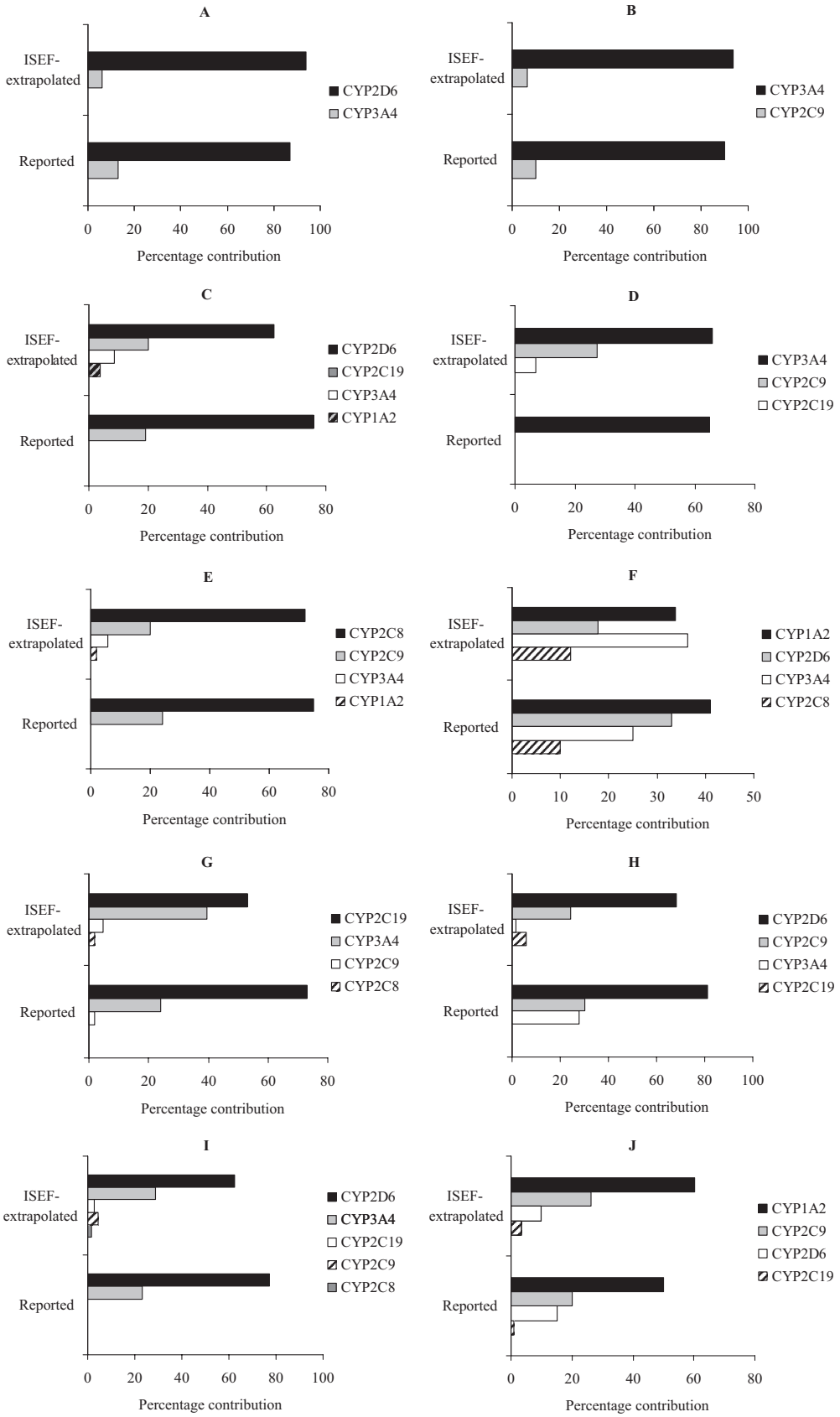


FIG. 1. Reaction phenotyping data for 10 validation drugs: comparison of ISEF-extrapolated data with reported data. A, metoprolol (Simcyp). B, sildenafil (Simcyp). C, imipramine (Simcyp). D, carbamazepine (Kerr et al., 1994). E, rosiglitazone (Simcyp). F, mirtazapine (Störmer et al., 2000b). G, omeprazole (Simcyp). H, fluoxetine (Margolis et al., 2000). I, tolterodine (Simcyp). J, harmine (Yu et al., 2003). The ISEFs used in the extrapolations are the value determined on the basis of substrate depletion. For P450s, when multiple probe substrates were used, the mean value of ISEF was used (Table 3).

On the basis of the level of confidence in the rhP450-ISEF extrapolation required, ISEF values can be derived using either substrate depletion or metabolite formation enzyme kinetics. For early reaction phenotyping, an approach that incorporates a moderate confidence level with higher throughput compatibility is more appropriate. In our study, for the first time, we used a single substrate concentration

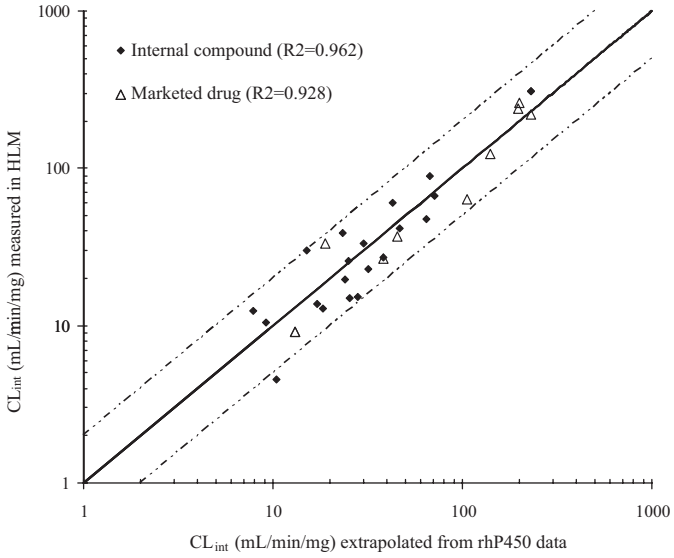


FIG. 2. Correlation between $CL_{int}$ extrapolated using ISEF-$CL_{int}$ from rhP450 data and measured in HLM for all validation drugs and internal compounds tested.

($<K_m$) and measured substrate depletion in both rhP450 and HLM as a primary method for ISEF determination. The substrate depletion method is based on the assumptions that the probe substrate is highly selective to the isozyme at the concentration below $K_m$, and the effect of nonspecific microsomal binding on the $K_m$ in rhP450 and HLM can be ignored. In our studies, an identical protein concentration was used in both incubations; therefore, the latter assumption is reasonable. The ISEFs for six P450s were generated on the basis of substrate depletion, ISEF-$CL_{int}$, and were compared with the ISEF determined using the more traditional method, metabolite formation (VISEF and CLISEF) (Table 4). The VISEF values tended to be slightly higher than those determined on the basis of measured substrate depletion (ISEF-$CL_{int}$) but were well within 2-fold for CYP1A2, CYP2C9, and CYP3A4 and slightly more than 2-fold for CYP2D6. The CLISEF values determined on the basis of $CL_{int}$ ($= V_{max}/K_m$) are generally lower than the VISEF values and were well within 2-fold of that determined on the basis of measured $CL_{int}$ (ISEF-$CL_{int}$) for all four P450s (Table 4).

The contributions of CYP3A4, CYP2C9, and CYP2D6 extrapolated using ISEF-$CL_{int}$, VISEF, and CLISEF were compared with directly measured data (using HLM inhibition assay or metabolite formation as reported) (Fig. 3). In addition, the overall extrapolation accuracy for each ISEF method was evaluated and is presented in Table 6. The average fold error values for the three different approaches in the ISEF determination and for all three P450s were less than 2-fold. For CYP3A4 and CYP2C9, extrapolation using ISEF-$CL_{int}$ had better accuracy, whereas for CYP2D6, the extrapolation accuracy using the three different ISEFs was similar (Table 6; Fig. 3). Use of the CLISEF yielded relatively poor extrapolation accuracy compared directly with the measured data for CYP2C9 (average fold error 1.96). These data suggest that the substrate depletion method is a suitable alternative to traditional approaches.

Considering the possible substrate dependence of ISEF, we tested multiple substrates for CYP2C9, CYP2D6, and CYP3A4 in its ISEF determination. With all other conditions set, the change of substrate alone resulted in a less than 2-fold difference in ISEF values for

TABLE 5

*Comparison of P450 phenotyping data using rhP450-ISEF extrapolation and HLM chemical inhibition for proprietary compounds*

| Compound | P450 | rhP450 $CL_{int}$ | HLM $CL_{int}$-ISEF Extrapolated | % Contribution | |
|---|---|---|---|---|---|
| | | | | Calculated | Measured (HLM with Inhibitor)[a] |
| | | *μl per min/pmol* | *μl per min/mg* | | |
| I | 2D6 | 3.50 | 5.04 | 65 | 51 |
| | 2C19 | 0.434 | 1.52 | 19 | 25 |
| | 1A2 | 0.141 | 1.25 | 16 | 24 |
| II | 3A4 | 0.708 | 15.5 | 100 | 75 |
| | 1A2 | N.D. | N.D. | | 12 |
| | 2C9 | N.D. | N.D. | | 13 |
| III | 3A4 | 0.932 | 20.4 | 85 | 100[b] |
| | 2C9 | 0.058 | 3.7 | 15 | N.A. |
| IV | 3A4 | 1.07 | 23.4 | 72 | 71 |
| | 2C9 | 0.08 | 5.10 | 16 | 29 |
| | 2C19 | 1.10 | 3.85 | 12 | N.A. |
| V | 3A4 | 0.770 | 16.8 | 96 | 94 |
| | 2C19 | 0.219 | 0.77 | 4 | 6 |
| VI | 3A4 | 2.02 | 44.2 | 100 | 100 |
| VII | 3A4 | 0.781 | 17.1 | 90 | 86 |
| | 2C8 | 0.045 | 1.52 | 8.1 | N.A. |
| | 2C19 | 0.078 | 0.27 | 1.4 | 14 |
| VIII | 3A4 | 1.064 | 23.3 | 94 | 81 |
| | 1A2 | 0.163 | 1.44 | 6 | 19 |
| IX | 3A4 | 1.412 | 30.9 | 79 | 67 |
| | 1A2 | 0.318 | 2.81 | 7.2 | 3.4 |
| | 2C19 | 0.547 | 1.91 | 4.9 | 8.8 |
| | 2C9 | 0.047 | 2.98 | 7.7 | 9.6 |
| | 2D6 | 0.193 | 0.28 | 0.7 | 11 |
| X | 3A4 | 0.912 | 19.4 | 77 | 80[c] |
| | 1A2 | 0.256 | 2.26 | 8.9 | 14[c] |
| | 2C8 | 0.091 | 3.08 | 12 | 4.5[c] |
| | 2C19 | 0.157 | 0.55 | 2.2 | 1.7[c] |

N.D., not detected; N.A., data not available.
[a] Experimental data normalized to 100%.
[b] Determined on the basis of hydroxylation metabolite formation.
[c] Determined on the basis of one major metabolite formation in $^{14}$C-X study.

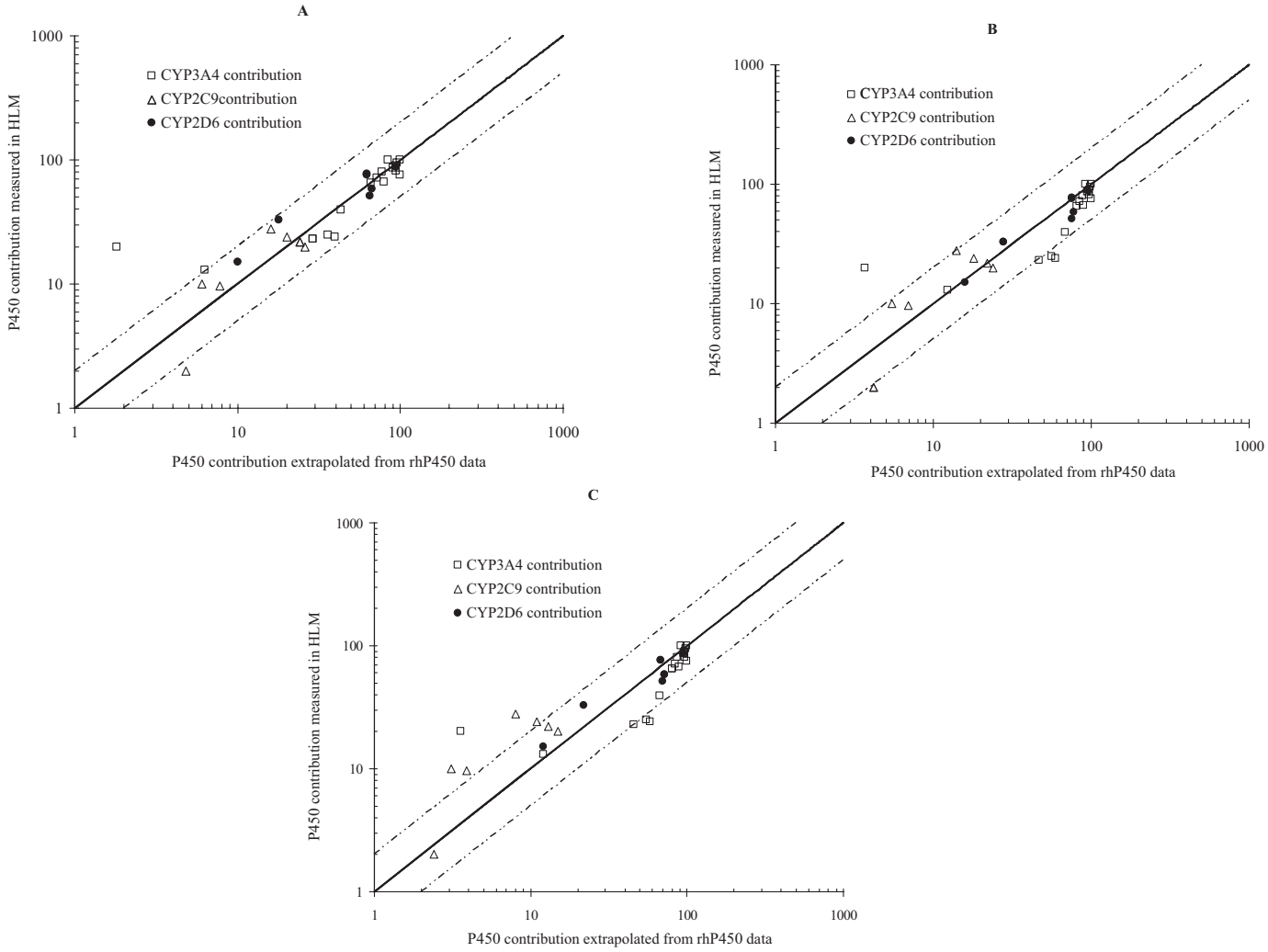


FIG. 3. Correlation between P450 contribution extrapolated using different ISEF methods and that measured using HLM inhibition methods. A, extrapolations based on ISEF-$CL_{int}$. B, extrapolations based on VISEF. C, extrapolations based on CLISEF.

CYP2C9, CYP2D6, and CYP3A4. The contributions of CYP3A4 extrapolated using ISEFs determined from three substrates, CYP2C9 from three substrates, and CYP2D6 from two substrates were compared with the directly measured data (Table 6). For CYP3A4 and CYP2D6, the extrapolation using ISEF determined from the different substrates provided a similar extrapolation accuracy (Table 6). For CYPC9, the extrapolation accuracy using the ISEF determined from diclofenac yielded a relatively poor extrapolation accuracy compared

TABLE 6

*Accuracy of extrapolation of P450 contributions using ISEF determined by different methods and substrates*

| | Average Fold Error (rhP450-ISEF Extrapolated/Directly Measured in HLM) | | |
|---|---|---|---|
| | CYP2C9 Contribution ($n$ = 7 Compounds) | CYP2D6 Contribution ($n$ = 7 Compounds) | CYP3A4 Contribution ($n$ = 17 Compounds) |
| ISEF determined using different methods | | | |
| ISEF-$CL_{int}$ | 1.03 | 1.05 | 1.05 |
| VISEF | 1.15 | 1.10 | 1.17 |
| CLISEF | 1.96 | 1.03 | 1.16 |
| ISEF-$CL_{int}$ determined using different substrates | | | |
| CYP2C9 | | | |
| Diclofenac | 1.55 | | |
| *S*-Warfarin | 1.14 | | |
| Tolbutamide | 1.11 | | |
| CYP2D6 | | | |
| Bufuralol | | 1.19 | |
| Dextromethorphan | | 1.08 | |
| CYP3A4 | | | |
| Midazolam | | | 1.03 |
| Testosterone | | | 1.14 |
| Nifedipine | | | 1.24 |

with that for the other two probe substrates. Because there are a number of factors that can differ in determining ISEF values, including the recombinant and HLM system, substrate, and assay conditions among each laboratory, the combination of these variants could easily cause a 10- to 100-fold difference in ISEF values (Proctor et al., 2004). For preliminary phenotyping determinations, an average ISEF from multiple substrate measurement can be used.

The relative contribution of each P450 isoform to the overall metabolism is a function of substrate concentration depending on the metabolic pathway. Reaction phenotyping at high substrate concentrations may not reflect the relative importance of the isoform at the therapeutically relevant concentrations. The ISEF extrapolation of $CL_{int}$ in individual P450 isoforms at low substrate concentrations provided a reasonable prediction of the major P450 isoform contributing to the metabolism in our data set. For example, the metabolism of mirtazapine is reported to involve five P450 isoforms through at least three metabolic pathways (Störmer et al., 2000a,b). These authors concluded that the relative contribution from three major isoforms, CYP1A2, CYP2D6, and CYP3A4, is concentration-dependent in each metabolic pathway, ranging from 15 to 50% at concentrations of 0.1 to 5 $\mu$M. CYP2C8 and CYP2C9 contribute less than 10% to mirtazapine metabolism (Störmer et al., 2000a,b). Our study using the ISEF approach based on mirtazapine substrate depletion at 1 $\mu$M in rhP450s predicted a 32% contribution of CYP1A2, 17% of CYP2D6, and 34% of CYP3A4. The ISEF extrapolation predicted an 11% contribution of CYP2C8 and less than 10% from CYP2C19. Our studies indicated no contribution of CYP2C9. The data presented in these studies demonstrate the utility of the ISEF approach in phenotyping of drugs with multienzyme biotransformation pathways.

A potential application of the ISEF approach in early drug discovery is the prediction of metabolic clearance in human liver microsome with information of predominant contributing isoforms to the intrinsic clearance. If we assume that all major metabolizing enzymes were identified and their contributions were quantified using rhP450-ISEF method, the addition of contribution of each P450 to the metabolism would be close to total $CL_{int}$ in HLM. On the basis of the data obtained from our studies, there is a good correlation between the ISEF-$CL_{int}$ extrapolated $CL_{int}$ and HLM measured data (Fig. 2). In addition, the correlation and variability between HLM measured data and extrapolated data using the ISEFs from different approaches for 22 compounds that are mainly metabolized (>90%) by CYP1A2, 2C9, 2D6, and 3A4 were analyzed (Table 7; Fig. 4). The extrapolated $CL_{int}$ using ISEF determined on the basis of substrate depletion [$R^2$ = 0.966 using ISEF-$CL_{int}$ mean value of multiple substrates (Fig. 4A) and $R^2$ = 0.901 using ISEF-$CL_{int}$ from a single substrate (Fig. 4B)] is better correlated with $CL_{int}$ measured in HLM than that extrapolated using ISEF on the basis of enzyme kinetics of a single substrate [$R^2$ = 0.888 using VISEF (Fig. 4C), and $R^2$ = 0.821 using CLISEF (Fig. 4D)]. Nearly 90% of extrapolated data using ISEF-$CL_{int}$ were within a 2-fold range of HLM data and near uniformly distributed about unity. Approximately 50% of extrapolated data using VISEF and CLISEF were more than 2-fold higher than those measured in HLM. In addition, the RMSE values for the extrapolations using ISEF-$CL_{int}$ were smaller than those using VISEF and CLISEF, indicating less variability between ISEF-$CL_{int}$ extrapolated data and HLM measured data (Table 7). Of the four ISEF approaches established in this study, the extrapolation using the mean value of ISEF determined on the basis of substrate depletion gave the best extrapolation accuracy (average fold error of 1.03). Overall, these data provided information for both HLM metabolic $CL_{int}$ and major P450 contributions to the metabolic clearance. Incorporating the rhP450 data and ISEF into modeling and simulation software, such as Simcyp, provides conve-



TABLE 7

*$CL_{int}$ measured in HLM and extrapolated from rhP450 data using ISEF determined by different methods*

Compounds delineated by letters are internal compounds. ISEF method 1: ISEF determined on the basis of substrate depletion (ISEF-$CL_{int}$), mean ISEF value for CYP2C9, CYP2D6, and CYP3A4 using multiple substrates. ISEF method 2: ISEF determined on the basis of substrate depletion (ISEF-$CL_{int}$) of single substrate for CYP2C9 (diclofenac), CYP2D6 (bufuralol), and CYP3A4 (midazolam). ISEF method 3: ISEF determined on the basis of metabolite formation enzyme kinetics, VISEF. ISEF method 4: ISEF determined on the basis of metabolite formation enzyme kinetics, CLISEF.

| Compound | $CL_{int}$ Measured in HLM | $CL_{int}$ Extrapolated Using ISEF | | | |
|---|---|---|---|---|---|
| | | Method 1 | Method 2 | Method 3 | Method 4 |
| A | 30 | 15 | 21 | 33 | 32 |
| B | 39 | 24 | 30 | 47 | 44 |
| C | 14 | 17 | 24 | 37 | 36 |
| D | 60 | 43 | 60 | 95 | 92 |
| E | 13 | 18 | 25 | 38 | 37 |
| F | 20 | 24 | 33 | 52 | 50 |
| G | 27 | 38 | 49 | 74 | 70 |
| H | 15 | 28 | 37 | 57 | 52 |
| I | 41 | 47 | 64 | 100 | 96 |
| J | 89 | 67 | 91 | 141 | 135 |
| K | 26 | 25 | 34 | 52 | 50 |
| L | 11 | 9 | 12 | 19 | 19 |
| M | 67 | 71 | 97 | 152 | 146 |
| N | 5 | 11 | 14 | 22 | 21 |
| O | 309 | 230 | 312 | 485 | 466 |
| P | 33 | 30 | 32 | 50 | 48 |
| Metoprolol | 9 | 13 | 11 | 24 | 17 |
| Carbamazepine | 0 | 5 | 6 | 9 | 8 |
| Fluoxetine | 27 | 38 | 31 | 58 | 41 |
| Sildenafil | 221 | 229 | 310 | 486 | 466 |
| Tolterodine | 260 | 201 | 199 | 366 | 296 |
| Harmine | 238 | 197 | 174 | 219 | 154 |
| $R^2$ | | 0.966 | 0.901 | 0.888 | 0.821 |
| Average fold error | | 1.03 | 1.26 | 1.95 | 1.79 |
| MRS | | −7.8 | 5.2 | 48 | 37 |
| RMSE | | 14 | 29 | 49 | 57 |

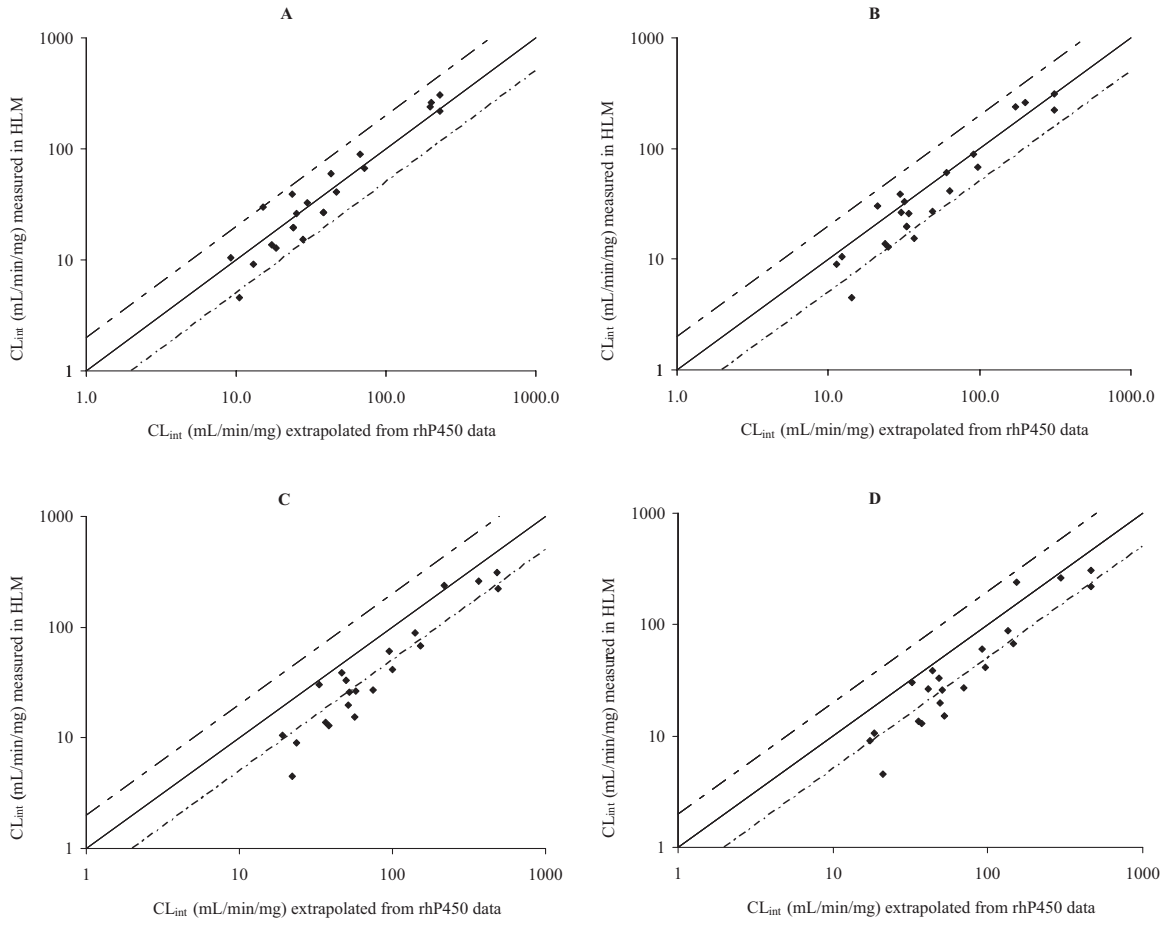


FIG. 4. Correlation between $CL_{int}$ extrapolated using the different ISEF approaches and measured in HLM for 22 compounds metabolized predominantly (>90%) by CYP1A2, 2C9, 2D6, and 3A4. A, ISEF determined on the basis of substrate depletion (ISEF-$CL_{int}$); mean ISEF value for CYP2C9, CYP2D6, and CYP3A4 using multiple substrates. B, ISEF determined based on substrate depletion (ISEF-$CL_{int}$) of a single substrate for CYP2C9 (diclofenac), CYP2D6 (bufuralol), and CYP3A4 (midazolam). C, ISEF determined on the basis of metabolite formation enzyme kinetics, VISEF. D, ISEF determined on the basis of metabolite formation enzyme kinetics, CLISEF.

nient tools for understanding the in vitro-in vivo correlation (Howgate et al., 2006).

Other approaches that have been used in scaling phenotyping data generated from rhP450 system to HLM include incorporation of CYP450 abundance factors and relative activity factor (RAF) (Crespi, 1995; Crespi and Penman, 1997; Crespi and Miller, 1999; Venkatakrishnan et al., 2000). When CYP450 abundance factors are used, typically the average abundance of each P450 in human liver is used, and the differences in intrinsic activity or accessory protein expression between the two systems are not corrected. This approach may lead to an under- or overestimation of contribution because of differences in activity between different recombinant isoforms. The RAF approach has been successfully used to estimate P450 isoform contribution to drug metabolism in several laboratories (Kobayashi et al., 1997; Venkatakrishnan et al., 1998; von Moltke et al., 1998; Nakajima et al., 1999). The use of RAF scaling factors in the prediction of metabolism due to variability of P450 abundance between the HLM and rhP450 systems can be difficult (Proctor et al., 2004).

In conclusion, this method does not provide a replacement for more traditional approaches using chemical inhibition or correlation analysis; rather it would serve as an additional tool for reaction phenotyping when traditional approaches are not feasible for compounds with low turnover in HLM. This approach would also provide quantitative extrapolated HLM intrinsic clearance data along with reaction phenotyping data. In addition, these studies provide added support for the ISEF approach in early reaction phenotyping but also suggest additional aspects that will aid in the characterization of drugs in later stages of development.

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#### Authorship Contributions

*Participated in research design:* Chen, Liu, Nguyen, and Fretland.
*Conducted experiments:* Liu and Nguyen.
*Performed data analysis:* Chen.
*Wrote or contributed to the writing of the manuscript:* Chen and Fretland.

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