Title: The Impact of the Hepatocyte-to-Plasma pH Gradient on the Prediction of Hepatic Clearance and Drug-Drug Interactions for CYP2D6 Substrates

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Abbreviations:

Cytochrome P450, CYP; Cytochrome P450 2D6, CYP2D6; acid dissociation constant, pKa; ionization factor, F; Michaelis-Menten constant, Km; Maximum rate of reaction, Vmax; Intrinsic clearance, CLint; Human liver microsomes, HLM; inhibitory constant for reversible inhibition, KI; inhibitory constant for time-dependent inhibitors, KT; maximum rate of enzyme inactivation, kinact; time-dependent inhibition, TDI; pharmacokinetic, PK; physiologically-based pharmacokinetic, PBPK; in vitro to in vivo extrapolation, IVIVE; Drug-drug interactions, DDI; area under the plasma concentration-time curve, AUC
Abstract

The proton gradient from the intracellular space to plasma creates an unbound drug gradient for weak acids and bases that could modulate apparent drug clearance and drug-drug interactions. Cytochrome P450 intrinsic clearance and inhibitor potency are routinely determined in vitro at the plasma pH of 7.4 rather than the intrahepatocyte pH of 7.0. We determined the impact of pH on in vitro enzyme kinetic parameters and inhibition potency for substrates (bufuralol, dextromethorphan), reversible inhibitors (quinidine, amiodarone, desethylamiodarone, clozapine) and mechanism-based inhibitors (paroxetine, desethylamiodarone), of the major drug metabolizing enzyme, CYP2D6. The lower intracellular pH 7.0 compared to pH 7.4 resulted in a 60 and 50% decrease in intrinsic clearance for the substrates bufuralol and dextromethorphan respectively. Reversible inhibition constants for three of the four inhibitors tested were unaffected by pH; while for the inhibitor quinidine, a 2-fold increase in the inhibition constant was observed at pH 7.0. For time-dependent inhibitors, desethylamiodarone and paroxetine, changes in time-dependent inhibition parameters were different for each inhibitor. These results were incorporated into physiologically-based pharmacokinetic models indicating that the changes in in vitro parameters determined at pH 7.0 offset the effect of increased unbound intracellular concentrations on apparent clearance and extent of drug-drug interactions. However, this offset between concentration and enzyme activity cannot be generalized for all substrates, inhibitors, and enzymes as the effect of a lower pH in vitro varied significantly, therefore it would be prudent to determine in vitro enzyme parameters at the hepatocyte appropriate pH 7.0.
Introduction

Physiologically-based pharmacokinetic (PBPK) models, of various types, are widely used to predict absorption, in vivo drug clearance, volume of distribution and the “shape” of plasma and tissue concentration versus time curves using in vitro and/or preclinical in vivo data (Jones and Rowland-Yeo, 2013). In these models, the dependence of factors such as the intestinal fluid solubility, apparent intestinal permeability and tissue membrane partitioning on pH has been taken into account (Rodgers et al., 2005; Chen et al., 2012). The intracellular pH is more acidic than that of plasma and interstitial fluid (Frieden, 1984; Hall and Guyton, 2011) and consequently the unbound intracellular concentration, consisting of ionized and unionized species, will differ to that in plasma for drugs that exist in ionized and unionized forms at physiological pH values (Shore et al., 1957). The ratio of the intracellular unbound concentration to the unbound plasma concentration, $K_{puu}$, can be readily calculated using the Henderson-Hasselbalch relationship under the assumptions that the permeability of the cell membrane to ionized drug is negligible and the distribution of unionized drug is only passive (Berezhkovskiy, 2011; Mateus et al., 2013). However, the implications of the pKa dependent $K_{puu}$ for prediction of in vivo clearance from in vitro data are poorly understood.

The ionization factor ($F_I$) was developed to modify the in vitro derived drug intrinsic clearance to account for the pKa dependent $K_{puu}$ in the prediction of in vivo clearance (Berezhkovskiy, 2011). The use of $F_I$ assumes that the only effect of the lower intracellular pH, relative to plasma, is to change the rate of elimination by modulating the product of unbound intracellular concentration and intrinsic clearance (Berezhkovskiy, 2011; Berezhkovskiy et al., 2012). However this approach assumes that the in vitro intrinsic clearance of the eliminating pathway is estimated at the appropriate intracellular pH or that intrinsic clearance is not affected.
by pH over the range of 7.0 to 7.4. In addition, the F_{i} approach assumes that the rate of elimination is a function of the sum of the ionized and unionized forms of the unbound drug. Similarly when the pKa dependent K_{p_{u}} is employed to modify the inhibition potency of a drug it is assumed that the inhibitory constant is appropriately estimated and that the sum of the ionized and unionized forms of the unbound drug are equally inhibitory.

The determination of Michaelis-Menten constant (K_{m}), maximum rate of reaction (V_{max}), intrinsic clearance (CL_{int}) and inhibition constants (inhibitory constant for reversible inhibitors (K_{i}), inhibitory constant for time-dependent inhibition (K_{i}) and maximum rate of enzyme inactivation (k_{i\text{inac}})) for cytochrome P450 (CYP) catalyzed reactions are routinely performed at pH 7.4 despite in vitro and in vivo observations that hepatocyte intracellular pH, including the cytosol and endoplasmic reticulum, is 7.0 (Park et al., 1979; Pollock, 1984; Bonventre and Cheung, 1985; Andersson et al., 1987; Fitz et al., 1992; Durand et al., 1993; Strazzabosco et al., 1995; Gerweck and Seetharaman, 1996; Kim et al., 1998; Vidal et al., 1998). Ionizable groups within CYP enzymes have the potential to modulate catalytic and inhibitory parameters as pH varies, and the relative affinity of the active site for ionized and unionized drug is unclear (Denisov et al., 2005). Berezhkovskiy et al. (2012) investigated the difference in fraction unbound and intrinsic clearance, through parent degradation, in rat liver microsomes at pH 7.0 and pH 7.4 for a set of seven proprietary compounds. Although differences were detected between the pH values, it was concluded that the results were within assay variability and that the prediction of hepatic clearance using F_{i} could be used with intrinsic clearance values determined at pH 7.4. However, this interpretation was based on a small chemical space and unknown elimination pathways and therefore, extending this conclusion to all in vitro metabolism studies is not presently justified.
The current study was undertaken to establish the impact of pH on these in vitro determinations for one of the major drug metabolizing enzymes, CYP2D6. Enzyme kinetic parameters used for clearance (Km, Vmax, CLint) were determined by metabolite formation in human liver microsomes (HLM) using two prototypical probe substrates, bufuralol and dextromethorphan. The inhibitory potential for reversible and time-dependent inhibitors (Ki, KI and k_inact) towards these substrates was also investigated. The in vitro findings were incorporated into PBPK models to predict exposure and drug-drug interactions (DDI) in an effort to understand the significance of pH dependence of enzymes in the in vitro system in combination with the F_l correction.
Materials and Methods

Materials. Chemical reagents were obtained commercially: dextromethorphan, NADPH, quinidine, amiodarone, clozapine, paroxetine, and metoclopramide (Sigma Aldrich Ltd., St. Louis, MO); bufuralol, 1’-hydroxybufuralol, and desethylamiodarone (Toronto Research Chemicals Inc., Toronto, ON, Canada); dextrorphan (Cerilliant, Round Rock, TX); sodium phosphate monobasic, sodium phosphate dibasic, phosphoric acid, and sodium hydroxide (Fisher Scientific, Pittsburgh, PA). A single lot of HLM pooled from 150 individuals (UltraPoolTM®, Lot. #38290, equal proportion male and female, Corning, Tewksbury, MA) was used for all experiments.

In vitro incubations. Reactions were conducted in a shaking water bath at 37°C. Incubations were carried out in 100 mM sodium phosphate (NaPO₄) buffers adjusted to pH of interest (6.0, 6.5, 7.0, 7.2, 7.4, 8.0, 8.5, 9.0) with phosphoric acid or sodium hydroxide as necessary. Linear conditions for metabolite formation with respect to time and protein at pH 6.0, pH 7.4, and pH 9.0 were determined for bufuralol and dextromethorphan, and are reflected in the final conditions described.

Substrates, buffer, and microsomes were premixed, and incubations were initiated by addition of the NADPH cofactor. In the case of reversible inhibition studies, inhibitor and cofactor were added at the initiation of the reaction simultaneously. Time-dependent inhibition studies were performed using the dilution method (Mohutsky and Hall, 2014). All incubations were carried out in 96-well plates in triplicate. Product formation (1’-hydroxybufuralol dextrorphan, 3-hydroxyquinidine) assays were performed on three separate occasions. Methanol (MeOH) was used as a solvent for all experiments at a final percent solvent less than 2% v/v.
1'-hydroxybufuralol formation. Formation of 1'-hydroxybufuralol was determined using bufuralol (0.78-100 μM) and HLM (0.125 mg/ml) in 100 mM NaPO₄ buffer over a range of pH values between 6.0 and 9.0 (Crespi et al., 1998). The mixture was pre-incubated for 3 min at 37°C prior to reaction initiation through the addition of NADPH (1 mM, prepared in corresponding pH buffer), for a total volume of 150 μl. The reaction was allowed to proceed for 20 min at 37°C, at which time a 50 μl aliquot was added to 100 μl of 90:10 v/v (MeOH:H₂O) containing internal standard to stop the reaction. Plates were sealed with Easy Pierce 20 μm foil (Thermo Fisher Scientific; Waltham, MA), vortexed for 20 s, centrifuged (3500 g for 10 min) and analyzed by LC-MS/MS (Supplemental Materials). Reactions were performed in triplicate wells on three separate occasions. Metabolite formation was determined using a standard curve of 1'-hydroxybufuralol generated under identical conditions (0.5-1000 nM).

Dextrorphan formation. Formation of dextrorphan from dextromethorphan (Schmider et al., 1997) was determined using the same procedure as described above for 1'-hydroxybufuralol with the following changes: dextromethorphan (0.39-50 μM) and HLM (0.1 mg/ml) in 100 mM NaPO₄ buffer at pH (6.0 to 9.0) were used; after pre-incubation and initiation, reactions proceed for 10 min at 37°C before being stopped. Metabolite formation was determined using a standard curve of dextrorphan generated under identical conditions (0.5-500 nM).

3-hydroxyquinidine formation. Formation of 3-hydroxyquinidine from quinidine was measured as it is the major metabolite of quinidine metabolism and is only formed via CYP3A4 in vitro (Nielsen et al., 1999). Formation was determined at pH 7.0 and pH 7.4 as described for
1'-hydroxybufuralol formation with the following changes: quinidine (1.95-250 μM) and HLM (0.1 mg/ml) in 100 mM NaPO₄ buffer at pH 7.0 or 7.4 were incubated at 37°C for 20 min after reaction initiation. Reaction aliquots (30 μl) was added to 150 μl of 90:10 v/v (MeOH:H₂O) containing internal standard to stop the reaction. Metabolite formation was determined using a standard curve of 3-hydroxyquinidine generated under identical conditions (0.98-2000 nM).

**Reversible inhibition (Kᵢ determination).** The equilibrium inhibition constant (Kᵢ) was determined at pH 7.0 and pH 7.4 for four reversible inhibitors (quinidine, amiodarone, desethylamiodarone, clozapine). Bufuralol or dextromethorphan (3.125, 6.25, 12.5, 50 μM), and HLM (0.125 and 0.1 mg/ml respectively) in 100 mM NaPO₄ buffer at pH 7.0 or 7.4 were prepared and kept on ice. After pre-incubation (3 min at 37 °C), inhibitor (quinidine (0-0.2 μM), amiodarone (0-200 μM), desethylamiodarone (0-100 μM), or clozapine (0-100 μM)) was added with NADPH (1 mM prepared in corresponding buffer) (Fowler and Zhang, 2008). Reactions proceeded for 20 min for bufuralol and 10 min for dextromethorphan. Reaction (50 μl) was stopped through addition to 100 μl of 90:10 v/v (MeOH:H₂O) containing internal standard. Plates were sealed with Easy Pierce 20 μm foil (Thermo Fisher Scientific; Waltham, MA), vortexed for 20 s, centrifuged (3500 g for 10 min) and analyzed by LC-MS/MS (Supplemental Materials).

**Time-dependent inhibition (Kᵢ and kᵢₘₐₓ determination).** The maximum rate of enzyme inhibition (kᵢₘₐₓ) and inhibitor concentration at which half the maximal rate of inactivation occurs (Kᵢ) were determine for CYP2D6 using the dilution method (Mohutsky and Hall, 2014). Briefly, perpetrator compounds (desethylamiodarone (0-50 μM), paroxetine (0-5 μM),...
metoclopramide (0-200 μM)) were incubated at 37°C with pooled HLM (0.5 mg/ml) and NADPH (1 mM) in 100 mM NaPO₄ buffer at pH 7.0 or 7.4 for 0, 2.5, 5, 15 and 30 min. After pre-incubation, a 10-fold dilution into a secondary incubation containing 50 μM bufuralol or 50 μM dextromethorphan with NADPH (1 mM) in corresponding buffer at pH 7.0 or 7.4 was performed. The secondary incubation was allowed to proceed for 10 min at 37°C, then an aliquot (50 μl) was added to 100 μl of 90:10 v/v (MeOH:H₂O) containing internal standard to stop the reaction. Plates were sealed with Easy Pierce 20 μm foil (Thermo Fisher Scientific; Waltham, MA), vortexed for 20 s, centrifuged (3500 g for 10 min) and analyzed by LC-MS/MS (Supplemental Materials).

Fraction unbound in microsomes. The fraction unbound in microsomes (fu_mic) was determined using a 96-well Micro-Equilibrium Dialysis Device HTD 96 (HTDialysis LLC, Gales Ferry, CT) per the manufacturer’s instructions at HLM protein concentrations of 0.1 mg/ml for dextromethorphan, 0.5 mg/ml for metoclopramide and paroxetine and 0.125 mg/ml for all other compounds. HLM were prepared in 100 mM NaPO₄ buffer at pH 7.0 or 7.4 containing 1 μM of test drug and 100 μl added to one side of the dialysis membrane. The corresponding buffer (pH 7.0 or pH 7.4; 100 μl) was placed on the opposite side of the dialysis membrane. Samples (n=6 replicates per day; repeated on three separate runs) were incubated in a shaking incubator (400 rpm) at 37°C for 4 h using regenerated cellulose dialysis membranes (molecular weight cut off 6-8 K). After incubation, aliquots from microsomes and buffer sides were taken and quenched into 90:10 v/v (MeOH:H₂O) containing internal standards. Plates were sealed with Easy Pierce 20 μm foil (Thermo Fisher Scientific; Waltham, MA), vortexed for 20 s, centrifuged (3500 g for 10 min) and analyzed by LC-MS/MS (Supplemental Materials).
**PBPK simulations.** The population-based PBPK simulator Simcyp (V14; Certara, Princeton, NJ) was used to simulate exposure and DDI. Three separate simulations scenarios were performed based on: 1) baseline Simcyp compound file (SimpH 7.4); 2) baseline Simcyp compound file with the incorporation of F₁ correction (SimpH 7.4, F₁); 3) baseline Simcyp compound file corrected as described below, using in vitro results from pH 7.0 and 7.4 in the current study to correct baseline compound file (SimpH 7.0, F₁).

The ionization factor was calculated as described by Berezhkovskiy (2011) as the ratio of ionized drug fractions in plasma to intracellular hepatocyte space by Eq. 1

\[
F_I = \frac{1 - f_p^i}{1 - f_h^i}
\]  

(1)

Where \( f_p^n \) and \( f_h^n \) are the fraction of neutral (unionized) drug and \( f_p^i \) and \( f_h^i \) are the fraction of charged (ionized) drug in the plasma (p) and intracellular hepatocyte space (h) respectively. The fraction of ionized drug was determined using the Henderson-Hasselbalch equation for monoprotic bases (Eq. 2)

\[
f_{base}^i = \frac{1}{1 + 10^{pH - pK_a}}
\]  

(2)

and the diprotic bases (Eq. 3) quinidine and metoclopramide

\[
f_{diprotic}^{i, base} = \frac{1}{1 + \left[10^{pK_{a1} - pH} + 10^{pK_{a2} - pH} + 10^{pK_{a1} + pK_{a2} - 2pH}\right]^{-1}}
\]  

(3)

The pH values were 7.4 for plasma and 7.0 for the intracellular hepatocyte space.

The pH specific in vitro CL_{int} (CL_{int in vitro pH}) was calculated as shown in equation 4 using \( K_m, f_u_{mic} \) and \( V_{max} \) values determined at pH 7.0 or 7.4.
To account for the kinetic parameter changes ($K_m$, $V_{max}$) and $f_{umic}$ at the specific pH, assuming that all bottom up data in the Simcyp files were generated at pH 7.4, equation 5 describes the calculations employed for the victim drugs, dextromethorphan and bufuralol, intrinsic clearances. For dextromethorphan, the CYP2D6 HLM $CL_{int}$ ($CL_{int,Simcyp}$) value provided in the elimination characteristics of the baseline Simcyp compound file and our own in vitro data were used to calculate an adjusted $CL_{int}$ for the pH 7.0 simulations

$$CL_{int \; in \; vitro \; pH} = \frac{V_{max \; in \; vitro \; pH}}{K_m \; in \; vitro \; pH \times f_{umic \; pH}}$$

(4)

In the case of bufuralol, the baseline Simcyp bufuralol file was used as a starting point. Enzyme specific pharmacokinetic (PK) parameters ($V_{max}$ and $K_m$), determined in a recombinant system, for the conversion of bufuralol to either the metabolite 1’hydroxybufuralol and 6’-hydroxybufuralol are provided in the elimination characteristics of the compound file. These PK parameters were used to calculate a $CL_{int}$ ($V_{max}/K_m$) for each enzyme isoform and metabolite pathway combination (e.g., a separate $CL_{int}$ for CYP2D6 conversion of bufuralol to 1’hydroxybufuralol and 6’-hydroxybufuralol were determined). Since the contribution of the 6’-hydroxybufuralol metabolic pathway towards the total clearance is minor (~3%), the calculated $CL_{int}$ for the 1’hydroxybufuralol and 6’-hydroxybufuralol for each isoform were combined to provide a total $CL_{int}$ ($CL_{int\; total}$) for each of the individual recombinant enzyme isoforms. Each enzyme isoform $CL_{int\; total}$ was converted to a HLM $CL_{int\; total}$ by using the relative abundance of the enzyme isoform per mg protein from the Healthy Volunteers population. This provided a fraction metabolized ($f_m$) for CYP2D6 of 0.617, which is much lower than literature calculated $f_m$ of 0.83 based on EM/PM ratios (Dayer et al., 1985; Dayer et al., 1986). To compensate,
CYP2D6 CL\textsubscript{int total} was increased to achieve an $f_m$ of 0.83, while CYP2C19 CL\textsubscript{int total} was decreased to maintain the same total clearance of all the isoforms combined. This new value for CYP2D6 CL\textsubscript{int total} was used for the simulations, and equation 1 was used to calculate changes with regard to pH 7.0.

Simcyp baseline files for clozapine and paroxetine were used, and only the inhibition parameters ($K_i$, $K_t$, $k_{inact}$, $f_{mic}$) determined in vitro at the different pH values were altered in these files. In the case of quinidine, the baseline Simcyp compound file was used and the changes in inhibition parameters made. Additionally, the elimination through the major pathway (formation of 3-hydroxyquinidine by CYP3A4) was altered, based on our in vitro findings at pH 7.0 and pH 7.4, in the same manner as described above for dextromethorphan.

Compound files for amiodarone and desethylamiodarone were built as described by Chen et al. (Chen et al., 2015) with the following exceptions: amiodarone and desethylamiodarone were categorized as monoprotic bases with pKa values of 8.47 and 9.4 respectively, and the fraction available from dosage form ($f_a$) was increased from 0.6 to 0.8 as it provided a better fit to the clinical data from Andreasen et al. (1981) and Haffajee et al. (1983).

The ionization correction proposed by Berezhkovskiy (2011) was calculated based on the physicochemical properties of the compounds (acid/base; pKa) and the pH of the intracellular (7.0) and extracellular (7.4) compartments (Table 1). This $F_i$ value was then incorporated in the elimination section of the compound file as an active uptake into hepatocytes. This value alters the intracellular concentration of the compound by changing the $K_{puu}$ to compensate for the difference in pH between the extracellular and intracellular compartments.

Simulation dosing schedules were chosen to mimic clinical dosage and/or clinical DDI studies, if available; however, for bufuralol a literature search did not identify any DDI studies.
and therefore the dextromethorphan DDI study design information was used for bufuralol. All simulations were performed using the Simcyp Healthy Volunteer Population using a 10 by 10 subject trial set up (total population size 100; equal portion male/female, age range 20-50 yr) in the fasted state.

Inhibition by quinidine followed the study design from Abdul Manap et al. (1999) whereby a single dose of quinidine (50 mg) was given 1 h prior to administration of the bufuralol (60 mg) or dextromethorphan (30 mg) and followed for 96 h. For clozapine, a single dose (12.5, 400 or 900 mg to represent possible dosing range) was given simultaneously with bufuralol (60 mg) or dextromethorphan (30 mg) and simulated for a 24 h period (Perry et al., 1991). Interaction with amiodarone followed the study by Funck-Brentano et al. (1991) where a 1000 mg loading dose was given every day (QD) for 10 days followed by 400 mg maintenance dose QD for 10 days. On day 20, a single dose of bufuralol (60 mg) or dextromethorphan (40 mg) was given and the simulation proceeded for 24 h after victim drug dosing. Paroxetine interaction followed the study format of Liston et al. (2002) where paroxetine (20 mg) was given QD for 10 days followed by dextromethorphan (30 mg) or bufuralol (60 mg) on day 10 and followed for 24 h.

**Statistical analysis.** The HLM enzyme kinetic parameters ($V_{\text{max}}$, $K_m$, $CL_{\text{int}}$) for bufuralol, dextromethorphan and quinidine, as well as $f_u\text{nic}$ values for all drugs, represent the mean of triplicate runs. Mean values of the replicates for pH 7.0 and 7.4 were analyzed for statistical differences with an unpaired, two-tail t-test using GraphPad Prism version 6.05 (GraphPad Software, San Diego, CA) using a $\alpha = 0.05$. The ratio of the values obtained at pH 7.0 to values obtained at pH 7.4 are represented in the text of the results section as $\text{Ratio}_{7.0:7.4}$. 
Results

**Impact of pH on CYP2D6 enzyme kinetics.** Enzyme kinetic parameters $K_m$, $V_{max}$ and $CL_{int}$ varied with incubation pH, but the changes did not correlate with an increased abundance of ionized or unionized bufuralol and dextromethorphan (Fig. 1). The greatest affinity (lowest $K_m$) was observed at pH 8.0 for both substrates (Fig. 1C, D), while $V_{max}$ peaked at pH 7.4 and pH 7.2 for bufuralol and dextromethorphan respectively (Fig. 1E, F). This resulted in $CL_{int}$ being highest at pH 8.0 for bufuralol and pH 7.4 for dextromethorphan (Fig. 1G, H). Comparing pH values 7.0 and 7.4, represented as the ratio of the values obtained at pH 7.0 to values obtained at pH 7.4 ($Ratio_{7.0:7.4}$), $K_m$ was increased (lower affinity) at pH 7.0 compared to pH 7.4 with a $Ratio_{7.0:7.4}$ of 1.3 and 2 for bufuralol and dextromethorphan respectively. Conversely, the $V_{max}$ was decreased at pH 7.0 compared to pH 7.4 for bufuralol ($Ratio_{7.0:7.4} = 0.6$) but remained relatively unchanged for dextromethorphan ($Ratio_{7.0:7.4} = 1.1$). The calculated $CL_{int}$ was lower at pH 7.0 compared to pH 7.4 for both substrates, with $Ratio_{7.0:7.4}$ of 0.4 and 0.5 for bufuralol and dextromethorphan respectively. This decrease in $CL_{int}$ was statistically significant between the two pH values for both bufuralol ($p = 0.004$) and dextromethorphan ($p < 0.001$).

Although $fu_{mic}$ differed between pH values, no statistical significance was reached in the fraction unbound between pH 7.0 and pH 7.4 for bufuralol, dextromethorphan, clozapine, quinidine, desethylamiodarone, and metoclopramide. A statistically significantly higher $fu_{mic}$ was determined for pH 7.0 compared to pH 7.4 for amiodarone ($Ratio_{7.0:7.4} = 1.3; p = 0.001$) and paroxetine ($Ratio_{7.0:7.4} = 1.3; p = 0.003$). When pH 7.0 and pH 7.4 results were corrected for fraction unbound in microsomes, the ratios described above were unaffected, with the exception of the $Ratio_{7.0:7.4}$ for the $K_m$ for bufuralol which increased from 1.3 to 1.4.
Impact of pH on quinidine metabolism. The enzyme kinetic parameters for quinidine metabolism were significantly different (p value < 0.001 for $K_m$ and $V_{max}$ respectively) between pH 7.0 ($K_m = 398.8 \mu M; V_{max} = 671.8 \text{ pmol/min/mg protein}$) and pH 7.4 ($K_m = 72.3 \mu M; V_{max} = 1094.0 \text{ pmol/min/mg protein}$). This resulted in a statistically significant decrease (p < 0.001) in the CL_{int} at pH 7.0 (1.7 $\mu l/min/mg$ protein) compared to CL_{int} at pH 7.4 (15.3 $\mu l/min/mg$ protein) with a Ratio_{7.0:7.4} of 0.1.

Impact of pH on CYP2D6 inhibition. Reversible inhibition, described by unbound corrected Ki ($K_{i,u} = K_i \cdot f_{umic}$), did not appear to be significantly affected by the change in pH for the victim compound bufuralol with perpetrators amiodarone, desethylamiodarone and clozapine, or dextromethorphan with clozapine (Table 2). A slight increase in inhibitor potency at pH 7.0 was observed for the perpetrator/victim combination of amiodarone/dextromethorphan (Ratio_{7.0:7.4} = 0.6), while a slight decrease in inhibitor potency at pH 7.0 was determined for desethylamiodarone/dextromethorphan (Ratio_{7.0:7.4} = 1.3). However, the $K_{i,u}$ for quinidine was approximately 2-fold higher (weaker inhibition) at pH 7.0 compared to pH 7.4 for both bufuralol (Ratio_{7.0:7.4} = 2.2) and dextromethorphan (Ratio_{7.0:7.4} = 2.0).

Despite reports of metoclopramide as a time-dependent inhibitor (TDI) (Desta et al., 2002; Berry and Zhao, 2008), in our hands, no evidence of time-dependent inhibition of CYP2D6 was observed. The unbound $K_i$ ($K_{i,u} = K_i \cdot f_{umic}$) of paroxetine was higher (decreased potency) at pH 7.0 compared to pH 7.4 (Ratio_{7.0:7.4} of 1.7 and 2.4 for bufuralol and dextromethorphan respectively as the victim drug), while $k_{inact}$ was unaffected for bufuralol (Ratio_{7.0:7.4} = 0.9) and dextromethorphan (Ratio_{7.0:7.4} = 1.0) as the victim drug (Table 3; Fig. 2). This trend was reversed with the TDI perpetrator, desethylamiodarone, where $K_{i,u}$ was unaffected (Ratio_{7.0:7.4} of
1.1 and 0.9 for bufuralol and dextromethorphan respectively), but $k_{\text{inact}}$ was higher (greater rate of enzyme inactivation) at pH 7.0 for both bufuralol ($\text{Ratio}_{7.0:7.4} = 1.4$) and dextromethorphan ($\text{Ratio}_{7.0:7.4} = 1.7$) (Table 3; Fig. 2). No reversible inhibition at time zero for the secondary incubation was observed for desethylamiodarone or metoclopramide TDI experiments. However, some reversible inhibition was observed for the two highest concentrations of paroxetine (5 and 2.5 $\mu$M having activity inhibited by 45 and 30% respectively compared to controls). This was expected due to final concentrations of paroxetine in the secondary incubation being close to $K_i$ after dilution.

**PBPK simulations using in vitro parameters.** The simulations including the $F_I$ correction alone for bufuralol and dextromethorphan predicted a 2-fold lower exposure of drug given by the area under the concentration-time profile curve (AUC). A 0.4- and 0.5-fold difference between $\text{Sim}_{\text{pH 7.4, FI}}$ and $\text{Sim}_{\text{pH 7.4}}$ respectively was observed when the ionization correction was employed, as a result of an increase in the rate of elimination due to a greater intracellular unbound concentration available in the hepatocyte (Fig. 3; Table 4). When pH 7.0 in vitro data were combined with the ionization correction, the calculated exposure for $\text{Sim}_{\text{pH 7.0, FI}}$ was within twenty percent of the $\text{Sim}_{\text{pH 7.4}}$ with an AUC fold difference of 0.8 for both bufuralol and dextromethorphan (Fig. 3; Table 4). The increase in intracellular unbound concentration due to the ionization correction was offset by the decrease in $CL_{\text{int}}$ determined at pH 7.0.

The AUC ratios (AUC in the presence of inhibitor to absence of inhibitor) for the victim-perpetrator combinations are listed in Table 5. No significant change in AUC ratio was observed for clozapine, regardless of dosage (single dose of 12.5, 400, or 900 mg). Under the $\text{Sim}_{\text{pH 7.4, FI}}$ conditions, interaction studies showed a slight increase in AUC ratio for both bufuralol and
dextromethorphan compared to SimpH 7.4 (Ratio7.0:7.4 ranging from 1.2-1.5) for the amiodarone (including desethylamiodarone metabolite), quinidine and paroxetine perpetrators. However, when SimpH 7.0, FI conditions were used, the AUC ratio returned to the SimpH 7.4 observations for all perpetrators with the exception of quinidine, which maintained the higher AUC ratio with dextromethorphan, and paroxetine where a lower AUC ratio was determined for both bufuralol and dextromethorphan.

The lack of clinically observed DDI observations makes it difficult to assess the predictability of the models. The only clinical observations reported for dextromethorphan dosed alone as the victim are with quinidine as the perpetrator. Two studies which followed the same dosing design described in the methods (single 30 mg dose dextromethorphan with single 50 mg dose quinidine) observed a dextromethorphan AUC ratio of 16.2 (Abdul Manap et al., 1999) and 43 for extensive metabolizers (Capon et al., 1996). These interactions are significantly higher than the current model predictions ranging from 3.3 to 4.5 (Table 5). Additional clinical studies of various designs were modeled and resulted in an under prediction of the quinidine interaction (Supplemental Material). The under prediction of quinidine suggests that other factors may be influencing the level of inhibition observed in vivo and highlights the importance of understanding all pharmacokinetic attributes of the victim and perpetrator compounds.
Discussion

Intracellular pH is consistently reported in the range of 6.8 to 7.2 depending on the cell and tissue type, with hepatocytes having an intracellular pH 7.0 in humans and animal species (Waddell and Bates, 1969; Park et al., 1979; Roos and Boron, 1981; Pollock, 1984; Bonventre and Cheung, 1985; Andersson et al., 1987; Fitz et al., 1992; Durand et al., 1993; Strazzabosco et al., 1995; Gerweck and Seetharaman, 1996; Vidal et al., 1998; Schmitt, 2008; Sanchez and Lopez-Zapata, 2015). Since plasma pH is 7.4, this results in a pH differential between plasma and the intracellular environment that theoretically would lead to a pKa dependent change in the ratio of intracellular unbound concentration to the unbound plasma concentration, or $K_{puu}$. In vitro evidence has been presented demonstrating the differential accumulation of diverse acid, base and neutral compounds in support of the pH partitioning theory (Shore et al., 1957; Mateus et al., 2013). Since the liver is the major route of drug elimination, and given the phenomenon of $K_{puu}$ changing based on the physicochemical properties of the drug and the pH of plasma and the hepatocyte, hepatic models for clearance can be modified to account for changes in intracellular concentration through an $F_1$ correction (Berezhkovskiy, 2011).

Use of the $F_1$ correction assumes that metabolism of a drug is driven by the sum of the ionized and unionized forms, and that the rate of reaction is not driven solely by a single species. This is not the case for the monoamine oxidase B (MAO B) enzyme that has been shown to predominantly metabolize the protonated (ionized) form of β-phenylethylamine (Jones et al., 2007). However, in our current study, a wide range of pH values (6.0 to 9.0) were used to investigate metabolism through CYP2D6. This range included considerable changes in the ratio of ionized and unionized forms of bufuralol and dextromethorphan. Evidence to suggest that a single species of either substrate was responsible for the metabolism was not observed.
Therefore, the assumption was made that the total concentration of drug, the sum of the ionized and unionized species, drives the reaction and the total concentration of intracellular drug can be used in the adjusted models for predictions.

An important caveat of studying the influence of pH is that both the charged state of the compound and the enzyme can be altered, which can result in differences in interactions. Enzyme activity changes with respect to pH have been demonstrated in the literature for CYP enzymes and vary between isoforms and substrates investigated. For example, activity of 7-hydroxyethoxycoumarin O-deethylase peaks at pH 7.4 over the pH range of 7.0 to 8.5 in mice liver fractions (Greenlee and Poland, 1978), and while 6β-hydroxytestosertone formation in HLM is higher at pH 7.4 compared to pH 7.1, different oxidative pathways for testosterone metabolism peak at different pH values between 6.8 to 8.0 (Gemzik et al., 1990; Fisher et al., 2000). Our findings show that pH significantly impacts CYP2D6 as evidenced through changes in affinity and the rate of reaction, given by $K_m$ and $V_{max}$, towards the substrates with respect to pH. These observations represent the net effect of the changes in the enzyme as well as changes in the substrate ionization ratio in the reaction. Therefore, regardless of which species (ionized or unionized) is metabolized or state of the enzyme, if the in vitro measurements mimic the in vivo physiology, then these changes are accounted for and the results can be scaled without correcting for an in vitro bias as they provide a more physiologically accurate measure of $CL_{int}$.

Additionally, the buffering capacity, ionic strength and buffer constituents can all influence the activity of enzymes differently (Gemzik et al., 1990; Maenpaa et al., 1998) and further investigations are needed to understand the influence of different buffer conditions in conjunction with changing pH.
This is in contrast to the work done by Berezhkovskiy who investigated the difference in protein binding and intrinsic clearance at pH 7.0 and pH 7.4 for a set of seven proprietary compounds (Berezhkovskiy et al., 2012). Although changes were detected between the different pH values, Berezhkovskiy concluded the results to be within assay variability and the novel equations using F1 could be applied to in vitro values determined at pH 7.4. However, work presented here illustrates that significant changes in the measured in vitro parameters at pH 7.0 compared to pH 7.4 are reproducible and outside of assay variability. Changes appear to be on a case by case basis and therefore no general conclusion for all clearance pathways and drugs can be made. Further implementation of F1 with in vitro hepatocyte data improved in vivo predictions and led the author to question whether the intracellular pH of hepatocytes in vitro is maintained (Berezhkovskiy, 2011). He hypothesized that a disproportionately large ratio of incubation buffer to hepatocyte cell surface area would destroy the pH gradient, resulting in an intracellular pH equivalent to the extracellular environment. Metabolic stability of six commercial drugs in hepatocytes incubated in buffer at pH 7.0 compared to pH 7.4 were not different. Berezhkovskiy argued that depletion would theoretically be altered if hepatocytes were able to maintain an intracellular pH 7.0 with a higher external pH due to an increase or decrease in intracellular concentration, and lack of difference between the two incubation conditions indicated that intracellular pH of the hepatocytes reflected the extracellular pH (Berezhkovskiy et al., 2013). However, this conclusion is based on the assumption that the rate of metabolism is identical at each pH since the drug concentration would theoretically be the same in the intracellular and extracellular compartments if buffer pH matched intracellular pH. Our current results indicate that intrinsic clearance can be altered between the two pH values. Therefore, Berezhkovskiy’s conclusion can only be supported by determining whether the intrinsic clearance of the
compounds investigated is unaltered between the two pH values. Further work in our lab has indicated that one of the compounds investigated, diclofenac, has metabolism sensitive to pH in HLM (data publication in process) that would explain the difference observed for this compound (Berezhkovskiy et al., 2013).

Enzyme inhibition can also show a similar dependence on pH. While differences in reversible inhibition between pH values were observed, these were mostly eliminated when correcting for fraction unbound at the specific pH with the exception of quinidine, which retained a twofold weaker inhibition at pH 7.0 compared to pH 7.4 for both bufuralol and dextromethorphan. Application of the Ft correction increased the intracellular concentration of quinidine and this in isolation increased its inhibition potential. However, it was realized that the clearance of the inhibitor via CYP3A could also be affected at the different pH. Upon implementation of the change in metabolism and inhibition potential of quinidine, the change in metabolism of the victim drugs, and the ionization correction for both the victim and the perpetrator into the model, we found that the changes in intracellular concentration put forth by Ft were offset by the changes in PK and inhibition parameters at the intracellular pH 7.0. Furthermore, changes in TDI parameters varied inversely between the perpetrators investigated. Combined with the lack of change in the inhibition potential of some inhibitors, these findings reinforce that differences with respect to pH are not consistent and preclude generic conclusions towards inhibition in general.

Finally, PBPK models represent a convenient tool to incorporate the in vitro parameters necessary to make in vivo clearance and DDI predictions. Implementation of the Ft correction into these models resulted in a higher predicted intracellular concentration and hence an increase in the predicted rate of elimination, as well as a decreased plasma exposure and inhibition AUC.
ratio when using in vitro values determined at pH 7.4. Conversely, when using values determined at pH 7.0, the F\textsubscript{1} increase in intracellular concentration was offset by the decrease in in vitro parameters measured at the lower pH and resulted in a plasma exposure and inhibition AUC ratio closer to that predicted without accounting for intracellular trapping or in vitro intrinsic clearance. This demonstrates the assumption that in vitro intrinsic clearance is not affected by pH is inaccurate, at least for these enzyme and substrate combinations. Therefore, the implementation of a pKa dependent Kp\textsubscript{uu} correction to account for a change in the rate of elimination of an ionizable drug must be applied to in vitro data generated at the correct hepatic intracellular pH 7.0. In the case of inhibitors, such as quinidine, in which in vitro assays tend to under predict inhibition potency, the reasons remain unclear. We have demonstrated that the F\textsubscript{1} correction cannot completely explain these in vitro to in vivo correlation disparities when combined with changes in the parameters measured at pH 7.0. This reveals the importance of using an integrated system that takes into account all the different in vitro factors involved in making predictions (clearance of the compound, clearance of the inhibitor, fraction unbound, and inhibition potential) at the correct intracellular pH to make predictions based on physiologically accurate characteristics. It is recommend that in vitro parameters in drug discovery be determined at the physiologically appropriate intracellular pH 7.0, combined with an ionization correction, to provide a correct starting point in which to further explore other potential reasons for disparities between in vitro and in vivo outcomes.
Authorship Contributions

Participated in research design: Rougée, Mohutsky, Hall

Conducted experiments: Rougée, Mohutsky, Bedwell, Ruterbories

Contributed new reagents or analytical tools: Rougée, Mohutsky, Bedwell, Ruterbories

Performed data analysis: Rougée, Mohutsky, Bedwell, Ruterbories, Hall

Wrote or contributed to the writing of the manuscript: Rougée, Mohutsky, Bedwell, Ruterbories, Hall
References


Figure Legends

Figure 1. Effect of pH on CYP2D6 enzyme kinetic parameters. A) Calculated percentage of bufuralol (base, pKa 9.0) ionized and unionized species; B) Calculated percentage of dextromethorphan (base, pKa = 8.3) ionized and unionized species; C) $K_m$ of 1'-hydroxybufuralol formation from bufuralol; D) $K_m$ of dextrorphan formation from dextromethorphan; E) $V_{max}$ of 1'-hydroxybufuralol formation from bufuralol; F) $V_{max}$ of dextrorphan formation from dextromethorphan; G) $CL_{int}$ of 1'-hydroxybufuralol formation from bufuralol; H) $CL_{int}$ of dextrorphan formation from dextromethorphan. Dashed line represents the percent ionized of the substrate; solid line represents the percent unionized of the substrate. Symbols represent the mean of triplicate determinations, with vertical bars representing SEM.

Figure 2. Pseudo first order inhibition rate constants for loss of CYP2D6 activity versus inhibitor concentration performed at pH 7.0 (dashed line with square symbols) and pH 7.4 (solid line with circle symbols). A) Paroxetine inhibition of 1'-hydroxybufuralol formation from bufuralol; B) desethylamiodarone inhibition of 1'-hydroxybufuralol formation from bufuralol; C) paroxetine inhibition of dextrorphan formation from dextromethorphan; D) desethylamiodarone inhibition of dextrorphan formation from dextromethorphan. Symbols represent mean of triplicates.

Figure 3. Simulated plasma concentration time curves of A) bufuralol and B) dextromethorphan in the absence and presence of quinidine. Black lines represent baseline Simcyp compound file with pH 7.4 in vitro findings only; red lines represent baseline Simcyp compound file with pH 7.4 in vitro findings and the incorporation of $F_i$ correction; blue lines represent baseline Simcyp compound file with pH 7.0 in vitro correction and the incorporation of $F_i$ correction (see methods.
for details). Dashed lines represent substrate concentration in the absence of quinidine; solid lines represent substrate concentration in the presence of quinidine.
Table 1. Physicochemical properties of the CYP2D6 substrates and inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pKa1/pKa2</th>
<th>Ionization Factor</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufuralol</td>
<td>8.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.472</td>
<td>Substrate</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>8.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.343</td>
<td>Substrate</td>
</tr>
<tr>
<td>Quinidine</td>
<td>4.2/8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.456</td>
<td>Reversible inhibitor</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>8.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.393</td>
<td>Reversible inhibitor</td>
</tr>
<tr>
<td>Desethylamiodarone</td>
<td>9.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.497</td>
<td>Reversible inhibitor; Time-dependent inhibitor</td>
</tr>
<tr>
<td>Clozapine</td>
<td>7.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.045</td>
<td>Reversible inhibitor</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>9.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.504</td>
<td>Reversible inhibitor; Time-dependent inhibitor</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>0.6/9.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.493</td>
<td>Reversible inhibitor; Time-dependent inhibitor</td>
</tr>
</tbody>
</table>

pKa = acid dissociation constant; Source: <sup>a</sup>Simcyp; <sup>b</sup>The Human Metabolome Database (http://www.hmdb.ca); <sup>c</sup>Foye et al. (2013).
Table 2. Reversible CYP2D6 inhibition kinetic parameters at pH 7.0 and pH 7.4.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$f_{u, \text{mic}}$ pH 7</th>
<th>$K_{i,u}$ pH 7</th>
<th>$f_{u, \text{mic}}$ pH 7.4</th>
<th>$K_{i,u}$ pH 7.4</th>
<th>$K_{i,u}$</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bufuralol</td>
<td>Quinidine</td>
<td>0.934</td>
<td>0.090</td>
<td>0.939</td>
<td>0.041</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td></td>
<td>0.0020</td>
<td>0.096</td>
<td>0.0016</td>
<td>0.112</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Desethylamiodarone</td>
<td></td>
<td>0.0046</td>
<td>0.078</td>
<td>0.0043</td>
<td>0.087</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td></td>
<td>0.843</td>
<td>28.6</td>
<td>0.830</td>
<td>28.4</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Paroxetine</td>
<td></td>
<td>0.493$^a$</td>
<td>0.46</td>
<td>0.410$^a$</td>
<td>0.33</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Quinidine</td>
<td>0.946$^a$</td>
<td>0.051</td>
<td>0.951$^a$</td>
<td>0.026</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>Amiodarone</td>
<td></td>
<td>0.0026$^a$</td>
<td>0.083</td>
<td>0.0020$^a$</td>
<td>0.134</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Desethylamiodarone</td>
<td></td>
<td>0.0057$^a$</td>
<td>0.043</td>
<td>0.0054$^a$</td>
<td>0.034</td>
<td>1.27</td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td></td>
<td>0.870$^a$</td>
<td>14.9</td>
<td>0.859$^a$</td>
<td>15.6</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Paroxetine</td>
<td></td>
<td>0.549$^a$</td>
<td>0.32</td>
<td>0.465$^a$</td>
<td>0.28</td>
<td>1.14</td>
<td></td>
</tr>
</tbody>
</table>

Ratio between pH 7.0 and pH 7.4 results are given. $^a f_{u, \text{mic}}$ for inhibitor under in vitro assay conditions calculated from Austin (2002).
Table 3. Time-dependent CYP2D6 inhibition kinetic parameters at pH 7.0 and pH 7.4.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$f_{u_{mic}}$ pH 7</th>
<th>$K_{I,u}$ pH 7</th>
<th>$k_{inact}$ pH 7</th>
<th>$f_{u_{mic}}$ pH 7.4</th>
<th>$K_{I,u}$ pH 7.4</th>
<th>$k_{inact}$ pH 7.4</th>
<th>$K_{I,u}$</th>
<th>$K_{inact}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nM</td>
<td>min$^{-1}$</td>
<td></td>
<td>nM</td>
<td>min$^{-1}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bufuralol</td>
<td>Paroxetine</td>
<td>0.196</td>
<td>106.43</td>
<td>0.163</td>
<td>0.148</td>
<td>62.60</td>
<td>0.189</td>
<td>1.70</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Desethylamiodarone</td>
<td>0.0011$^a$</td>
<td>1.47</td>
<td>0.021</td>
<td>0.0011$^a$</td>
<td>1.41</td>
<td>0.015</td>
<td>1.05</td>
<td>1.40</td>
</tr>
<tr>
<td>Dextromethorphan</td>
<td>Paroxetine</td>
<td>0.196</td>
<td>167.38</td>
<td>0.190</td>
<td>0.148</td>
<td>70.30</td>
<td>0.196</td>
<td>2.38</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Desethylamiodarone</td>
<td>0.0011$^a$</td>
<td>2.19</td>
<td>0.019</td>
<td>0.0011$^a$</td>
<td>2.38</td>
<td>0.011</td>
<td>0.92</td>
<td>1.73</td>
</tr>
</tbody>
</table>

Ratio between pH 7.0 and pH 7.4 results are given. $f_{u_{mic}}$ for inhibitor under in vitro assay condition prior to dilution (0.5 mg/ml).

$^a$f$_{u_{mic}}$ for inhibitor under in vitro assay conditions calculated from Austin (2002).
Table 4. Simulated PK parameters for bufuralol and dextromethorphan.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>Simulation</th>
<th>$CL_{int,u}$ $^a$</th>
<th>$CL$ (l/h)</th>
<th>$C_{max}$ (μg/l)</th>
<th>$AUC$ (μg/l·h)</th>
<th>$CL_{int,u}$ $^a$</th>
<th>$CL$ (l/h)</th>
<th>$C_{max}$ (μg/l)</th>
<th>$AUC$ (μg/l·h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sim$_{pH \ 7.4}$</td>
<td>20.0</td>
<td>49.02</td>
<td>209.02</td>
<td>1223.91</td>
<td></td>
<td>36.0</td>
<td>500.47</td>
<td>5.16</td>
<td>59.94</td>
</tr>
<tr>
<td>Sim$_{pH \ 7.4, FI}$</td>
<td>20.0 (1.0)</td>
<td>118.70 (2.4)</td>
<td>116.84 (0.6)</td>
<td>505.47 (0.4)</td>
<td></td>
<td>36.0 (1.0)</td>
<td>1085.39 (2.2)</td>
<td>2.61 (0.5)</td>
<td>27.64 (0.5)</td>
</tr>
<tr>
<td>Sim$_{pH \ 7, FI}$</td>
<td>8.3 (0.4)</td>
<td>64.72 (1.3)</td>
<td>180.45 (0.9)</td>
<td>927.01 (0.8)</td>
<td></td>
<td>19.4 (0.5)</td>
<td>630.14 (1.3)</td>
<td>4.28 (0.8)</td>
<td>47.61 (0.8)</td>
</tr>
<tr>
<td>Observed</td>
<td>-</td>
<td>66.96</td>
<td>164$^b$</td>
<td>896$^b$</td>
<td></td>
<td>-</td>
<td>845.1</td>
<td>3.49$^c$</td>
<td>35.5$^c$</td>
</tr>
</tbody>
</table>

Dose of bufuralol (60 mg) or dextromethorphan (30 mg) simulation for 24 h. Geometric means reported with fold difference between simulation and traditional Sim$_{pH \ 7.4}$ given in parentheses. $CL_{int,u}$ = in vitro intrinsic clearance corrected for fraction unbound in microsomes; $CL$ = clearance calculated as Dose/AUC; $C_{max}$ = maximum serum concentration reached.

$^a$CL$_{int,u}$ units in μl/min/mg protein; $^b$Dayer et al. (1982); $^c$Chi et al. (2013)
Table 5. Ratio of AUC in the presence and absence of inhibitor from simulations.

<table>
<thead>
<tr>
<th>Simulation</th>
<th>Bufuralol</th>
<th>Dextromethorphan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clozapine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Amiodarone</td>
</tr>
<tr>
<td>Sim&lt;sub&gt;pH 7.4&lt;/sub&gt;</td>
<td>1.04</td>
<td>4.43</td>
</tr>
<tr>
<td>Sim&lt;sub&gt;pH 7.4, FI&lt;/sub&gt;</td>
<td>1.06 (1.0)</td>
<td>5.50 (1.2)</td>
</tr>
<tr>
<td>Sim&lt;sub&gt;pH 7, FI&lt;/sub&gt;</td>
<td>1.04 (1.0)</td>
<td>3.33 (0.8)</td>
</tr>
</tbody>
</table>

<sup>a</sup>AUC ratios for 900 mg clozapine dose given. Ratio between simulation and traditional Sim<sub>pH7.4</sub> given in parentheses.
Figure 3

(A) Systemic Concentration (mg/l) vs. Time (h)

(B) Systemic Concentration (mg/l) vs. Time (h)