UTILITY OF RECOMBINANT ENZYME KINETICS IN PREDICTION OF HUMAN CLEARANCE – IMPACT OF VARIABILITY, CYP3A5 AND CYP2C19 ON CYP3A4 PROBE SUBSTRATES

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Running title: Quantitative prediction of clearance from recombinant CYPs

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Abbreviations used are: CL_{int}, intrinsic clearance; CL_{max}, maximum clearance, rCYP, HLM, human liver microsomes; RAF, Relative activity factor; rCYP, recombinant cytochrome P450
ABSTRACT

A systematic kinetic analysis of the metabolism of five benzodiazepines (low to high clearance compounds) was performed in CYP3A4, CYP3A5 and CYP2C19 baculovirus-expressed recombinant systems. The data obtained in the expression systems were scaled and compared to human liver microsomal predicted clearance and observed in vivo values, using either CYP relative activity factors (RAF) or the relative abundance approach. Inter-individual variability, both in content (CYP3A4, CYP3A5) and activity (CYP3A4, CYP3A5 and CYP2C19), were incorporated in the clearance prediction by bootstrap analysis. These re-sampling Monte Carlo based simulations were performed in order to justify any distribution assumptions in the generated range of the predicted clearance due to a limited sample size. Therefore, this approach allowed extrapolation of the recombinant clearance data to specific population groups and investigation of the role of 'minor' forms like CYP3A5 and CYP2C19 in comparison to the most prolific CYP3A4. The use of quinidine 3-hydroxylation and alprazolam 1'-hydroxylation, as RAF markers for CYP3A4 and CYP3A5 activity, respectively, and the incorporation of variability improved the clearance prediction of the selected benzodiazepines (apart from flunitrazepam) to within the 2-fold of the in vivo value. Clearance estimates from the immunoquantified protein levels was approximately 8-fold lower in comparison to the RAF approach. The differences observed in the benzodiazepines metabolite pathway ratios between CYP3A4 and CYP3A5, particularly for 1’- to 4-hydroxymidazolam and alprazolam provided useful measure of inter-individual differences within the CYP3A family.
Recombinant CYP450 enzymes (rCYP) are a convenient and successful in vitro tool for the identification of the metabolic pathway(s) and the quantification of the individual CYP enzyme(s) contributing to the overall metabolism of a drug (Crespi, 1995, Ito et al., 1998, Nakajima et al., 2002). In a large number of cases CYP3A enzymes are found to be the enzyme of paramount importance. However, recent studies have placed emphasis on other CYP3A family members, notably CYP3A5, which demonstrate either similar or reduced metabolic activity in comparison to CYP3A4 (Williams et al., 2002, Patki et al., 2003), and the clinical significance of hepatic and intestinal CYP3A5 is currently under debate (Paine et al., 1997, Lin et al., 2002, Thummel, 2003, Westlind-Johnsson, 2003, Wong et al., 2004, Xie et al., 2004).

Recombinant enzymes also represent an alternative in vitro metabolic system to hepatic microsomes or hepatocytes to predict the in vivo clearance of drugs (Ito et al., 1998, Hirota et al., 2001). This approach offers an advantage in allowing incorporation and customisation of inter-individual variation of CYP expression, frequently a confounding element in human prediction from hepatic microsomes, particularly for CYP3A substrates. In many microsomal prediction studies, inter-individual variability issues are ignored as predictions are based on kinetic data generated in a limited number of livers or using a ‘representative’ liver pool rather than a characterized liver bank. Approaches to introduce population variability in CYP expression into in vivo predictions from hepatic tissues have yet to be established.

Recently a number of authors have questioned the degree of CYP3A variability in vivo. A collated database of 17 independent studies by Lin et al. (2001) indicated only a 4-fold inter-individual variability in 85% of healthy subjects. Floyd et
al. (2003) observed a similar degree of variability with the probe midazolam, regardless of the route of administration, reflecting therefore hepatic (intravenous route) and combined hepatic and intestinal activity (oral route). However, one could argue that bias in the estimate of variability due to strict study criteria (healthy volunteers, low dose of midazolam, certain ethnic groups) could reduce the incidence of extreme data. In addition to intrinsic variability, in vitro differences between the liver banks can be associated with storage and degradation during harvesting (possible differential degradation between CYP3A4 and CYP3A5 indicated by Floyd et al., 2003). Therefore, it is not surprising that the range of midazolam CLint estimates have been reported to differ 5-fold between two liver banks (Rawden et al., submitted for publication). In addition, the contribution of both enzymes is rarely included in the actual prediction of clearance of CYP3A substrates (Hirota, et al., 2001). Both relative activity factors (RAF) and relative abundance of certain CYPs have been proposed for the quantitative prediction of clearance from heterologous expression systems (Störmer et al., 2000, Venkatakrishnan et al., 2000). The application of both approaches in clearance prediction and the selection of the appropriate marker substrate for determination of RAF, both for CYP3A4 and CYP3A5, are assessed here.

The overall aim of the current study is to investigate the utility of recombinant enzymes for human clearance prediction and to incorporate a range of complex issues associated with CYP3A – well-documented atypical kinetics (Galetin et al., 2003), importance of parallel pathways and multiple CYP involvement, in particular the contribution of two polymorphic, ‘minor’ enzymes (CYP3A5 and CYP2C19). Additionally, approaches to introduce inter-individual variability in clearance estimates obtained by both RAF and CYP abundance approaches are investigated and
compared with the prediction obtained using human liver microsomes and in vivo
data collated from a number of clinical studies (Rawden et al., submitted for
publication). Five benzodiazepines – midazolam, diazepam, triazolam, flunitrazepam
and alprazolam were selected due to the 100-fold difference in their microsomal
clearance, the availability of an extensive in vivo database previously collated in our
laboratory (Rawden et al., submitted for publication) and the involvement of
CYP3A4, CYP3A5 and CYP2C19 in their metabolism (Bertilsson et al., 1989,
Materials and Methods

Chemicals. Midazolam, triazolam, alprazolam, diazepam, flunitrazepam, quinidine, mephenytoin, NADP, isocitric dehydrogenase were purchased from Sigma Chemicals Co. (Poole, Dorset, UK). (3S)-3-hydroxyquinidine, 4’-hydroxymephenytoin and midazolam metabolites were obtained from Ultrafine Chemicals (Manchester, UK). Alprazolam and triazolam metabolites were purchased from Biomol International (PA, USA) and flunitrazepam metabolites were a gift from Roche (Basel, Switzerland). All other reagents and solvents were of high analytical grade. Microsomes from baculovirus-insect-cell-expressed CYP3A4 with coexpressed NADPH-cytochrome P-450 reductase (CYP3A4/OR) were obtained from BD Gentest Co. (Woburn, MA, USA).

Incubation conditions. The kinetic studies were performed in SUPERSONES®, baculovirus-insect-cell-expressed systems, containing no cytochrome b5. Incubation times (5 mins – midazolam, 10 mins - mephenytoin and 20 mins for all the other benzodiazepines and quinidine) and protein concentrations (0.05-0.2 mg/ml) were within the linear range for each individual substrate. Protein concentration was equivalent to the relative content of 13.5-22 pmol CYP/incubation system and the equivalent amounts of all three enzymes were used. Microsomes were suspended in phosphate buffer (0.1 M, pH 7.4) with the final incubation volume of 0.25 mL. Samples were pre-incubated for 5 min in a shaking water bath at 37°C and each reaction was initiated with an NADPH regenerating system (1mM NADP+, 7.5 mM isocitric acid, 10 mM magnesium chloride and 0.2 units isocitric dehydrogenase).

No significant microsomal binding was observed (<10%) for neither of the substrates investigated. The final concentration of the organic (either methanol or acetonitrile) in incubation media was 0.1 % v/v. The substrate concentrations ranged from 2.5-1000
µM (alprazolam, triazolam), 2.5-500 µM (diazepam), 10 -1000 µM (flunitrazepam, mephenytoin), 1-200 µM (midazolam) and 1-500 µM (quinidine). The reaction was terminated by 0.25 mL of ice-cold acetonitrile with 1µM of the appropriate internal standard samples were centrifuged at 13,400g for 10 min and further analyzed by LC-MS/MS.

**LC-MS/MS Methods.** Each metabolite pair, together with either diazepam (for 1'- and 4-hydroxyalprazolam and triazolam), triazolam (for 3-hydroxy and nordiazepam), clobazam (for 3-hydroxy and desmethylflunitrazepam), alprazolam (for 1’- and 4-hydroxymidazolam) or dextromethorphan (for 3-hydroxyquinidine) as internal standard, were separated on a Luna C18(2) 50 x 4.6 mm 3µm column (Phenomenex, UK) at 40 °C using either a binary or ternary gradient maintained at 1 ml/min by a Waters Alliance 2795 HT LC system.

For 1’- and 4-hydroxyalprazolam, an initial mobile phase of 90 % 0.001 M ammonium acetate/ 10 % acetonitrile was ramped immediately to 66 % 0.001 M ammonium acetate/ 34 % acetonitrile at 1 minute and immediately to 34 % 0.001 M ammonium acetate/ 66 % acetonitrile at 4 minutes. The initial ratio was immediately re-established at 5 minutes and maintained to 6 minutes. The retention times were approximately 4.1 (4-hydroxyalprazolam), 4.4 (1’-hydroxyalprazolam) and 5.7 (diazepam) minutes. For 3-hydroxy and nordiazepam, an initial mobile phase of 90 % 0.001 M ammonium acetate/ 10 % acetonitrile was ramped linearly to 18 % 0.001 M ammonium acetate/ 82 % acetonitrile from 1 to 4 minutes. The initial ratio was immediately re-established at 4 minutes and maintained to 5 minutes. The retention times were approximately 4.2 (triazolam) and 4.4 (3-hydroxy, nordiazepam) minutes. For 3-hydroxy and desmethylflunitrazepam, an initial mobile phase of 90 % 0.001 M ammonium acetate/ 10 % acetonitrile was ramped linearly to 10 % 0.001 M
ammonium acetate/ 90 % acetonitrile from 1 to 5 minutes. The initial ratio was immediately re-established at 5 minutes and maintained to 5.5 minutes. The retention times were approximately 4.3 (3-hydroxy, desmethylflunitrazepam) and 4.7 (clobazam) minutes. For 1’- and 4-hydroxymidazolam, an initial mobile phase of 66 % 0.001 M ammonium acetate/ 34 % acetonitrile was ramped linearly to 50 % 0.001 M ammonium acetate/ 50 % acetonitrile between 1 and 4 minutes. The initial ratio was immediately re-established at 4 minutes and maintained to 5 minutes. The retention times were approximately 3.2 (4-hydroxymidazolam), 3.3 (alprazolam) and 3.5 (1’-hydroxymidazolam) minutes. For 1’- and 4-hydroxytriazolam, an initial mobile phase of 90 % 0.001 M ammonium acetate/ 10 % acetonitrile was ramped immediately to 66 % 0.001 M ammonium acetate/ 34 % acetonitrile at 1 minute and immediately to 34 % 0.001 M ammonium acetate/ 66 % acetonitrile at 4 minutes. The initial ratio was immediately re-established at 5 minutes and maintained to 6.5 minutes. The retention times were approximately 4.4 (1’-hydroxytriazolam), 4.5 (4-hydroxytriazolam) and 5.7 (diazepam) minutes. For 3-hydroxyquinidine, an initial mobile phase of 90 % 0.001 M ammonium acetate/ 10 % acetonitrile was ramped linearly to 90 % 0.01 M formic acid/ 10 % acetonitrile between 1 and 4 minutes. The initial ratio was immediately re-established at 5 minutes and maintained to 6 minutes. The retention times were approximately 2.9 (3-hydroxyquinidine) and 3.2 (dextromethorphan) minutes.

The compounds were detected and quantified by atmospheric pressure electrospray ionisation MS/MS using a Micromass Quattro Ultima triple quadrupole mass spectrometer. The LC column eluate was split and ¼ was delivered into the MS where the desolvation gas (nitrogen) flow rate was 600 l/hr, the cone gas (nitrogen) flow rate was 100 l/hr and the source temperature was 125 °C. Using positive ion
mode, protonated molecular ions were formed using a capillary energy of 3.5 kV and cone energies of 39 V (3-hydroxydiazepam), 70 V (diazepam, 3-hydroxyquinidine), 71 V (3-hydroxyflunitrazepam), 74 V (nordiazepam), 76 V (desmethylflunitrazepam), 78 V (clobazam), 80 V (1'- and 4-hydroxymidazolam, 1'- and 4-hydroxytriazolam, triazolam), 89 V (dextromethorphan) and 90 V (alprazolam). Product ions formed in argon at a pressure of 2 x10^{-3} mbar and at collision energies of 12 eV (3-hydroxydiazepam, m/z 301.1→254.9), 15 eV (3-hydroxyflunitrazepam, m/z 330.05→284.35), 20 eV (4-hydroxyalprazolam, m/z 325.05→280.10; diazepam, m/z 285.0→257.00; clobazam, m/z 301.05→259.35), 22 eV (desmethylflunitrazepam, m/z 300.00→254.35), 25 eV (nordiazepam, m/z 270.95→208.00; 4-hydroxymidazolam, m/z 342.00→234.30; alprazolam, 309.00→281.30; triazolam, 343.00→308.00), 28 eV (3-hydroxyquinidine, 341.05→226.05) 30 eV (1’-hydroxyalprazolam, m/z 325.05→297.1; 1’-hydroxymidazolam, m/z 342.00→203.30; 1’-hydroxytriazolam, m/z 359.05→176.0), 35 eV (4-hydroxytriazolam, m/z 359.05→273.00) and 40 eV (dextromethorphan, 272.05→170.90) were monitored as ion chromatograms which were integrated and quantified by quadratic regression of standard curves using Micromass QuanLynx 3.5 software.

Quantitative Western blotting. The absolute amounts of CYP3A4 and CYP3A5 in 12 tissue samples from in HL Bank 2 were measured by SDS-PAGE in 12% precast gel (BioRad, Hercules, CA), followed by Western blotting with specific anti CYP3A4 and CYP3A5 antibodies (BD Gentest, Woburn, USA). Heterologously expressed CYP3A4 and CYP3A5 in baculovirus (BD Gentest) were used for calibration. CYP3A protein levels were estimated by comparison of the sample band integrated optical density with the appropriate standard curve, with the limit of quantitation of 10 and 1 pmol/mg protein for CYP3A4 and CYP3A5, respectively.
Data Analysis. The kinetic parameters for each substrate were obtained from untransformed data by nonlinear least squares regression using GraFit 4 (Erithacus Software, Horley, Surrey, UK). In case of 3-OH quinidine, 1’ and 4-OH midazolam (CYP3A4), flunitrazepam N-demethylation (CYP3A5) and 3-hydroxylation (CYP2C19) the Michaelis-Menten equation with the weighting factor of 1/y was used for kinetic analysis. Kinetic parameters \( V_{\text{max}}, K_s \) (substrate dissociation constant), \( \alpha \) (defining changes in binding affinity - homotropic cooperativity) and \( \beta \) (changes in catalytic rate constant, \( K_p \)) were calculated from untransformed data using the two-site model (Houston et al., 2003). This type of analysis was performed for the cases of positive and negative cooperativity, i.e. sigmoidal and substrate inhibition kinetic profiles, respectively. When the metabolic profile was consistent with positive homotropic behaviour the \( CL_{\text{max}} \), the maximum clearance when the enzyme is fully activated, was calculated by the following equation:

\[
CL_{\text{max}} = \frac{V_{\text{max}}}{K_s} \times \frac{\sqrt{(1/\alpha) - 1}}{2(1-\alpha)}
\]

The \( CL_{\text{max}} \) estimate was derived from the two-site kinetic model (See Appendix), assuming that \( \beta=2 \) (\( V_{\text{max}} \) is equivalent to \( 2K_p[E]_t \), where \( [E]_t \) is the total enzyme concentration (Segel, 1975)).

**Clearance prediction from rCYP – relative abundance approach.**

Immunquantified levels of CYP3A4 and CYP3A5 enzymes in a HL Bank of 12 livers (Rawden et al., submitted for publication) were used to predict the clearance by the following equation:

\[
CL \ (\mu L/\text{min/mg protein}) = CL_{\text{CYP3A4}} + CL_{\text{CYP3A5}}
\]
\[
\text{CL}_{\text{CYP3A4}} (\mu\text{L/min/nmol CYP}) \times \text{pmolCYP3A4/mg protein} + \text{CL}_{\text{CYP3A5}} (\mu\text{L/min/nmol CYP}) \times \text{pmolCYP3A5/mg protein}
\]

(2)

No information on the CYP2C19 abundance in this HL Bank was available and therefore this enzyme was not included in the prediction via this approach.

**Relative activity of CYPs.** To obtain meaningful quantitative comparison with the human liver microsomal (HLM) data, Crespi et al. (1995) have suggested the utilization of RAF, which represents the ratio of the rate of a specific marker reaction in HLM to the rate of the same metabolic pathway catalysed by the specific cDNA-expressed isoform (Eq. 3). Although this activity-based approach enables the correlation between the two systems, it assumes the possibility of extrapolation from the marker substrate to the other substrates for the same isoform, which can be questionable for CYP3A4. In contrast to the CYP content, RAF approach accounts for differences in activity per unit enzyme between recombinants and HLM and may therefore represent a better scaling estimate than immunoquantified protein levels. Similar substrate specificity between CYP3A enzymes and yet not defined specific marker for CYP3A5 represent an additional complicating issue.

\[
\text{RAF} = \frac{V_{\text{max HLM}} (\text{nmol/min/mg protein})}{V_{\text{max recombinant CYP}} (\text{nmol/min/nmolCYP})} = \frac{\text{nmol CYP}}{\text{mg protein}}
\]

(3)

The value obtained can be expressed either in units nmolCYP/mg protein or if the abundance of a particular CYP/mg protein is incorporated RAF is unitless, with the overall calculation of CL\text{pred} being the same.

**Clearance prediction from rCYP – relative activity approach.** Based on
the metabolite formation observed in both rCYP3A4 and CYP3A5, quinidine 3-hydroxylation was used as a selective CYP3A4 marker and the RAF was calculated using either our human liver bank or BD Gentest liver pool as liver sources. Alprazolam 1’-hydroxylation was the most sensitive probe to generate CYP3A5 RAF as described in the Discussion. CYP2C19 RAF estimate using $V_{\text{max}}$ approach (same as for all the other isoforms) was determined using mephenytoin 4’-hydroxylation.

Contribution of a particular enzyme to the overall CL is calculated as shown in Eq. 4 (Ito et al., 1998); the units for $\text{CL}_{\text{rCYP}}$ and $\text{RAF}_{\text{CYP}}$ are $\mu$L/min/nmol CYP and nmol CYP/mg protein, respectively.

\[
\text{CL (}\mu\text{L/min/mg protein}) = \text{CL}_{\text{CYP3A4}} + \text{CL}_{\text{CYP3A5}} + \text{CL}_{\text{CYP2C19}} = \text{CL}_{\text{rCYP3A4}} \times \text{RAF}_{\text{CYP3A4}} + \text{CL}_{\text{rCYP3A5}} \times \text{RAF}_{\text{CYP3A5}} + \text{CL}_{\text{rCYP2C19}} \times \text{RAF}_{\text{CYP2C19}} \quad (4)
\]

In addition to CYP3A4, the contribution of CYP3A5 and CYP2C19 (where applicable) to the formation of both 1’- and 4-hydroxy (midazolam, alprazolam and triazolam) and 3-hydroxy and N-demethyl (diazepam and flunitrazepam) was incorporated by bootstrapping (Armitage et al., 2002). This re-sampling analysis with replacement (1000 simulations in S-Plus 2000, MathSoft, Inc., MA, USA) was performed in order to obtain an estimate of the standard error of the predicted clearance due to relatively small initial sample size (n=12).

Bootstrap predicted clearance values from both the RAF and abundance approach (mean ± sd) were scaled to in vivo clearance applying physiologically-based scaling factor of 856 mg protein/kg, obtained as the average recovery of microsomal protein per gram of liver (40 mg protein/g liver) determined using 38 human livers multiplied by the average liver weight in humans (21.4 g liver/kg) (Ito and Houston,
submitted for publication). A literature database of in vivo CL_int values for benzodiazepines used to assess predictions and variability were collated from plasma clearances reported for 4 – 20 datasets and 38 – 237 individual subjects per drug (Rawden et al., submitted for publication).
Results

General kinetics of benzodiazepines in rCYPs. The kinetic properties of 5 benzodiazepines were determined in human CYP3A4, CYP3A5 and CYP2C19 Supersomes™ enzymes baculovirus-insect cell-expressed with coexpressed OR (Table 1 and Table 2). Less than 10% of the substrate depletion was noted and secondary metabolism was minimal throughout the course of incubation. In each case 2 metabolites were formed but with substantial quantitative differences between CYPs.

Positive cooperativity, commonly reported with CYP3A4 (Houston et al., 2003), was also observed in the kinetic profiles obtained with CYP3A5 for alprazolam, 4-triazolam, and 3-hydroxylation of diazepam and flunitrazepam. However, negative cooperativity (substrate inhibition) was observed for 1’- and 4-hydroxymidazolam (CYP3A5) and mephenytoin 4’-hydroxylation and diazepam and flunitrazepam N-demethylation with recombinant CYP2C19, but not CYP3A4. Kinetic profiles obtained for quinidine for both CYP3A enzymes showed standard Michaelis-Menten kinetics, whereas 4-hydroxyalprazolam and both pathways for midazolam were hyperbolic only in CYP3A4 (Fig. 1). Two-site kinetic analysis of the atypical profiles showed that the extent of cooperative binding to the CYP3A5 binding site was similar to CYP3A4 for most of the substrates investigated (comparable α values, Table 1 and 2). The only exception was diazepam where a higher affinity for the second substrate molecule was observed for both 3-hydroxy and N-demethylation pathways in comparison to CYP3A4, resulting in higher clearance via CYP3A5.

In contrast to the positive homotropy observed with CYP3A4 and CYP3A5, both diazepam pathways and flunitrazepam N-demethylation with CYP2C19 were
consistent with substrate inhibition (Fig. 1B). Of the substrates investigated, diazepam pathways showed the most pronounced substrate inhibition with the increasing substrate concentrations by CYP2C19 ($\beta < 0.1$), in contrast to a 0.22-0.45 range obtained for flunitrazepam N-demethylation, mephenytoin 4’-hydroxylation and midazolam 1’- and 4-hydroxylation.

Midazolam, triazolam and 4-hydroxyalprazolam metabolite formation by CYP2C19 was insignificant in comparison to both CYP3A enzymes (maximum of 0.2-4% of the rate obtained by CYP3A4/A5 over a range of substrate concentrations). In case of 1’-hydroxyalprazolam formation, CYP2C19 activity at very low substrate concentrations (e.g., 10 $\mu$M) was comparable to CYP3A4, whereas the rates generated with CYP3A5 were over 2-fold higher. However, with the increasing alprazolam concentrations the contribution of CYP2C19 was minor in comparison to CYP3A enzymes.

Pathway ratio. Marked differences in pathway ratios (major:minor) between CYP3A5 and CYP3A4 (2-10 fold) were observed for all benzodiazepines except for triazolam; these differences showed noticeable substrate concentration-dependence for diazepam and flunitrazepam (Fig. 2). The most characteristic changes in pathway ratios between CYP3A4 and CYP3A5 were observed for midazolam and alprazolam - over the range of substrate concentrations the 1’- to 4-hydroxymidazolam ratio increased 4-10 fold. However, alteration in alprazolam regioselectivity was observed as the ratio of 4- to 1’-hydroxyalprazolam decreased in CYP3A5 (5-10 fold) in comparison to CYP3A4. The latter represented the only case of a pathway switch, i.e., the minor pathway by CYP3A4 was the dominant one by CYP3A5, as in other cases regioselectivity was maintained.
Clearance by recombinant CYPs. The ratio of clearances in recombinant CYP3A5 and CYP3A4, calculated using either $CL_{int}$ or $CL_{max}$ (Eq. 1) where appropriate, varied between substrates (Fig 3). Clearance obtained for 3-hydroxyquinidine, 4-hydroxyalprazolam, 4-hydroxymidazolam and nordiazepam formation was 1.3 – 2.3 higher in CYP3A4, whereas for the six other metabolic pathways clearance in recombinant CYP3A5 exceed clearances in recombinant CYP3A4 by 1.2 to 5.2-fold. Comparable activity between CYP3A4 and CYP3A5 was observed for 1’-OH midazolam formation. Higher binding affinity for CYP3A5 (e.g. 3-hydroxydiazepam), higher $V_{max}$ values (1’- and 4-hydroxytriazolam) or a combination of both effects (3-hydroxyflunitrazepam) were responsible for the higher clearance observed by recombinant CYP3A5. 3-hydroxylation of diazepam and flunitrazepam by CYP3A5 was dominant (55 and 68%, respectively), whereas CYP2C19 was more significant for N-demethylation (Fig 4A and 4B for diazepam and flunitrazepam, respectively).

Of the substrates investigated quinidine and alprazolam showed the largest differential in the contribution of either CYP3A4 or CYP3A5 to the overall kinetic profile. The relative ratio of quinidine 3-hydroxylation in CYP3A4 to CYP3A5 increased more than 4-fold between 5 and 500 µM quinidine concentrations. Differences in clearance values (Fig. 3) were not that pronounced due to 3-fold higher binding affinity for CYP3A5 (34 vs. 102 µM). In contrast, rate of alprazolam 1’-hydroxylation, particularly at higher substrate concentrations, was 10-fold higher by CYP3A5 than CYP3A4 (Fig. 1A), increasing the clearance ratio (Fig. 3). Based on metabolite formation observed with both recombinant CYP3A, quinidine 3-hydroxylation was used as a selective CYP3A4 marker and the RAF values obtained in both liver sources are shown in Table 3. The higher RAF obtained using BD...
Gentest HL Pool was in agreement with the previous report by Störmer et al. (2000). Large inter-individual variability in the CYP3A4 activity in our liver bank was substantiated by a 10-fold difference in the individual RAF values, consistent with study by Venkatakrishnan et al (2000). However, the resulting mean value for CYP3A4 RAF was 4-fold higher than the estimate by Venkatakrishnan et al (2000) using diazepam as a CYP3A4 marker. This discrepancy could be attributed to the arguable specificity of previously used markers for CYP3A4 (Störmer et al., 2000, Venkatakrishnan et al., 2000). Alprazolam 1’-hydroxylation was the most sensitive probe to generate CYP3A5 RAF, not reported previously in the literature. CYP2C19 RAF estimate was determined using mephenytoin 4’-hydroxylation ($V_{\text{max}}$ in rCYP2C19 = 4.95 ± 0.49 pmol/min/pmolCYP) and was within the previously reported range of 0.9-10.5 pmol CYP/mg protein (Ito et al., 1998).

**Comparison of recombinant enzymes and HLM.** The predicted total clearance from the recombinant enzyme data incorporated each enzyme and both pathways for midazolam, triazolam, diazepam, flunitrazepam and alprazolam metabolism, by applying either relative abundance or RAF approach (Eq. 2 and 4, respectively). For all the substrates investigated 10-40 % higher clearance values were obtained using BD Gentest HL Pool as a liver source for RAF determination (data not shown). Using the activity approach clearance estimate for flunitrazepam and diazepam increased by 26 and 29%, respectively when all three enzymes were included in the prediction in comparison to clearance when only CYP3A4 was considered. Clearance predicted using the relative abundance of CYP3A4 and CYP3A5 in our human liver bank (0.4 - 14 % of total CYP3A) indicated only a minor contribution of hepatic CYP3A5 to the overall clearance of the prototypical CYP3A substrates (e.g., midazolam, quinidine). The only exception was alprazolam 1’-
hydroxylation where the CYP3A5 contribution varied between 3-47%. The rank order of the predicted clearance estimates was RAF > HLM > relative abundance (Metabolite formation data for five benzodiazepines in liver microsomes and the prediction to in vivo CL_{int} is shown in Rawden et al., submitted for publication). For the benzodiazepines investigated RAF clearance estimates were 2-8 fold higher than HLM with the exception of flunitrazepam where the prediction from HLM and by RAF were similar (6.0 and 6.3 µL/min/mg protein, respectively).

Clearance prediction from rCYP data for five benzodiazepines was performed by bootstrap analysis (1000 simulations). This re-sampling analysis was carried out in order to overcome the relatively limited information on CYP3A abundance and activity (data taken from n=12), rationalize any distribution assumptions and incorporate the variability of both CYP content and RAF estimates. An example of the bootstrap-generated distribution range of predicted clearance for midazolam and a 7.3-fold difference observed between the RAF and relative abundance approach is illustrated in Fig. 5

**Prediction of CL_{int} in vivo from recombinant systems.** The bootstrap generated range of predicted clearance with the incorporated variability of the relative abundance and CYP activity was scaled to clearance in vivo. Figure 6 illustrates the comparison of both scaled in vitro clearance (from rCYP3A4 and rCYP3A5 data) and in vivo CL_{int} values (ml/min/kg) for 5 benzodiazepines investigated. Predicted clearance applying range of immunoquantified CYP3A4 and CYP3A5 abundance (from human liver bank, n=12) was 7-9 fold lower in comparison to the RAF approach. High variability in CYP3A4/CYP3A5 abundance in human liver bank was reflected in the high coefficients of variation of the CL_{pred} obtained for each individual compound (28-99%); in contrast, variation by RAF approach although high, were
more consistent (65-68 %). For the benzodiazepines investigated RAF clearance prediction was within the 2-fold of the reported in vivo value with the exception of the 4-fold underestimation of flunitrazepam clearance.
Discussion

To date kinetic studies with recombinant enzymes have concentrated on achieving parity with hepatic microsomal preparations (Rodrigues, 1999, Venkatakrishnan et al., 2000, Störmer et al., 2000), with only a few reports of using such data for in vivo clearance predictions (Ito et al., 1998, Hirota et al., 2001). We have expanded the predictive utility of recombinant enzyme kinetic parameters by incorporating frequency distribution of particular CYPs and their activity as assessed by RAF estimates. An approach to building in variability to the scaling process (via either CYP abundance and activity approach) has been explored by using benzodiazepines as well-characterized drugs extensively metabolised by CYP3A4. In addition, the potential impact of ‘minor’ CYPs (CYP3A5 and CYP2C19) and of the complex kinetic properties seen for all three enzymes has been assessed.

Kinetic issues. The systematic kinetic analysis performed for five benzodiazepines showed no significant differences in substrate-specificity between CYP3A4 and CYP3A5, in agreement with recent reports (Williams et al., 2002, Patki et al., 2002, Huang et al., 2003). However, for certain substrates the relative CYP3A5 to CYP3A4 clearance ratio varied from previous reports e.g., Williams et al. (2002) observed higher 4-hydroxytriazolam formation clearance by CYP3A4, whereas in our study clearance by CYP3A5 was higher (Fig. 3). The use of a more precise method of clearance estimation (CL\text{max} when the enzyme is fully activated (Eq. 1) rather than the slope of the velocity curve at the low range of substrate concentrations applied by Williams et al., 2002) may provide the possible explanation. Maximum clearance for 4-hydroxy triazolam was observed at a substrate concentration of 50 µM and if consideration is not given to the phenomenon of activation then clearance is likely to be underestimated. Clearance estimates obtained by both the two-site (CL\text{max}) and the
standard Michaelis-Menten approach (CL_{int}) showed a wide range for CYP3A4 (0.9-728 µL/min/nmolCYP) and CYP3A5 (1.1-626 µL/min/nmolCYP).

The two-site mechanistic kinetic model employed (Houston et al., 2003) showed that the atypical kinetic profiles were consistent between two enzymes for most of the pathways (Fig. 1A and 1B). Positive cooperativity in substrate binding to the active site was apparent in CYP3A5 (Table 1 and 2), supporting the existence of multiple binding sites on this enzyme comparable to CYP3A4.

**Pathway ratio.** Regioselectivity of midazolam, diazepam and flunitrazepam metabolic pathways was maintained in CYP3A5 but the relative pathway ratio was increased up to 10-fold in this enzyme relative to CYP3A4. A similar ratio has been reported for midazolam in both CYP3A5 positive livers (Gorski et al., 1994, Foti and Fisher, 2003) and intestines (Lin et al., 2002) indicating the value of this ratio as a measure of CYP3A4:CYP3A5 content in HLM. However, no significant correlation could be established between either the content or relative contribution of CYP3A5 to the total CYP3A and 1’- to 4-hydroxymidazolam ratio in our liver bank due to a small sample size. In addition, CYP3A5*3/*3 genotype was dominant in our livers, with only 4 out of 12 livers heterozygous for the CYP3A5*1 allele and none with the most active CYP3A5*1/*1 genotype (Rawden et al., submitted for publication). This is consistent with the findings by Lin et al. (2002) that the median ‘functional’ CYP3A5 was 4-fold higher in CYP3A5*1/*3 livers than in CYP3A5*3/*3.

Our data on 3-hydroxy:desmethylflunitrazepam pathway ratios in CYP3A4 and CYP3A5 contradict the findings by Huang et al. (2003). However, the possible significance of the pathway ratio for flunitrazepam (and diazepam) is likely to be limited due to important involvement of CYP2C19 in N-demethylation.
Pathway regioselectivity was not maintained for all the benzodiazepines (Fig. 2). In the case of alprazolam, the switch in pathway importance was noted as the 1'-hydroxyalprazolam clearance by CYP3A5 was increased by 5.5 fold relative to CYP3A4, whereas a decrease by 2-fold was observed for the 4-hydroxylation. Hirota et al. (2001) have reported an increase in formation of 1'-hydroxyalprazolam, but to a smaller extent (3.4-fold). Due to importance of CYP3A5 in 1'-hydroxyalprazolam formation observed in our and in previous studies (Gorski et al., 1999, Hirota et al., 2001) this pathway was used as the most selective marker for CYP3A5 RAF estimation. A similar rationale was the basis for application of quinidine 3-hydroxylation to differentiate CYP3A4 from CYP3A5 activity.

**Clearance prediction from recombinant enzymes.** The predicted clearance from immunoquantified CYP protein levels was up to 9-fold lower in comparison to the RAF approach for all the benzodiazepines studied; the extent of under-prediction was similar to that observed with the prediction from HLM data (Rawden et al., submitted for publication). The activity based approach proved to be a better scaling estimate than both HLM and CYP abundance, as the predicted clearance was within the 2-fold in vivo range for midazolam, triazolam, diazepam and alprazolam, although flunitrazepam clearance was under-predicted (Fig. 6). Accurate quantitative estimates of the CYP3A expression levels are highly dependent on the antibody and protein-standards used (Hustert et al., 2001). The considerable variation observed in the amount of non-functional apoprotein and inability of Western blotting technique to distinguish between the active and non-active CYP may result in the underestimation of CYP abundance and clearance prediction via this approach.
The concentration of accessory proteins (e.g., NADPH P450-oxidoreductase and cytochrome b5) and their relative ratio to the CYP protein can differ considerably between recombinant enzymes and HLM (Venkatakrishnan et al., 2000). CYP3A4 activity can be affected by the lack/addition of these proteins, particularly as cytochrome b5 has been reported to show substrate-dependent stimulatory effect on CYP3A4 activity (Yamazaki et al., 1996, Voice et al., 1999). Nakajima et al. (2002) have reported that differences observed in NADPH P450-oxidoreductase/CYP ratio in the expression systems were not a critical factor for the quantitative clearance prediction. Based on Hirota et al. (2001) data, RAF estimates +/-cytochrome b5 (0.14 and 0.68, respectively) for alprazolam 4-hydroxylation were obtained and applied for the prediction of midazolam CLint in vivo. Predictions observed were in good agreement with the in vivo value for midazolam (422 ± 280 ml/min/kg) (Rawden et al., submitted for publication) as RAF differences counterbalanced the difference in clearance obtained in recombinants +/- cytochrome b5. Therefore, estimation of the RAF value and clearance prediction of test compounds are valid if determined under the same conditions (either +/- cytochrome b5).

The contribution of CYP2C19 to the overall predicted clearance was minor in comparison to CYP3A4/5. No information on PM/EM status in our liver bank was available; however a 28-fold range in mephenytoin 4'-hydroxylation activity observed in our HL Bank encompasses mephenytoin S/R ratio documented between PM and EM subjects (Bertilsson et al., 1989).

**Interindividual variability.** CYP3A4 inter-individual variability reflects the combined effects of modulation by endogenous compounds, various drugs and other environmental as well as genetic factors (Thummel and Wilkinson, 1998, Ozdemir et al., 2000). CYP3A4 activity follows unimodal population distribution in contrast to
genetic polymorphism seen with CYP2C19 (Wedlund, 2000) as most of the single nucleotide polymorphisms show frequency of <1-2% (Lamba et al., 2002). In contrast, polymorphic expression of CYP3A5 gene (CYP3A5*3, CYP3A5*6 or CYP3A5*7) (Hustert et al., 2001, Floyd et al., 2003) contributes significantly to variable abundance and activity of this enzyme in both liver and small intestine (Kuehl et al., 2001, Lin et al., 2002, Xie et al., 2004).

High variability in CYP3A relative abundance (73.2 ± 78.2 and 2.1 ± 1.7 pmolCYP/mg protein for CYP3A4 and CYP3A5, respectively) and in their activity assessed by RAF (coefficient of variation = 71 and 92% for CYP3A4 and CYP3A5, respectively) in our HL Bank was issue of concern in the prediction of clearance from rCYP. To overcome the sample size limitations (n=12) and possible bias in the quantitative prediction of in vivo clearance, bootstrap analysis (1000 simulations) was performed in order to justify any distribution assumptions in the generated values for the predicted clearance.

Our study indicates a significant contribution of CYP3A5 when present in equivalent amounts to CYP3A4; however due to low hepatic relative abundance in our liver data set (0.5 – 6.15 pmolCYP/mg protein) its in vivo significance is probably minor, as shown in a number of studies (Shih and Huang, 2002, Floyd et al., 2003, Westlind-Johnssson, 2003). Recent characterization of CYP3A5 genotype and phenotype in a large panel of livers and small intestines by Lin et al. (2002) revealed that CYP3A5 protein content accounted for 31% of the variability in hepatic 1'-hydroxylation of midazolam and a better correlation between total midazolam hydroxylation activity and CYP3A content when contribution of CYP3A5 was included. A similar tendency was observed in our data – incorporation of CYP3A5 relative abundance improved the clearance prediction for midazolam by 13%. Also, a
3-fold increase in the predicted clearance for midazolam was observed when CYP3A5 distribution is assumed to be in the higher range (e.g., up to 50% - Lin et al., 2002, data not shown). Therefore, the CYP3A5 population distribution in liver and gut, and the ability to accurately distinguish CYP3A5 from CYP3A4, from the activity and abundance point of view, needs to be evaluated in order to make the final decision on the contribution of this enzyme to the overall clearance.

In conclusion, this study indicates a more accurate in vivo clearance prediction from recombinant enzymes by using the RAF approach rather than the relative abundance. In addition, the ability to incorporate the inter-individual variability of the corresponding CYPs was demonstrated. However, further refinement will be necessary in order to encompass the CYP3A population distribution both in liver and intestinal abundance and activity as this information becomes available.
Acknowledgements. The Authors would like to thank Dr. Leon Aarons and Kayode Ogungbenro for advice on bootstrap analysis and Drs. Julie Andrews and Elena Paskaleva on their work in estimating CYP3A relative abundance in the liver bank.
References:


Ito K and Houston JB Prediction of human drug clearance from in vitro and preclinical data using physiologically-based and empirical approaches (submitted for publication)


Rawden HC, Carlile DJ, Tindall A, Hallifax D, Galetin A, Ito K and Houston JB. Microsomal prediction of *in vivo* clearance and associated inter individual variability of six benzodiazepines in humans (submitted for publication).


FOOTNOTES

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Figure Legends

Fig. 1  Comparison of CYP3A4 and CYP3A5 metabolite formation profiles for six substrates.

A: CYP3A4 (△) and CYP3A5 (▲) substrates: Alprazolam, Midazolam, Triazolam (1’-OH and 4-OH pathways).

B: CYP3A4 (△), CYP3A5 (▲) and CYP2C19 (■) substrates: Diazepam and Flunitrazepam (3-OH and N-demethylation pathways).

C: Quinidine - RAF marker for CYP3A4, where (△) and (▲) represent CYP3A4 and CYP3A5, respectively. Mephénytoin – RAF marker for CYP2C19 activity.

Data points represent the mean of duplicate determinations.

Fig. 2  Comparison of metabolic pathway ratio for 4 benzodiazepines using CYP3A4 (△) and CYP3A5 (▲). No significant difference between the CYPs was observed for triazolam (data not shown).

Fig. 3  CYP3A5 to CYP3A4 clearance ratio obtained in SUPERSOMES® for the range of the substrates investigated and their respective pathways.

Fig. 4  Contribution of CYP3A5, CYP3A5 and CYP2C19 to diazepam (A) and flunitrazepam (B) clearance in rCYPs.

Fig. 5  Predicted CL using relative abundance (A) and RAF (B) approach using bootstrap analysis – example of midazolam

Contribution of both 1’- and 4-hydroxy pathways via CYP3A4 and CYP3A5 included.
Fig. 6  

Comparison of clearances predicted (mean ± sd) from recombinant enzymes and clearance observed in vivo for the 5 benzodiazepines

Inter-individual variability in CYP3A4/CYP3A5 abundance and activity (RAF) were incorporated in the prediction by bootstrap analysis (1000 simulations). In vivo data used from Rawden et al. (submitted for publication).
TABLE 1

Kinetic parameters for midazolam, triazolam and alprazolam metabolite formation in CYP3A4 and CYP3A5 baculosomes (mean ± se)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pathway</th>
<th>Enzyme</th>
<th>$V_{\text{max}}$ pmol/min/pmolCYP</th>
<th>$K_m$ or $K_s$ µM</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\text{CL}<em>{\text{int}}$ or $\text{CL}</em>{\text{max}}$ µL/min/nmolCYP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midazolam</td>
<td>1'-hydroxy</td>
<td>CYP3A4</td>
<td>1.96 ± 0.08</td>
<td>2.69 ± 0.53</td>
<td>-</td>
<td>-</td>
<td>728</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>6.7 ± 0.5</td>
<td>10.7 ± 1.2</td>
<td>-</td>
<td>0.22 ± 0.04</td>
<td>626</td>
</tr>
<tr>
<td></td>
<td>4-hydroxy</td>
<td>CYP3A4</td>
<td>2.52 ± 0.16</td>
<td>29 ± 5</td>
<td>-</td>
<td>-</td>
<td>86.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>0.52 ± 0.09</td>
<td>12.1 ± 2.7</td>
<td>-</td>
<td>0.45 ± 0.13</td>
<td>43</td>
</tr>
<tr>
<td>Triazolam</td>
<td>1'-hydroxy</td>
<td>CYP3A4</td>
<td>1.40 ± 0.03</td>
<td>16.5 ± 1.4</td>
<td>-</td>
<td>-</td>
<td>84.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>2.63 ± 0.13</td>
<td>25.1 ± 4.3</td>
<td>-</td>
<td>-</td>
<td>104.8</td>
</tr>
<tr>
<td></td>
<td>4-hydroxy</td>
<td>CYP3A4</td>
<td>3.70 ± 0.16</td>
<td>186 ± 38</td>
<td>0.29 ± 0.11</td>
<td>-</td>
<td>21.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>4.02 ± 0.22</td>
<td>150 ± 31</td>
<td>0.33 ± 0.10</td>
<td>-</td>
<td>27.8</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>1'-hydroxy</td>
<td>CYP3A4</td>
<td>0.13 ± 0.005</td>
<td>144 ± 51</td>
<td>0.27 ± 0.08</td>
<td>-</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>1.28 ± 0.15</td>
<td>226 ± 34</td>
<td>0.45 ± 0.18</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>4-hydroxy</td>
<td>CYP3A4</td>
<td>2.48 ± 0.07</td>
<td>187 ± 18</td>
<td>-</td>
<td>-</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>1.96 ± 0.09</td>
<td>338 ± 61</td>
<td>0.36 ± 0.08</td>
<td>-</td>
<td>6.03</td>
</tr>
<tr>
<td>Substrate</td>
<td>Pathway</td>
<td>Enzyme</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; pmol/min/pmolCYP</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; or K&lt;sub&gt;s&lt;/sub&gt; µM</td>
<td>α</td>
<td>β</td>
<td>CL&lt;sub&gt;int&lt;/sub&gt; or CL&lt;sub&gt;max&lt;/sub&gt; µL/min/nmolCYP</td>
</tr>
<tr>
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<td>---------------------------------</td>
<td>-------------------------------</td>
<td>--------</td>
<td>-------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3-hydroxy</td>
<td>CYP3A4</td>
<td>5.55 ± 0.21</td>
<td>318 ± 44</td>
<td>0.11 ± 0.03</td>
<td>-</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>5.77 ± 0.09</td>
<td>339 ± 27</td>
<td>0.025 ± 0.004</td>
<td>-</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C19</td>
<td>1.14 ± 0.05</td>
<td>74 ± 6</td>
<td>-</td>
<td>0.07 ± 0.02</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>N-demethylation</td>
<td>CYP3A4</td>
<td>0.67 ± 0.05</td>
<td>157 ± 55</td>
<td>0.28 ± 0.05</td>
<td>-</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>0.32 ± 0.05</td>
<td>125 ± 45</td>
<td>0.13 ± 0.02</td>
<td>-</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C19</td>
<td>1.95 ± 0.13</td>
<td>74 ± 9</td>
<td>-</td>
<td>0.04 ± 0.02</td>
<td>26.3</td>
</tr>
<tr>
<td>Flunitrazepam</td>
<td>3-hydroxy</td>
<td>CYP3A4</td>
<td>1.29 ± 0.05</td>
<td>694 ± 104</td>
<td>0.03 ± 0.01</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>2.38 ± 0.05</td>
<td>337 ± 32</td>
<td>0.07 ± 0.02</td>
<td>-</td>
<td>13.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C19</td>
<td>0.064 ± 0.003</td>
<td>89 ± 13</td>
<td>-</td>
<td>-</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>N-demethylation</td>
<td>CYP3A4</td>
<td>0.20 ± 0.01</td>
<td>224 ± 44</td>
<td>0.36 ± 0.19</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>0.14 ± 0.01</td>
<td>128 ± 21</td>
<td>-</td>
<td>-</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP2C19</td>
<td>0.50 ± 0.07</td>
<td>289 ± 48</td>
<td>-</td>
<td>0.28 ± 0.10</td>
<td>1.7</td>
</tr>
</tbody>
</table>
TABLE 3

RAF estimates (nmolCYP/mg protein) for CYP3A4, CYP3A5 and CYP2C19 using 2 different liver sources

<table>
<thead>
<tr>
<th>Liver source</th>
<th>Marker</th>
<th>Human liver bank*</th>
<th>BD Gentest human liver pool</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP3A4</td>
<td>Quinidine 3-hydroxylation</td>
<td>0.46 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>CYP3A5</td>
<td>Alprazolam 1'-hydroxylation</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>CYP2C19</td>
<td>Mephenytoin 4'-hydroxylation</td>
<td>0.0042 ± 0.0037</td>
</tr>
</tbody>
</table>

* Values represent mean ± sd of RAF estimates obtained in 12 livers
APPENDIX

In the case of autoactivation, clearance is defined by the following equation (derived from two-site model, Houston et al., 2003), assuming that $\beta=2$ ($V_{\text{max}}$ is equivalent to $2K_p[E]_t$, where $[E]_t$ is the total enzyme concentration (Segel, 1975)).

$$
\frac{v}{[S]} = \frac{V_{\text{max}} \left( \frac{1}{K_s} + \frac{[S]}{\alpha K_s^2} \right)}{1 + \frac{2[S]}{K_s} + \frac{[S]^2}{\alpha K_s^2}}
$$

(5)

Similar principles used for the derivation of $CL_{\text{max}}$ from the Hill equation (Houston and Kenworthy, 2000) were applied here, whereas the first derivative $d v/S/d S$ gives the slope of the clearance plot (eq. 6):

$$
d \frac{v/S}{d S} = \frac{V_{\text{max}} \left( \frac{1}{K_s} + \frac{2[S]}{K_s} + \frac{[S]^2}{\alpha K_s^2} \right) - V_{\text{max}} \left( \frac{1}{K_s} + \frac{[S]}{\alpha K_s^2} \right) \left( \frac{2}{K_s} + \frac{2[S]}{\alpha K_s^2} \right)}{1 + \frac{2[S]}{K_s} + \frac{[S]^2}{\alpha K_s^2}}
$$

(6)

To calculate the x and y coordinates for the inflection point ($CL_{\text{max}}$) the slope ($d v/S/d S$) is set to zero and the following equation is obtained:

$$
\frac{1}{\alpha} - 2 - \frac{2}{\alpha K_s} S - \frac{1}{\alpha^2 K_s^2} S^2 = 0
$$

(7)

where $S$ is defined as $S= -\alpha K_s \left( 1 \pm \sqrt{(1/\alpha)-1} \right)$

(8)

Solving for $S$ under the condition that $(1 \pm \sqrt{(1/\alpha)-1})<0$ in equation 8

$$
S= -\alpha K_s \left( 1 - \sqrt{(1/\alpha)-1} \right), \text{ where } \alpha<0.5
$$

(9)

After substitution of the $S$ value into the equation 6 the $y$ value ($v/S$) of the inflection point is obtained:

$$
CL_{\text{max}} = \frac{V_{\text{max}}}{K_s} \sqrt{(1/\alpha)-1} \frac{\sqrt{(1/\alpha)-1}}{2(1-\alpha)}
$$

(10)
Figure 1

A

Alprazolam

Midazolam

Triazolam

B

Diazepam

Flunitrazepam

C

Quinidine

Mephenytoin

Figure 1
A) 1'-hydroxy : 4-hydroxymidazolam

B) 4-hydroxy : 1'-hydroxyalprazolam

C) 3-hydroxy : nordiazepam

D) 3-hydroxy : desmethyflunitrazepam

Figure 2
Figure 3

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Figure 5

A

B

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 6

Intrinsic clearance (mL/min/kg)

- Midazolam
- Diazepam
- Triazolam
- Flunitrazepam
- Alprazolam

Relative abundance

RAF

In vivo

Relative abundance