IMPACT OF INCUBATION CONDITIONS ON BUFURALOL HUMAN CLEARANCE PREDICTIONS: ENZYME LABILITY AND NONSPECIFIC BINDING

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ABSTRACT:

Human liver microsomes (HLMs) are frequently utilized in drug discovery to predict the human clearance of a compound. The extent to which the incubation conditions affect the accuracy of a human clearance prediction was determined for bufuralol. HLMs were preincubated at 37°C for varying times (5–120 min) with and without NADPH, and the remaining enzyme activity was determined by incubating compounds that have been characterized to be selective for individual cytochromes P450 or flavin-containing monooxygenase 3. CYP2D6, the high-affinity component of bufuralol metabolism, was shown to be the least stable of the isoforms studied. The loss of CYP2D6 activity was further examined by determining the kinetics of 1'-hydroxybufuralol formation after different preincubation time periods, by using reactive oxygen species (ROS) scavengers, and by utilizing Western blotting techniques. A 3-fold decrease in \( V_{\text{max}} \) was observed over 2 h, whereas the \( K_{\text{m}} \) remained constant. ROS scavengers were able to block enzyme lability, and Western blots revealed no apparent loss of immunoreactive enzyme. The protein binding of bufuralol was determined in HLMs, recombinant CYP2D6, and human plasma. A prediction of theoretical bufuralol concentrations over a 120-min incubation that incorporated enzyme lability was performed and shown to be closer to actual data than if enzyme lability were ignored. Finally, a similar prediction using literature bufuralol data, coupled with the observed protein binding data, was used to illustrate that the most accurate predictions of bufuralol clearance are obtained when the amount of protein in the incubation is kept to a minimum and the overall incubation time is less than 20 min.

The ability to accurately predict the in vivo clearance of a compound is an essential aspect of the drug discovery process (Houston and Carlile, 1997; McGinnity and Riley, 2001) due to the rates of attrition from unacceptable pharmacokinetics. One of the most common reagents used to predict hepatic intrinsic clearance is liver microsomes (Obach et al., 1997). They are inexpensive and require less preparation when compared with other in vitro sources, such as hepatocytes and liver slices. They can also be successfully cryopreserved until use. Liver microsomes contain the major enzymes responsible for the phase I metabolism of many therapeutic compounds, including the cytochromes P450 (Wrighton et al., 1993; McGinnity et al., 2000), and methods currently exist to scale this metabolism to a predicted in vivo clearance (Obach et al., 1997).

An acceptable human clearance predicted from liver microsomes is often one of the first absorption, distribution, metabolism, and elimination (ADME) hurdles a compound must pass in a drug discovery program. In drug discovery, metabolites are often unknown and metabolite standards are not available, rendering determination of \( V_{\text{max}} \) and \( K_{\text{m}} \) values not feasible. Consequently, clearance is usually determined by monitoring substrate depletion in a standardized microsomal screen (Rodrigues, 1997). Although in vitro techniques have been greatly enhanced in recent years, error is still apparent after extrapolating these data to in vivo parameters. There are a number of factors that can introduce error into microsomal clearance predictions, including but not limited to enzymatic polymorphisms, in vitro enzyme thermal stability, and nonspecific binding to microsomal protein (Obach, 1997; McLure et al., 2000). Enzymatic polymorphisms, such as the debrisoquine/sparteine-type genetic polymorphism in cytochrome P450 2D6 (CYP2D6), can be a cause of interindividual differences in the phase I metabolism of a given compound (Yamazaki et al., 1994). The thermal stability of an in vitro system can also affect the integrity of enzymes involved in a compound’s metabolism, depending on the enzymes present and the incubation conditions chosen (Nakamura et al., 2002). Finally, the extent of nonspecific binding to microsomal protein has been shown to affect a number of pharmacokinetic parameters, depending on the class of compound (acidic versus basic), including in vitro clearance values and the resulting ability to successfully scale to an in vivo clearance (Obach, 1999).

Bufuralol is a β-adrenergic receptor antagonist that has been reported to be metabolized predominantly to 1'-hydroxybufuralol by CYP2D6 (Kronbach et al., 1987). An extensive amount of work has
been performed to characterize this metabolic pathway. Data are available on the formation of 1’-hydroxybufuralol in recombinant enzymes (Mankowski, 1999), on the contributions of various CYP2D6 alleles to this pathway (Marucci et al., 2002), and on species differences that this pathway exhibits (Mimura et al., 1994; Hiroi et al., 2002). However, to our knowledge, no data are available on the scale of 1’-hydroxybufuralol kinetic parameters to a predicted in vivo clearance (Cl<sub>in</sub>).

The significance of trying to understand the Cl<sub>in</sub> of bufuralol in human liver microsomes exists in the fact that it is catalyzed by CYP2D6 and in the protein binding characteristics of the compound. Catalysis of the metabolism by CYP2D6 presents a unique challenge since the enzyme has been shown to be highly polymorphic (Yu et al., 2002). The same enzyme has also been shown to exhibit varying degrees of thermal stability at higher temperatures in vitro, depending on the CYP2D6 allele involved (Nakamura et al., 2002). Finally, bufuralol has been shown to be bound to differing extents in human liver microsomes, depending on the amount of microsomal protein present (Margolis and Obach, 2003), a fact that makes the predicted clearance of bufuralol highly dependent on the incubation parameters chosen.

The primary goal of this study was to determine the effects of the above factors on the Cl<sub>in</sub> of bufuralol. CYP2D6 thermal stability in human liver microsomes and Baculosomes was evaluated at 37°C for varying time periods and under different preincubation conditions. Next, bufuralol protein binding was examined in human liver microsomes, Baculosomes, and human plasma. Finally, incubations to determine the metabolic turnover of bufuralol in human liver microsomes and Baculosomes were performed, utilizing the data obtained from the thermal stability and protein binding experiments. These data were then scaled, accounting for enzyme activity, along with literature bufuralol enzyme kinetics, to predict a human in vivo clearance, and compared with reported human in vivo clearance values.

### Materials and Methods

**Chemicals.** Magnesium chloride, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), β-nicotinamide adenine dinucleotide phosphate (NADP), α,α'-isocitric acid, isocitric dehydrogenase, superoxide dismutase (SOD), catalase (CAT), deferoxamine (DEF), caffeine, 1,7-dimethylxanthine, coumarin, 7-hydroxycoumarin (umbelliferone), chlorozoxazone, benzodyamine, and peroxidase-conjugated anti-mouse secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO). (S)-Warfarin, 7-hydroxywarfarin, (S)-mephenytoin, 4-hydroxymephenytoin, bufuralol, 1’-hydroxybufuralol, 6-hydroxylchlorozoxazone, midazolam, 1-hydroxymidazolam, and 4-hydroxydiazemolol were purchased from BD Gentest. Western blotting detection reagents were obtained from Amersham Biosciences Inc. (Piscataway, NJ). High performance liquid chromatography (HPLC) grade acetonitrile, HPLC grade methanol, and HPLC grade water were purchased from J. T. Baker (Phillipsburg, NJ). Benzodyamine N-oxide was a generous gift from Allan Rettie (University of Washington).

**Human Liver Microsomes.** Microsomal preparations were obtained from a Pfizer Inc. in-house supply. These microsomes were prepared from a mixture of 56 different livers (Kalvass et al., 2001). The human liver microsome mixture represents average activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. Microsomal lots from individual livers (HL-1038, HL-1056, and HL-1057-2), chosen for their high CYP2D6 activity as well as their CYP2D6/CYP2C19 ratio, were utilized to determine interliver variability.

**Regenerating System.** An NADPH regenerating system was used for the incubations that were initiated with the addition of substrate (protocol A). NADP (0.54 mM in incubation), 125 mM MgCl<sub>2</sub> (11 mM in incubation), α,α'-isocitric acid (6.2 mM in incubation), and isocitric dehydrogenase (0.5 U/ml) were mixed immediately before beginning the assay and kept on ice until use.

**Microsomal Incubations.** Incubations to determine thermal stability were initiated with the addition of either a marker substrate (protocol A) or NADPH (0.5 mM final concentration, protocol B); the two protocols are highly representative of those commonly used in discovery ADME laboratories. For protocol A, a human liver microsomes, potassium phosphate buffer (100 mM, pH = 7.4) and the NADPH regenerating system were mixed and preincubated for 5, 10, 15, 30, 45, 60, and 120 min. For protocol B, human liver microsomes, MgCl<sub>2</sub> (1 mM in incubation), marker substrate, and potassium phosphate buffer (100 mM, pH = 7.4) were mixed and preincubated over the same time course as noted above. Specific reaction conditions are shown in Table 1. ROS scavengers such as SOD (800 U/ml in incubation) and CAT (800 U/ml in incubation) and chelating agents such as DEF (100 μM in incubation) were added to some incubations. The microsomal incubations were allowed to run for 20 min with aliquots (100 μl) taken at 0, 5, 10, 15, and 20 min. Aliquots were quenched with 2 volumes of cold acetonitrile containing a proprietary internal standard [molecular weight 434, logP 4.44, multiple reaction monitoring (MRM) 435 → 109 (positive ionization) or 433 → 182 (negative ionization)] and centrifuged to remove any insoluble material. A portion of the supernatant (20 μl) was injected for HPLC-tandem mass spectrometry analysis.

**Analysis of all samples except 4-hydroxymephenytoin was carried out on a Hewlett Packard 1100 quaternary gradient (Hewlett Packard, Palo Alto, CA) HPLC system (H<sub>2</sub>O/acetonitrile/0.1% acetic acid mobile phase) equipped with a Gilson Medical Electronics (Middleton, WI) 215 Liquid Handler autoinjector interfaced to an MDS Scies (Concord, ON, Canada) API-3000 triple quadrupole mass spectrometer for compound detection. Analysis of 4-hydroxymephenytoin was carried out on an HP1100 HPLC system (10 mM ammonium acetate/acetonitrile mobile phase) equipped with a Leap HTS PAL autoinjector (LEAP Technologies Inc., Carboro, NC) interfaced to a Micro mass (Manchester, UK) Quattro Ultima mass spectrometer. Both platforms utilized a Phenomenex (Torrance, CA) Primesep 5-μm C18-HC 30 × 2 mm column. Substrate depletion and metabolite formation were quantitated by comparing peak area ratios in the incubation to standard curves containing known amounts of substrate and metabolite. Incubation conditions for enzyme kinetics were slightly modified to use less protein (0.4 mg/ml in incubation) and shorter incubation time (15 min) to assure linearity as well as CYP2D6 selectivity. Reaction linearity was confirmed by a minimal observed change in substrate concentration as well as linear 1’-hydroxybufuralol formation.

### TABLE 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Marker Substrate</th>
<th>RM</th>
<th>Metabolite</th>
<th>RM</th>
<th>Mobile Phase System</th>
<th>MS Polarity</th>
<th>[Substrate]</th>
<th>[Protein]</th>
<th>Time</th>
</tr>
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<tbody>
<tr>
<td>CYP1A2</td>
<td>Caffeine</td>
<td>195/138</td>
<td>1,7-Dimethylxanthine</td>
<td>181/124</td>
<td>1 +</td>
<td>1000</td>
<td>0.8</td>
<td>20</td>
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<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>147/103</td>
<td>7-OH-coumarin</td>
<td>163/101</td>
<td>1 +</td>
<td>200</td>
<td>0.8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>S(-)–Warfarin</td>
<td>307/250</td>
<td>7-OH-warfarin</td>
<td>323/177</td>
<td>1 –</td>
<td>20</td>
<td>0.8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S(+)-Mephenytoin</td>
<td>N/A</td>
<td>4’-OH-mephenytoin</td>
<td>233/161</td>
<td>2 –</td>
<td>100</td>
<td>0.8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol</td>
<td>262/188</td>
<td>1-OH-bufuralol</td>
<td>278/186</td>
<td>1 +</td>
<td>27</td>
<td>0.8</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozoxazone</td>
<td>168/132</td>
<td>6-OH-chlorozoxazone</td>
<td>184/120</td>
<td>1 +</td>
<td>42</td>
<td>0.8</td>
<td>20</td>
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<tr>
<td>CYP3A4</td>
<td>Midazolam</td>
<td>326/291</td>
<td>1- and 4-OH-midazolam</td>
<td>342/203,234</td>
<td>1 +</td>
<td>13</td>
<td>0.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>FMO3</td>
<td>Benzodyamine</td>
<td>310/866</td>
<td>Benzodyamine N-oxide</td>
<td>326/102</td>
<td>2 +</td>
<td>200</td>
<td>0.1</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* Mobile phase system 1 = H<sub>2</sub>O/CH<sub>3</sub>CN/0.1% acetic acid; mobile phase system 2 = 10 mM ammonium acetate/CH<sub>3</sub>CN.

* N/A, S(+)-mephenytoin was not detected analytically.
netic parameters were estimated using a nonlinear regression analysis program (WinNonlin; Pharsight, Mountain View, CA).

Recombinant CYP2D6 Incubations. Baculosomes (recombinant P450 + NADPH-P450 reductase) were obtained from Invitrogen (Carlsbad, CA). Incubations utilizing recombinant CYP2D6 were performed in the same manner as the human liver microsome incubations. The concentration of CYP2D6 was 6.2 pmol/ml, to reflect the average specific content of this isoform in our previous incubations (approximately 3% of total P450; Shimada et al., 1994; Madani et al., 1999). All recombinant incubations were carried out using protocol A. Incubations carried out to determine the effect of protein concentrations on the CLint of bufuralol contained the same amount of recombinant CYP2D6 as noted above with varying amounts of control Baculosomes added to the incubation.

CYP2D6 Immunoblot. An immunoblot utilizing human liver microsomes that had been incubated for either 3 or 120 min was conducted using a monoclonal antibody human CYP2D6 immunoblotting kit. The immunoblot was carried out on HL-1056, HL-1057-2, and recombinant CYP2D6. Samples were incubated for the set time period, flash frozen using acetone and dry ice, and stored at −20°C until the immunoblot was performed. The samples were then added to an equal volume of sodium dodecyl sulfate sample buffer (60 mM Tris, pH = 6.8, 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 0.001% bromphenol blue) and heated at 70°C for 5 min before being loaded onto the gel. The final amount of protein added to each well was 20 µg. Conditions and procedures provided by BD Gentest were incorporated into the immunoblot assay. Enhanced chemiluminescence procedures were utilized, using a CYP2D6 primary and horseradish peroxidase-conjugated goat antimouse secondary antibody. Band development was performed with Western blotting detection reagents 1 and 2 (equal volumes mixed to 0.125 ml/cm² membrane). Visualization was performed on a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Protein Binding Incubations. Protein binding incubations for bufuralol in human plasma, human liver microsomes, and recombinant CYP2D6 were performed using 96-well equilibrium dialysis and a previously described method (Banker et al., 2002). Fresh blood was obtained from a single donor, heparinized, and centrifuged to obtain plasma. Human liver microsome and recombinant incubations were performed at protein concentrations ranging from 0 to 2 mg/ml. All incubations utilized a bufuralol concentration of 20 µM. Equilibrium dialysis was performed at 37°C for 4 h.

Prediction of Bufuralol Concentrations in Human Liver Microsomes. The theoretical concentration of bufuralol remaining in a human liver microsomal incubation was predicted each minute for 120 min, correcting for the lability of enzymes in the incubation. The contributions of CYP2D6 (major, CLint = 37.7 ml/min/mmol) and CYP2C19 (minor, CLint = 1.02 ml/min/mmol) to the metabolism of bufuralol were taken into consideration (Mankowski, 1999). The slope (Δ t = 5–120 min) of a log-linear trend line derived from Fig. 1 was used to estimate the enzyme inactivation rate constant (kmax) of the two enzymes. The decreasing CLint contributions of CYP2D6 and CYP2C19 at each minute of the incubation were calculated using:

\[
[\text{CL}_{\text{int}}] = [\text{CL}_{\text{int}}]_0 - ([\text{CL}_{\text{int}}]_0 \times k_{\text{max}} \times (t - t_0))
\]

where \(t_0\) is the beginning of each minute of incubation, and \(t\) is the end of that minute. The time in vitro half-life of bufuralol was then calculated from the in vitro half-life of bufuralol and the reaction is initiated with substrate; and protocol B, in which preincubation is performed with substrate present and the reaction is initiated with NADPH. Table 1 illustrates substrate and protein concentrations, marker substrates and metabolites monitored, and the precursor and product ions for the CYP2D6 and CYP2C19 incubations.

The preincubation of human liver microsomes at 37°C for periods of time from 5 min to 120 min illustrated degrees of thermal stability of CYP1A2, CYP2A6, CYP2C9, CYP2E1, CYP3A4, and FM30 that were dependent on the incubation conditions selected and varied significantly among the different enzymes (39–110% of control at 120 min; Fig. 1). CYP2D6 and CYP2C19, the most labile of the P450s, demonstrated thermal stability dependent on both the time of incubation and the protocol used. CYP2D6 appeared to be the more labile of the two, with less dependence on the protocol used (CYP2D6 activity 20–44% of control at 120 min). CYP2C19 appeared to be much more dependent on the incubation protocol chosen. CYP2C19 activity was at 21% of that in the control incubation after 120 min when protocol A was utilized, but maintained 87% of activity after 120 min when protocol B was chosen. Figure 1 illustrates a percentage of activity remaining compared with control for all of the cytochrome P450s tested as well as FM30.

The interliver variation of CYP2D6 thermal stability was also assessed, along with the thermal stability of recombinant CYP2D6. Three lots of human liver microsomes (HL-1038, HL-1056, and HL-1057-2) that exhibit various levels of 1'-hydroxybufuralol activity, and possess a high ratio of CYP2D6 activity to CYP2C19 activity to maximize the contribution of CYP2D6 to bufuralol hydroxylation, were preincubated for 5 to 120 min with NADPH present (protocol A). Recombinant CYP2D6 reactions were also preincubated for 5 to 120 min with NADPH present and initiated in the same manner as the three lots of liver microsomes. Incubations were initiated with the addition of bufuralol (27 µM incubation). CYP2D6 activity remaining exhibited approximately 2.5-fold variability among the three lots of liver microsomes (20–48% of control at 120 min). HL-1057-2 exhibited an initial increase in CYP2D6 activity, followed by the expected loss of activity after approximately 15 min of preincubation. Recombinant CYP2D6 showed a degree of degradation at 120 min
Fig. 1. Rates of formation of metabolites from substrates selective for individual microsomal enzymes after 37°C preincubation with protocol A (a) and protocol B (b).

Rates of formation (average of duplicate incubations; no pair showing more than 15% difference) for each preincubation time period were normalized to formation rates following a 5-min preincubation (control), as follows: 1,7-dimethylxanthine, 100 pmol/min/mg (a), 75 pmol/min/mg (b); 7-hydroxycoumarin, 458 pmol/min/mg (a), 677 pmol/min/mg (b); 7-hydroxywarfarin, 5 pmol/min/mg (a), 3 pmol/min/mg (b); 4-hydroxymephenytoin, 54 pmol/min/mg (a), 30 pmol/min/mg (b); 1'-hydroxybufuralol, 79 pmol/min/mg (a), 131 pmol/min/mg (b); 4-hydroxychloroxazone, 775 pmol/min/mg (a), 451 pmol/min/mg (b); 1'-hydroxymidazolam, 811 pmol/min/mg (a), 687 pmol/min/mg (b); 4-hydroxynidazolam, 141 pmol/min/mg (a), 114 pmol/min/mg (b); and benzoydamine N-oxide, 3300 pmol/min/mg (a), 7 pmol/min/mg (b). c, rates of formation of 1'-hydroxybufuralol and 4-hydroxymephenytoin after 37°C preincubation with protocol A.
that correlated well with the various lots of human liver microsomes (Fig. 2).

The apparent $K_m$ and $V_{max}$ values for the 1'-hydroxylation of bufuralol were determined in human liver microsomes preincubated for 3 and 120 min. Incubation conditions were slightly modified for the enzyme kinetics studies to assure linearity. The apparent $K_m$ of bufuralol hydroxylation remained essentially unchanged between the 3- and 120-min preincubations (7.28 ± 0.96 μM and 6.68 ± 1.63 μM, respectively), whereas the $V_{max}$ value decreased approximately 3-fold over the 120-min preincubation period (118.9 ± 7.53 pmol/min/mg at 3 min; 35.2 ± 3.99 pmol/min/mg at 120 min; Fig. 3), approximately correlating with the loss in CYP2D6 activity observed in Figs. 1 and 2.

The ability of ROS scavengers to attenuate the lability of CYP2D6 in preincubations with NADPH present was determined using SOD, CAT, and DEF. Figure 4 illustrates the rate of formation of 1'-hydroxybufuralol under conditions with and without ROS scavengers present. Including SOD and CAT in the incubation prevented all of the lability over a 120-min preincubation. Adding DEF to the incubation with SOD and CAT also prevented enzyme lability over 120

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**Fig. 2.** Rates of formation of 1'-hydroxybufuralol in human liver microsomes (four lots) and recombinant CYP2D6 following preincubation with NADPH (protocol A).

Rates of formation for each preincubation time period were normalized to formation rates following a 5-min preincubation (control), as follows: HL-1038, 104 pmol/min/mg; HL-1056, 131 pmol/min/mg; HL-1057-2, 88 pmol/min/mg; HL-MIX-13, 79 pmol/min/mg; rCYP2D6, 5352 pmol/min/mg.

**Fig. 3.** Enzyme kinetics of 1'-hydroxybufuralol after a 3- or 120-min preincubation with NADPH present.

The $K_{app}$ of bufuralol 1'-hydroxylation in human liver microsomes was determined to be 7.28 ± 0.96 μM, with a $V_{max}$ of 118.9 ± 7.53 pmol/min/mg (3 min) and 6.68 ± 1.63 μM with a $V_{max}$ of 35.2 ± 3.99 pmol/min/mg (120 min).
min; however, the overall rate of 1'-hydroxybufuralol formation was less than in the control incubations. To further examine the loss of CYP2D6 activity in human liver microsomes and recombinant enzymes and probe for a decrease in band density, an immunoblot analysis was performed on samples that had been incubated for 3 and 120 min. Samples were taken from HL-1056 (least stable over 120 min), HL-1057 (most stable), and recombinant CYP2D6. Only one band was observed for all of the samples at ~50 kDa, with no significant difference in band density between the two preincubation time periods, indicating no significant apparent proteolysis or loss of apoprotein over 120 min at 37°C (Fig. 5).

An attempt to model the changes in bufuralol metabolism due to CYP2D6 and CYP2C19 thermal stability was performed using the observed degradation rates from Fig. 1c. The concentration of bufuralol remaining in the incubation was under-predicted by approximately 10% at 30 min and by as much as 30% at 60 min if enzyme lability is ignored. This illustrates that a higher correlation is observed between the theoretical and observed data if these rates are incorporated in the calculation (Fig. 6).

In an attempt to predict bufuralol CL int using a high-throughput, standardized in-house human liver microsome screen (incubation time = 30 min; [protein] = 1.6 mg/ml), bufuralol was incubated in four lots of pooled human liver microsomes ([P450] in incubation = 0.5 μM; [Bufuralol] = 1 μM). CL int values obtained from the disappearance half-life for bufuralol in all four lots of microsomes were below detectable limits (CL int < 3.50 ml/min/kg; data not shown).

The effect of nonspecific protein binding in recombinant CYP2D6 incubation was assessed by incubating bufuralol in a concentration of recombinant CYP2D6 Baculosomes that approximately equals the amount of CYP2D6 present in a standard liver microsome incubation (6.2 pmol/ml; 0.8 mg/ml in incubation) with control Baculosomes (no activity) added to bring the final protein concentration in the incubation to a range of 0.8 to 2.0 mg/ml. Incubations were run at a bufuralol concentration of 0.3 and 1.0 μM. 1/2 and CL int values were relatively consistent (0.2–0.46 ml/min/mg) for all protein and substrate combinations run (Table 2).

To further assess the impact of nonspecific protein binding on bufuralol clearance, the protein binding of bufuralol in human plasma, liver microsomes, and recombinant CYP2D6 was determined. Data for human plasma was obtained from a single donor, and human liver microsome and recombinant CYP2D6 protein levels ranged from 0 to

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**Fig. 4.** Rates of formation of 1'-hydroxybufuralol in human liver microsomes with ROS scavengers present.

Rates, calculated as the average of duplicate incubations, were normalized to the control rate of formation (28 pmol/min/mg).

**Fig. 5.** Immunoblot analysis of CYP2D6 after 37°C preincubations for various times.

Samples in wells 1 to 5 were incubated at 37°C for 3 min, with samples in wells 6 to 10 having been incubated for 120 min. Contents of the wells were as follows: 1 and 6, HL-1056 + NADPH; 2 and 7, HL-1056 + bufuralol; 3 and 8, HL-1057-2 + NADPH; 4 and 9, HL-1057-2 + bufuralol; 5 and 10, rCYP2D6 + NADPH.
2.0 mg/ml in incubation. Bufuralol had a fraction unbound in plasma \( (f_{u,\text{plasma}}) \) of 0.19 ± 0.06. Protein binding in human liver microsomes agreed well with previously published work (Margolis and Obach, 2003). The fraction unbound of bufuralol in microsomes \( (f_{u,\text{mic}}) \) appeared linear over a microsomal protein range of 0 to 0.8 mg/ml protein \( (f_{u,\text{mic}} = 0.06 - 0.57) \). In recombinant CYP2D6, bufuralol appeared to be essentially unbound in incubations with recombinant protein concentrations up to 1 mg/ml. The fraction unbound in recombinant CYP2D6 \( (f_{u,\text{2D6}}) \) was 0.84 when incubated with 2 mg/ml recombinant protein. Figure 7 illustrates the \( f_{u,\text{mic}} \) and the \( f_{u,\text{2D6}} \) over a range of protein concentrations.

Finally, a prediction of in vivo bufuralol clearance was made for extensive and poor metabolizers using 1'-hydroxybufuralol kinetic parameters from the literature and compared with reported in vivo bufuralol clearance values (Dayer et al., 1985). In general, more accurate predictions were obtained from experiments that used lower liver microsomal protein concentrations and kept their incubation time under 20 min, thus minimizing nonspecific binding and enzyme lability (Table 3). Given the observed CYP2D6 and CYP2C19 lability observed in Fig. 1c, the decrease in \( CL_{\text{int}} \) was modeled for each of the experiments examined. Whereas all previously published data, as well as our own work, show 1'-hydroxybufuralol formation to be approximately linear through 1 h, the simulation demonstrated that the depletion of bufuralol in these incubations began to lose linearity after approximately 20 min (data not shown). Finally, the \( CL_{\text{int}} \) values from Table 3 were plotted against their respective incubation times and compared with reported in vivo clearance data (Fig. 8). It appears that the ideal incubation time for predicting bufuralol clearance is less than 20 min, when both microsomal and plasma protein binding are taken into consideration.

### Discussion

The ability to successfully predict the in vivo clearance of a compound from in vitro parameters in a discovery ADME laboratory is essential to avoid compound attrition due to poor pharmacokinetic parameters as well as to ensure reasonable dosing regimens later in the development process. Although scaling techniques have greatly improved in recent years, a good deal of variability still exists in making in vivo clearance predictions from in vitro data (Naritomi et al., 2001). As previously mentioned, liver microsomes are one of the most
common tools that a drug discovery laboratory utilizes to make such a prediction. They are most useful for characterizing phase I metabolism, the majority of which is characterized by the P450 enzymes (Wrighton and Stevens, 1992). In vitro liver microsome incubations are subject to a number of factors that can influence a microsomal clearance prediction, such as enzymatic polymorphisms, in vitro enzyme thermal stability, and nonspecific binding to microsomal protein. Enzymatic polymorphisms, such as those documented with CYP2D6, can introduce variability from one in vitro system to the next (Dayer et al., 1984, 1987; Yamazaki et al., 1994; Nakamura et al., 2002). Thermal stability can affect the amount of time that the enzyme in question remains active. For example, it has been reported that P450 activity is conserved through approximately 1 hour at 37°C (Gram and Fouts, 1966), whereas FMO activity is considered to be much more labile (Cashman, 1999). Finally, nonspecific binding to microsomal protein has been shown to affect the accuracy of a clearance prediction, depending on the class (basic/neutral/acidic) of the compound (Obach, 1999).

Although the current study did not attempt to determine the exact biochemical mechanisms underlying the in vitro enzyme lability, it did evaluate how this lability affects CLp values, both alone and in combination with other incubation parameters. The thermal stability of six P450 isoforms (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4) as well as FMO3 was determined in HLMs over 120 min at 37°C. Rather than being completely conserved over 1 h, a large degree of isof orm-dependent variability in remaining activity (39–110% at 120 min) was observed. CYP2D6, which is responsible for catalyzing the majority of bufuralol metabolism, was the least stable of the enzymes studied. After a 120-min incubation, formation of 1-OH-bufuralol was at approximately 20 to 44% of control. This decrease in activity was observed whether the liver microsomes were preincubated with NADPH and no substrate (protocol A) or with substrate present and no NADPH (protocol B). The initial increase in activity observed in HLM-1057-2 with incubation time may be attributable to this lot containing a variant form of CYP2D6 not present in the other lots; however, this phenomenon was not probed further. We were able to block the lability observed in the presence of NADPH by including the ROS scavengers SOD and CAT in the incubation. These results would seem to indicate that although ROS play a role in the degradation of CYP2D6, as has been reported previously for P450 enzymes (Goasduff and Cederbaum, 1999), they cannot be the only contributors, as is evident in the loss of CYP2D6 activity in the absence of NADPH. Heme oxygenase has also been shown to play a role in CYP2D6 degradation, albeit in a whole cell system (Ding et al., 2001). However, the loss in CYP2D6 activity observed here in the absence of NADPH implicated a non-ROS and nonheme oxygenase mechanism of enzyme lability. This is in contrast to data observed in CYP2C19 incubations, where much less enzyme

![Figure 7. Percentage of unbound bufuralol in human liver microsomes and recombinant CYP2D6.](image)

**TABLE 3**

<table>
<thead>
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<th>Reference</th>
<th>V/K</th>
<th>EM/PM</th>
<th>Cl_v,f</th>
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<th>Time</th>
<th>f_uni</th>
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<th>Cl (all f)^a</th>
<th>Percentage Accuracy</th>
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lability was observed when NADPH was left out of the preincubation step. The inclusion of DEF with SOD and CAT blocked CYP2D6 lability over time, although the overall rate of the reaction was lower than that in the control. DEF contains a basic amine side chain, making it possible that it may competitively inhibit CYP2D6 and result in an overall lower rate of formation of 1'-hydroxybufuralol. This possibility was not further investigated.

The theoretical concentration of bufuralol in a prolonged incubation (120 min) was modeled to illustrate the effect of enzyme lability on reaction linearity. When loss of enzymatic activity was factored into the equation, a loss of linearity was observed at approximately 20 min. Although the loss of linearity is evident when the concentration is calculated for each minute of the incubation, an experimental protocol that only incorporates an initial and final time point to determine half-life would under-predict the CL<sub>int</sub> of bufuralol in the incubation by approximately 10% at 30 min and by as much as 30% at 60 min. Interestingly, several of the drugs used by Obach (1999) were substrates for CYP2D6. The observation of an under-prediction in that report, even when incorporating nonspecific binding, suggests that some of the fold error in predicting clearance could have been due to enzyme lability. This enzyme lability may also account for the loss in CYP2D6 enzyme activity often observed in mechanism-based inactivation controls (R. S. Obach, personal communication; T. D. Podoll, personal communication).

To further probe the factors affecting the clearance of bufuralol in HLMs and to examine why bufuralol CL<sub>int</sub> values were below detectable limits in multiple lots of HLMs, the protein binding characteristics of bufuralol were determined. Bufuralol was over 2-fold more bound in a standard high-throughput HLM incubation (f<sub>u-mic</sub> = 0.45) than in any of the recombinant CYP2D6 incubations (f<sub>u-2D6</sub> = 1), possibly explaining why bufuralol clearance was more readily detectable in recombinant incubations than in HLMs, where no substrate turnover was detected.

Finally, bufuralol clearance predictions were made using the enzyme lability and nonspecific binding data mentioned above, as well as previously published 1'-hydroxybufuralol kinetic parameters. The vast majority of the calculated CL<sub>int</sub> values underestimated the reported in vivo bufuralol clearance by over 90% for both extensive and poor metabolizers. Not surprisingly, the most accurate predictions came from the experimental protocols in which the least amount of binding occurred or in which the total incubation time was kept to less than 20 min. Predictions derived from kinetic data reported by Nakamura et al. (2002) were 75.3% and 83.1% accurate for extensive and poor metabolizers, respectively. The conditions under which this experiment was run allowed for essentially no nonspecific binding, and the incubation was only run for 15 min. A second experiment, reported by Prueksaritanont et al. (1995), was run under conditions in which bufuralol was more highly bound (f<sub>u-mic</sub> = 0.534); however, the incubation was limited to 5 min, resulting in a 76.5% accurate clearance prediction. It is our belief that such techniques can also be applied to compounds metabolized by other labile enzymes as well, thereby increasing the accuracy of their clearance prediction in a fashion similar to the one presented in this article.

Results such as these show that as the understanding of the effects of incubation parameters increases, so does the ability to accurately predict human clearance values. It has been shown that, in general, including microsomal and plasma protein binding improves the accuracy of clearance predictions (Obach, 1999). This article illustrates that correcting for enzyme lability can further improve this accuracy. These data also support the importance of selecting the proper incubation conditions for a microsomal assay. Properly selecting the length of the assay, the amount of protein used, and the preincubation conditions should only serve to improve the accuracy of a compound’s human clearance predictions.

Acknowledgments. We acknowledge Drs. M. Byron Kneller and
Allan Rettie (University of Washington) for the generous gift of benzylamine N-oxide, Wansiank Yoon (Pfizer Global Research and Development) for analytical assistance, Kelly Magnuson (Pfizer Global Research and Development) for assistance with the CYP2D6 immunoblotting procedure, and Dr. Cliff Fisher and John O’Donnell for intellectual dialogue.

References


