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Use of a Physiologically Based Pharmacokinetic Model to Study the Time to Reach Brain Equilibrium: An Experimental Analysis of the Role of Blood-Brain Barrier Permeability, Plasma Protein Binding, and Brain Tissue Binding

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Abbreviations used are: BBB, blood-brain barrier; BP, brain-to-plasma concentration ratio; CNS, central nervous system; PBPK, physiologically based pharmacokinetics; PS, permeability-surface area product

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ABSTRACT

This study was designed (1) to examine the effects of blood-brain barrier (BBB) permeability (quantified as permeability-surface area product, PS), unbound fraction in plasma ($f_{u,plasma}$) and brain tissue ($f_{u,brain}$) on the time to reach equilibrium between brain and plasma and (2) to investigate the drug discovery strategies to design and select compounds that can rapidly penetrate the BBB and distribute to the site of action. The pharmacokinetics of seven model compounds: caffeine, CP-141938, fluoxetine, NFPS, propranolol, theobromine, and theophylline in rat brain and plasma after subcutaneous administration was studied. The in vivo logPS and $\log f_{u,brain}$ calculated using a physiologically based pharmacokinetic model correlates with in situ logPS ($R^2 = 0.83$) and in vitro $\log f_{u,brain}$ ($R^2 = 0.69$), where the in situ PS and in vitro $f_{u,brain}$ was determined using in situ brain perfusion and equilibrium dialysis using brain homogenate, respectively. The time to achieve brain equilibrium can be quantitated with a proposed parameter, intrinsic brain equilibrium half-life [$t_{1/2eq,in} = V_b \ln 2/(PS \bullet f_{u,brain})$, where V_b is the physiological volume of brain). The in vivo $\log t_{1/2eq,in}$ does not correlate with in situ logPS ($R^2 < 0.01$) but correlates inversely with $\log(PS \bullet f_{u,brain})$ ($R^2 = 0.85$). The present study demonstrates that rapid brain equilibration requires a combination of high BBB permeability and low brain tissue binding. A high BBB permeability alone cannot guarantee a rapid equilibration. The strategy to select compounds with rapid brain equilibration in drug discovery should identify compounds with high BBB permeability and low nonspecific binding in brain tissue.

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The blood-brain barrier (BBB) consists of a continuous layer of endothelial cells joined by tight junctions at the cerebral vasculature. It represents a physical and enzymatic barrier to restrict and regulate the penetration of compounds into and out of the brain and maintain the homeostasis of the brain microenvironment. Brain penetration is essential for compounds where the site of action is within the central nervous system (CNS), while BBB penetration needs to be minimized for compounds that target peripheral sites to reduce potential CNS-related side effects. Therefore, it is critical during the drug discovery phase to design and select compounds having appropriate brain penetration properties for drug targets that reside within and outside the CNS (Chen et al., 2003a; Golden and Pollack, 2003).

The kinetics of brain penetration consists of the extent of brain equilibrium and the time to achieve brain equilibrium. The extent of brain equilibrium is often quantified by brain-plasma partition coefficient (K_p), the ratio of total brain concentration and plasma concentration at steady state. This parameter depends upon drug binding in plasma and brain tissue, the uptake and efflux transporters at BBB, metabolism in the brain and the bulk flow of cerebrospinal fluid (Hammarlund-Udenaes et al., 1997). If active transporters, brain metabolism and the bulk flow of cerebrospinal fluid do not contribute significantly to brain drug disposition, the free drug concentration in brain equals to the free drug concentration in plasma and the K_p equals to the ratio of unbound fraction in plasma and unbound fraction in brain at equilibrium. Theoretically, the ratio of unbound brain concentration and unbound plasma concentration at steady state, $K_{p,free}$, is a better parameter to quantitate the extent of brain equilibrium (Tunblad et al., 2003). This parameter is not dependent upon plasma and brain tissue binding. In drug discovery it is important to identify CNS targeted compounds with $K_{p,free}$ of unity by screening out substrates of efflux transporters. Extensive screening efforts have been made in the drug discovery phase to identify the substrates of known efflux transporters, such as P-glycoprotein (P-gp) (Adachi et al., 2001; Polli et al., 2001; Chen et al., 2003b; Lin and Yamazaki, 2003).

Although the extent of brain penetration or achievement of high free brain concentration is the most critical property, the time to reach brain equilibrium is another important perspective of the kinetics of brain penetration. In CNS drug discovery, it is often desirable to identify compounds that can quickly penetrate into brain thereby the unbound drug concentration at the targeted site in brain tissue can reach plasma unbound concentration quickly after administration. The property of quick brain penetration can be

gauged by the time to reach brain equilibrium. Therefore, a short time to achieve brain equilibrium is a surrogate for a rapid achievement of active brain concentration. Rapid CNS penetration becomes essential for the compounds used for many diseases such as hypnosis (Hardman et al., 2001), status epilepsy (Li et al., 2000), and stroke (Adams and Leira, 1998). Empirical approaches to identify compounds with quick brain penetration during the discovery phase include screens to identify compounds with high BBB permeability (Di et al., 2003; Liu et al., 2004). In addition to BBB permeability, it has been implied in the literature that the time of brain equilibrium also depends upon the partition coefficient (Hammarlund-Udenaes et al., 1997). No study has been conducted to evaluate systematically the factors that determine the time to reach brain equilibrium.

The present study was designed to evaluate the effects of BBB permeability, plasma protein binding, and brain tissue binding on the time to reach brain equilibrium. The application of the results from the present study on strategies to select rapid brain penetration compounds in drug discovery is also discussed.

MATERIALS AND METHODS

Chemicals. Caffeine, fluoxetine, propranolol, theobromine, and theophylline were obtained from Sigma-Aldrich (St. Louis, MO). N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (NFPS) and methoxy-3-[(2-phenyl-piperadinyl-3-amino)-methyl]-phenyl-N-methyl-methane-sulfonamide (CP-141938) were synthesized at Pfizer Global Research and Development Laboratories (Groton, CT) with purity greater than 98%. All other chemicals used in the experiments were of the highest available grade.

Animal Experiments. Male Sprague-Dawley rats (250-280 g) were obtained from Charles River (Raleigh, NC). They were housed at controlled temperature and humidity in an alternating 12-h light and dark cycle with free access to food and water. Rats received caffeine (10 mg/kg), CP-141938 (5 mg/kg), fluoxetine (10 mg/kg), NFPS (10 mg/kg), propranolol (5 mg/kg), theobromine (10 mg/kg), or theophylline (10 mg/kg) subcutaneously. The doses were prepared in 0.9% saline and delivered in a volume of 2 mL/kg. Blood samples were collected in heparin treated tubes at designated time between 10 min and 24 h post dose via cardiac puncture. After centrifugation (3000 rpm, 10 min) of the blood, plasma was isolated. Brain tissue was harvested and rinsed with saline immediately after collection. The plasma and brain samples were stored at –20°C prior to analysis.

Protein Binding. The unbound fraction in plasma and brain homogenate was determined using a 96-well equilibrium dialysis method reported previously (Kalvass and Maurer, 2002). Briefly, fresh Sprague-Dawley rat plasma and brain tissue were obtained on the day of the study. Brain tissue was homogenized in two volumes (w/v) of 100 mM sodium phosphate buffer. Plasma, brain homogenate, and phosphate buffer (for equilibrium controls) were spiked with a compound (500 ng/mL) and 150 µL of these matrices were added to the dialysis apparatus. The receiver side contained 150 µL of phosphate buffer. The 96-well equilibrium dialysis apparatus was maintained on a shaking device in an incubator at 37°C for 5 h. Ten µL of plasma or brain homogenate and 50 µL of buffer were taken from the apparatus and added to high performance liquid chromatography (HPLC) vials containing 100 µL of acetonitrile. The samples were then vortexed, centrifuged and the supernatant was stored at –20°C prior to analysis. The unbound fractions determined from diluted brain tissue homogenates was corrected to yield an estimate of unbound fraction in the intact brain tissue using a previously published method (Kalvass and Maurer, 2002).

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Sample Analysis. The brain tissues were homogenized in three volumes (w/v) of water. Twenty µL of plasma or brain homogenate, 20 µL of DMSO and 200 µL of acetonitrile were mixed in silanized 96-well glass tubes. For the protein binding samples, 10 µL of plasma or brain homogenate samples was mixed with 50 µL of control buffer; and 50 µL of buffer samples mixed with 10 µL control brain homogenate or control plasma to yield identical matrix between buffer and non-buffer samples. The samples were then mixed with 100 µL of acetonitrile and 50 µL of methanol water mixture (50:50, v/v) in 96-well glass tubes. After centrifugation at 3000 rpm for 5 min, the supernatant was analyzed by HPLC-MS/MS methods.

The HPLC-MS/MS system consisted of either a Shimadzu ternary pump (Shimadzu LC-10A, Kyoto, Japan) or an Agilent quaternary pump HPLC system (Hewlett Packard, Palo Alto, CA), an autosampler and a PE Sciex API 3000 or 4000 (Perkin-Elmer Sciex Instruments, Foster City, CA) mass spectrometer with a turbo ion spray interface (PE-Sciex, Thornhill, Ontario, Canada). A 10-µL aliquot of each sample was injected onto a HPLC column. CP-141938 was run on a Phenomenex Synergi Polar column (50x4.6 mm, 4µm, Phenomenex, Torrance, CA) isocratically at 75% acetonitrile and 25% 5 mM ammonium acetate containing 0.01% formic acid and 0.1% isopropranol with a flow rate of 1 ml/min for 4 min. Propranolol, Fluoxetine, and NFPS were run on a Phenomenex Primesphere C18 HC column (30x2.0 mm, 5 µm, Phenomenex, Torrance, CA) using a gradient pump program beginning at 5-10% acetonitrile and 90-95% aqueous 5-20 mM ammomium acetate containting 0.1% isopropranol with a flow rate of 0.4 mL/min. The acetonitrile increased linearly from 10% to 90% from 1.0 to 1.2 min and then maintained at 90% from 1.2 to 4.0 min. The system returned to the initial conditions in a single step and was allowed to equilibrate for 1 min. Caffeine, theobromine, and theophylline were run on a Phenomenex Luna Phenyl Hexyl column (50x4.6mm, 5 µm, Phenomenex, Torrance, CA) isocratically at 20% acetonitrile and 80% 5 mM ammonium acetate containing 0.1% formic acid and 0.1% isopropranol for 5 min at a flow rate of 1 mL/min. All analytes were eluted between 1 and 4 min.

The compounds were monitored using the following mass transitions: CP-141938, 404.3→160.0 (declustering potential 51 V; collision energy 29 V); propranolol, 260.2→116.3 (36 V; 27 V); fluoxetine, 310.1→148.1 (26 V; 13 V); NFPS, 394.2→102.2 (26 V; 21 V); caffeine 195.2→138.1 (41 V; 27 V); theobromine, 181.1→138.1 (46 V; 25 V); and theophylline, 181.1→123.1 (46 V; 23 V). The low limit of

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quantitation for the plasma samples of caffeine, CP-141938, fluoxetine, NFPS, propranolol, theobromine, and theophylline was 10, 1, 2.5, 1, 2, 50, and 50 ng/mL, respectively. The low limit of quantitation for the brain samples was 40, 4, 10, 4, 5, 200, and 200 ng/mL, respectively. The relative accuracy was between 80% and 120%.

The contribution of residual blood in the brain tissue to the brain concentration was corrected by subtracting 3.7% of the plasma concentration from the corresponding brain concentration (Khor et al., 1991).

Brain PBPK Model. A hybrid brain PBPK model was constructed based upon a previously published whole-body PBPK model (Peng et al., 2001). The brain consists of two compartments, i.e., brain intravascular and extravascular compartments (Figure 1A). This model was based upon following assumptions: (1) only the unbound drug in vascular space is available to cross the BBB and the unbound and bound compound equilibrates instantaneously within each compartment, (2) no transporter contributes significantly to the brain disposition, consequently, the uptake and efflux clearance equals to PS, the product of BBB permeability-surface area product representing the distribution clearance across BBB via passive diffusion, and (3) cerebrospinal fluid does not significantly impact on the brain drug disposition (Wang et al., 1997).

The plasma of the hybrid PBPK model consists of one compartment for all compounds except fluoxetine. The plasma of fluoxetine consists of a central compartment and a peripheral compartment. It is assumed that the elimination only occurs in the central compartment.

For caffeine, CP-141938, NFPS, theobromine, and theophylline their mass balance rate equation for the central compartment consisting rapid perfusion organs is:

$$V_p \frac{dC_a}{dt} = k_a \bullet X_{dose} - Cl_p \bullet C_a - Q(C_a - C_{iv}) \qquad \text{Equation 1}$$

where V_p (mL/kg) is distribution volume of central compartment, C_a (ng/mL) is the concentration in central compartment, k_a (h^{-1}) is absorption rate constant, X_{dose} (ng/kg) is the dose administered, Cl_p (mL/h/kg) is systemic clearance, Q (mL/h/kg) is cerebral blood flow, and C_{iv} (ng/mL) is concentration in the intravascular space in brain.

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$$\frac{dX_{dose}}{dt} = -k_a \bullet X_{dose} \qquad \text{Equation 2}$$

For propranolol: $$V_p \frac{dC_a}{dt} = -Cl_p \bullet C_a - Q(C_a - C_{iv}) \qquad \text{Equation 3}$$

For fluoxetine: $$V_p \frac{dC_a}{dt} = -Cl_p \bullet C_a - Q(C_a - C_{iv}) - Cl_d(C_a - C_{sp}) \qquad \text{Equation 4}$$

where Cl_d (mL/h/kg) is distribution clearance between central and peripheral compartment and C_{sp} (ng/mL) is concentration in peripheral compartment. The initial conditions for Equation 1, 2, 3, and 4 is $C_a = 0$, X_{dose} = dose, C_a = dose/V_p , C_a = dose/V_p, respectively.

Its mass balance rate equation for the peripheral compartment consisting slow perfusion organs is:

$$V_{sp} \frac{dC_{sp}}{dt} = Cl_d(C_a - C_{sp}) \qquad \text{Equation 5}$$

where V_{sp} (mL/kg) is distribution volume of the peripheral compartment.

The mass balance rate equation for brain intravascular compartment is:

$$V_{iv} \frac{dC_{iv}}{dt} = Q(C_a - C_{iv}) - PS \bullet f_{u,plasma}(C_{iv} - \frac{C_{ev}}{K_p}) \qquad \text{Equation 6}$$

where V_{iv} (mL/kg) is physiological volume of intravascular space in brain, $f_{u,plasma}$ is unbound fraction in plasma determined using equilibrium dialysis, C_{ex} (ng/mL) is concentration in extravascular space, and K_p is equilibrium brain/plasma partitioning coefficient: $K_p = \frac{f_{u,plasma}}{f_{u,brain}}$. $F_{u,brain}$ is unbound fraction in brain tissue.

The mass balance rate equation for brain extravascular compartment is:

$$V_{ev} \frac{dC_{ev}}{dt} = PS \bullet f_{u,plasma}(C_{iv} - \frac{C_{ev}}{K_p}) \qquad \text{Equation 7}$$

where V_{ev} (mL/kg) is physiological volume of extravascular space. The initial conditions for Equation 5, 6, and 7 is $C_{sp} = 0$, $C_{iv} = 0$, and $C_{ev} = 0$, respectively.

The Q, V_{ev}, and V_{iv} was assumed to be 312 mL/h/kg, 6.56 mL/kg, and 0.24 mL/kg, respectively (Peng et al., 2001). The pharmacokinetic parameters for the plasma (k_a, V_p, V_{sp}, Cl_p, and Cl_d) were estimated by fitting Equation 1-5 to the observed plasma concentrations in Figure 2 and the

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pharmacokinetic parameters for the brain (PS, K_p, and $f_{u,brain}$) were estimated by fitting Equation 6-7 to brain concentrations using the nonlinear least-squares regression program WinNonlin™ (version 3.2, Pharsight Corporation, Mountain View, CA). The goodness-of-fit were assessed based on Akaike's Information Criterion (Akaike, 1976), the degree of co-linearity of parameters, the degree of bias in residual error, the standard error of parameter estimates, and visual inspection of the generated curves relative to the data. A weighting scheme of $1/Y_{Predicted}$ was used for all fitting procedures (Liu et al., 1999).

Plasma terminal elimination rate constants (k_{el}) for all compounds except fluoxetine were determined from Cl_p/V_p, where the Cl_p and V_p were calculated from the PBPK model. The k_{el} of fluoxetine was estimated from Equation 8, based on a standard 2-compartment open model with elimination from the central compartment, assuming the mass balance in brain is negligible:

$$k_{el} = \frac{1}{2}\left[(k_{12} + k_{21} + k_{10}) - \sqrt{(k_{12} + k_{21} + k_{10})^2 - 4k_{21}k_{10}}\right] \qquad \text{Equation 8}$$

where k_{12} is rate constant from central (plasma) compartment to peripheralcompartment, k_{21} is the rate constant from peripheralcompartment to central compartment, k_{10} is the elimination rate constant from plasma compartment (Gibaldi and Perrier, 1982). By definition, $k_{12}= Cl_d/V_p$, $k_{21}= Cl_d/V_{sp}$, and $k_{10}= Cl_p/V_p$.

Relationship between Brain and Plasma Concentrations. In order to obtain an explicit expression to describe the BP-time course, the brain PBPK model was simplified by removing the cerebral blood flow from the model under the assumption that BBB permeability is the rate-limiting step for brain penetration (Figure 1B) (Hammarlund-Udenaes et al., 1997). Since brain tissue weight is less than 1% of body weight in rats (Brown et al., 1997), the amount of drug in brain tissue is much less than that in the rest of the body. Therefore, the impact of efflux of drug from brain on plasma concentration can be considered negligible. After a compound is administrated by an intravenous bolus injection, the mass balance rate equation for plasma can be described as:

$$V_p \frac{dC_p}{dt} = -Cl_p \bullet C_p \qquad \text{Equation 9}$$

The mass balance rate equation for brain tissue can be described as:

$$V_b \frac{dC_b}{dt} = PS \bullet f_{u,plasma} \bullet C_p - PS \bullet f_{u,brain} \bullet C_b \qquad \text{Equation 10}$$

where V_b (mL/kg) is volume of brain tissue, C_b (ng/mL) is concentration in brain compartment.

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Integration of the rate equations yields plasma and brain concentration-time equations:

$$C_p = \frac{Dose}{V_p} e^{-\frac{Cl_p}{V_p}t} \qquad \text{Equation 11}$$

$$C_b = \frac{Dose \bullet PS \bullet f_{u,plasma}}{V_b \bullet V_p (\frac{PS \bullet f_{u,brain}}{V_b} - \frac{Cl_p}{V_p})} \left(e^{-\frac{Cl_p}{V_p}t} - e^{-\frac{PS \bullet f_{u,brain}}{V_b}t} \right) \qquad \text{Equation 12}$$

Let $k_{el} = \frac{Cl_p}{V_p}$ and $k_{out} = \frac{PS \bullet f_{u,brain}}{V_b}$, where k_{el} and k_{out} is the plasma elimination rate constant in and the brain elimination rate constant, respectively.

Brain to plasma concentration ratio (BP) can be obtained from Equation 11 and 12:

$$\frac{C_b}{C_p} = \frac{PS \bullet f_{u,plasma}}{V_b (k_{out} - k_{el})} \left(1 - e^{-(k_{out} - k_{el})t}\right) \qquad \text{Equation 13}$$

According to Equation 13, compounds can be separated into two classes. For Class I compounds, their k_{out} is less than k_{el}. Their BP increases over time after a single dose and cannot reach a plateau. Their brain terminal $t_{1/2}$ is longer than the plasma $t_{1/2}$. For Class II compounds, their k_{out} is greater than k_{el}. Their BP increases initially and then reaches a plateau and the terminal $t_{1/2}$ of the brain concentration equals to the plasma $t_{1/2}$. From Equation 13 the equilibration time can be quantified with a parameter, equilibration half-life ($t_{1/2eq}$), which is defined as the time to reach 50% of BP at equilibrium. It can be calculated by following formula:

$$t_{1/2eq} = \frac{\ln 2}{k_{out} - k_{el}} \qquad \text{Equation 14}$$

If the plasma concentration maintains constant by an intravenous bolus loading dose and a constant intravenous infusion maintaining dose, the C_p will be constant (Patlak and Pettigrew, 1976). The following equation can be obtained from Equation 10:

$$\frac{C_b}{C_p} = \frac{f_{u,plasma}}{f_{u,brain}} \left(1 - e^{-k_{out} \bullet t}\right) \qquad \text{Equation 15}$$

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From Equation 15, the equilibration time can be quantified with another parameter, intrinsic equilibration half-life ($t_{1/2eq,in}$), which is defined as the time to reach 50% of BP at equilibrium. It can be calculated by the following formula:

$$t_{1/2eq,in} = \frac{\ln 2}{k_{out}} = \frac{V_b \ln 2}{PS \bullet f_{u,brain}} \qquad \text{Equation 16}$$

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RESULTS

The PS values of caffeine, CP-141938, fluoxetine, NFPS, propranolol, theobromine, and theophylline determined previously using the in situ rat brain perfusion method are presented in Table 1 (Liu et al., 2004). The unbound fractions in rat plasma ($f_{u,plasma}$) and brain homogenate ($f_{u,brain}$) measured by an equilibrium dialysis method are also presented in Table 1. The determined unbound fraction in rat plasma is consistent with the values reported in the literature (Bonati et al., 1984; Chauvelot-Moachon et al., 1988; Yasuhara and Levy, 1988; Shum and Jusko, 1989; Caccia et al., 1990). We observed an inverse relationship between permeability and the nonspecific binding in brain homogenate for some of the model compounds. Fluoxetine and propranolol have high PS and low $f_{u,brain}$. CP-141948, theobromine, and theophylline have low PS and high $f_{u,brain}$.

Determination of PBPK Parameters. The dose-normalized plasma and brain concentrations and the corresponding BP time course of the seven compounds are presented in Figure 2. The absorption of caffeine, CP-141938, NFPS, theobromine, and theophylline was best described by a first-order kinetics process. Fluoxetine and propranolol were treated as an intravenous bolus administration due to their rapid absorption. The heterogeneous way of treating the absorption process was only used to describe the plasma concentration-time profiles and should have no impact on the calculation of parameters related to brain drug disposition. The in vivo $f_{u,brain}$ was calculated from $f_{u,plasma}/K_p$. Brain elimination rate constant (k_{out}) was determined from $PS \bullet f_{u,brain}/V_b$, where the PS and $f_{u,brain}$ were calculated from the PBPK model and V_b is the physiological volume of rat brain, 6.56 mL/kg body weight (Peng et al., 2001). Plasma elimination rate constants (k_{el}) for all compounds except fluoxetine were determined from Cl_p/V_p, where the Cl_p and V_p were calculated from the PBPK model. The terminal k_{el} of fluoxetine was estimated from Cl_p, Cl_d, V_p, and V_{sp} using Equation 8. All the pharmacokinetic parameters are shown in Table 2 and 3. Large variability of the estimated PS values for caffeine and theobromine was observed. This is likely due to the lack of data points before plasma and brain reached equilibrium (Figure 2). The PBPK model was able to describe the BP values for all the data except the last time point (7 h) for CP-141938 (Figure 2).

Determination of Brain Equilibrium Half-Life. The observed brain equilibrium half-life (Obs. $t_{1/2eq}$) was visually estimated from Figure 2 as the time for BP to reach 50% of the BP at the plateau between 4 and 6 h (Table 3). The plasma and brain concentrations of caffeine, propranolol, theobromine,

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and theophylline had a similar terminal $t_{1/2}$ and their BP reached a plateau in less than 0.2 h. Their Obs. $t_{1/2eq}$ was assigned as 0.1 h. For CP-141938 and fluoxetine, the plasma and brain concentrations showed a similar terminal $t_{1/2}$ and the BP reached a plateau in approximately 2 h. Their Obs. $t_{1/2eq}$ was assigned as 1 h. The brain concentration of NFPS exhibited a longer $t_{1/2}$ and their BP did not reach a plateau at 24 h. Its $t_{1/2eq}$ was assigned as greater than 24 h. The $t_{1/2eq}$ and $t_{1/2eq,in}$ values were obtained using Equation 14 and 16, respectively (Table 3).

Correlation of Pharmacokinetic Parameters. The relationship of in vivo logPS and the in situ logPS is presented in Figure 3A (correlation coefficient $R^2 = 0.83$). The regression equation is In Vivo LogPS = 1.16(In Situ LogPS) - 0.15. The relationship of in vivo $logf_{u,brain}$ and in vitro $logf_{u,brain}$ determined from brain homogenate is presented in Figure 3B ($R^2 = 0.69$). The data points appear to be clustered on the extremes of the range, therefore more data are needed to evaluate the correlation. The relationship of $logf_{u,plasma}$ and $logf_{u,brain}$ determined from brain homogenate is presented in Figure 3C with ($R^2 = 0.91$). The relationship of $Logt_{1/2eq,in}$ and $Log(PS \bullet f_{u,brain})$ is shown in Figure 4A ($R^2 = 0.85$). The relationship of $Logt_{1/2eq,in}$ and in situ LogPS is presented in Figure 4B ($R^2 < 0.01$).

Simulation of the Time Course of Brain-to-Plasma Concentration Ratio. BP time course data were simulated using the brain PBPK model (Equation 1, 2, 6, and 7) to assess the effect of PS, $f_{u,plasma}$, and $f_{u,brain}$ on the time to equilibrium (Figure 5). The pharmacokinetic parameters used for the simulation are presented in the figure legend. The simulations demonstrate that a decrease in PS (Condition 1 versus 2) or $f_{u,brain}$ (Condition 1 versus 3) prolongs the time to equilibrium. However, the time to equilibrium did not change when PS decreased and $f_{u,brain}$ increased or PS increased and $f_{u,brain}$ decreased as long as $PS \bullet f_{u,brain}$ was kept constant (data not shown). Therefore, compounds having similar $PS \bullet f_{u,brain}$ should exhibit similar time to equilibrium although they may have a much different PS. The simulations demonstrated $f_{u,plasma}$ has no impact on the time to equilibrium (data not shown).

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DISCUSSION

There has been a notion that a compound with high BBB permeability will in turn lead to rapid brain penetration. It was also implied in the literature that partition coefficient may play a role in the time to reach brain equilibrium (Hammarlund-Udenaes et al., 1997). However, the exact contribution of BBB permeability, plasma binding, and brain tissue binding on the time to equilibrium has not been assessed systemically. Caffeine, CP-141938, fluoxetine, NFPS, propranolol, theobromine, and theophylline were selected as the model compounds because they displayed a marked differences in their BBB permeability (160-fold), unbound fraction in plasma (14-fold) and brain tissue (560-fold).

The present study demonstrates the utility of using a hybrid brain PBPK model to calculate kinetic parameters that are directly related to physiological processes (Gibaldi and Perrier, 1982). The hybrid brain PBPK model instead of a whole-body PBPK model was used in the present study because the objective of this work was to examine the effects of plasma binding, brain tissue binding, and BBB permeability on the brain equilibrium time. The brain PBPK model describes the observed brain and plasma concentration-time course data and BP-time course data well except the last data point for CP-141938 (Figure 2). This may be due to an experimental variability or slow equilibrium compartments within brain tissue as suggested previously for another P-gp substrate (Chen and Pollack, 1998). The calculated in vivo logPS from the PBPK model correlates with the determined logPS using an in situ brain perfusion method (R^2 = 0.83, Figure 3A). This study is consistent with a previous study in which Takasato et al. (1984) showed that in vivo PS values of eight solvent-like compounds using pharmacokinetic analysis were consistent with the in situ PS values (Rapoport et al., 1979). In addition, the calculated in vivo log$f_{u,brain}$ from the PBPK model also correlates with the determined log$f_{u,brain}$ determined in brain homogenate (Figure 3B, R^2 = 0.69). However, the data points are clustered at the extremes of the range and more studies are needed to evaluate the correlation.

The present study demonstrates theoretically and experimentally that the time to equilibrium is governed by the product of PS and $f_{u,brain}$. The time to equilibrium can be assessed by the time to reach the plateau of BP-time profiles (Figure 2) (Rapoport et al., 1982). According to Equation 13, compounds can be separated into two classes. For Class I compounds, their k_{out} is less than k_{el}. Their BP increases over time after a single dose and cannot reach a plateau. Their brain terminal $t_{1/2}$ is longer than the plasma $t_{1/2}$. In this

study, NFPS exhibited this profile (Figure 2). Its k_{out} (0.14 h^{-1}) is less than its k_{el} (0.21 h^{-1}). Its BP kept increasing up to 24 h post dose and its brain terminal $t_{1/2}$ was longer than its plasma $t_{1/2}$ (Figure 2). For the Class II compounds, their k_{out} is greater than k_{el}. Their BP increases initially and then reaches a plateau and the terminal $t_{1/2}$ of the brain concentration equals to the plasma $t_{1/2}$. The BP-time profile after a single dose is similar to the plasma concentration-time profile after an intravenous infusion (Equation 13) (Gibaldi and Perrier, 1982). Six of the seven compounds belong to the Class II compounds. Their k_{out} is greater than k_{el} and their BP increased after administration then reached a plateau approximately between 10 min and 2 h. Their brain and plasma $t_{1/2}$ values were similar (Table 2 and Figure 2).

For Class II compounds, the time to reach equilibrium can be quantified with brain equilibration half-life ($t_{1/2eq}$), which is defined as the time for BP to reach 50% of its plateau level after single intravenous bolus dose. A similar approach has been used in literature to describe the rate of penetration into cerebrospinal fluid (Brodie et al., 1960). The $t_{1/2eq}$ calculated using Equation 14 is consistent with the visually observed $t_{1/2eq}$ (Obs. $t_{1/2eq}$) from the BP-time profiles (Table 3). Because $t_{1/2eq}$ is only applicable to Class II compounds, we propose using the intrinsic brain equilibrium half-life ($t_{1/2eq,in}$) to describe the time to equilibrium. The $t_{1/2eq,in}$ is defined as the time for BP to reach 50% of its plateau level under the condition of constant plasma concentration (Patlak and Pettigrew, 1976). According to Equation 16, $t_{1/2eq,in}$ is inversely proportional to the product of PS and $f_{u,brain}$. Compounds having the same PS•$f_{u,brain}$ should exhibit the same time to reach brain equilibrium. These predictions were in agreement with the simulated data.

The experimental results from this study also support that $t_{1/2eq,in}$ is determined by the product of PS and $f_{u,brain}$. Log$t_{1/2eq,in}$ does not correlate with in situ logPS ($R^2 < 0.01$), however, a correlation was observed between the in vivo log$t_{1/2eq,in}$ and log(PS•$f_{u,brain}$) ($R^2 = 0.85$, Figure 4A). In our theoretical analyses, it was assumed that a transporter does not contribute significantly to brain drug disposition, nevertheless it can be demonstrated that the effect will be the same if transporters do contribute to the disposition, in which case the efflux clearance from brain to plasma instead of PS should be used.

There is an apparent inverse relationship of PS and $f_{u,brain}$ for some of the model compounds. CP-141938 and theobromine have a low PS but high $f_{u,brain}$, fluoxetine and propranolol have a high PS but low $f_{u,brain}$ (Table 1 and 2). This inverse relationship is expected as highly lipophilic compounds tend to have a

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high permeability to cross the BBB and high binding with brain tissue. In contrast, less lipophilic compounds tend to have a low permeability across the BBB and less binding to brain tissue. The inverse relationship between PS and $f_{u,brain}$ may provide a mechanistic explanation for the observation made by Hammarlund-Udenase (1997) that compounds having much different BBB permeability are able to reach brain equilibrium quickly.

The inverse relationship between PS and $f_{u,brain}$ suggests that to search for rapid brain penetration compounds in drug discovery both PS and $f_{u,brain}$ or the product of PS and $f_{u,brain}$ should be considered. A lead compound should not be eliminated as a candidate compound with quick onset of action only because it shows low permeability. For example, theobromine is a low PS (23 mL/h/kg) and high $f_{u,brain}$ (0.61) compound, resulting in $PS \bullet f_{u,brain}$ of 14 mL/h/kg. In contrast, fluoxetine is a high PS (619 mL/h/kg) and low $f_{u,brain}$ (0.00094) compound, results in $PS \bullet f_{u,brain}$ of 0.6 mL/h/kg. As expected from the $PS \bullet f_{u,brain}$ but not from the PS, the observed $t_{1/2eq,in}$ for theobromine (~0.1 h) was shorter than that of fluoxetine (~1 h).

The theoretical analysis and PBPK simulation in this study demonstrate that plasma protein binding only determines the total brain drug concentration, not the equilibration time (Equation 16). Interestingly, $\log f_{u,plasma}$ correlates with $\log f_{u,brain}$ for the seven model compounds (R^2 = 0.91, Figure 3C). This apparent correlation is also supported by literature data. For a set of 18 compounds (Kalvass and Maurer, 2002), $\log f_{u,plasma}$ correlates with $\log f_{u,brain}$ after two outliers (compound 4 and 8) were removed (R^2 = 0.60). In addition, a good correlation was also observed for 32 compounds (R^2 = 0.85) (Maurer et al., 2004). Hence, in theory, plasma protein binding per se is not related to the time to equilibrium and should not be considered as a criterion to select rapid brain equilibrium compounds in drug discovery. However, due to the empirical correlation between plasma binding and brain tissue binding, low plasma protein binding may be used as a surrogate.

Two approaches, namely a hybrid brain PBPK model and a two-compartmental brain model, have been employed in this study. The hybrid brain PBPK model approach was used to calculate in vivo $f_{u,brain}$ and PS values and to simulate brain and plasma data. The two-compartmental brain model is a simplified form of the hybrid brain PBPK model and was used to derive the explicit expression of the theoretical relationship. These two approaches are consistent with each other. For example, NFPS did not reach equilibrium up to 24 h post administration. The PBPK model showed that its k_{el} is greater than its k_{out}. This

observation is consistent with the prediction from the analyses of the two-compartmental model that equilibrium cannot be achieved if its k_{el} is greater than k_{out}. For the other six compounds, the PBPK model showed that the k_{el} values are less than their k_{out}. This observation is also consistent with the prediction from the analyses of the two-compartmental model that the equilibrium can be achieved if its k_{el} is less than k_{out}.

In summary, the present study demonstrates the utility of PBPK model to calculate physiologically relevant pharmacokinetic parameters. We demonstrated that rapid brain equilibration requires a combination of high BBB permeability and low brain tissue binding. Importantly, high BBB permeability alone cannot guarantee a quick equilibration time. The strategy to select compounds with rapid brain penetration in drug discovery should identify those compounds with high PS and low nonspecific binding in brain tissue and plasma.

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FIGURE LEGENDS

Figure 1. A hybrid brain PBPK model (A) and a brain compartment model (B) were used to characterize the pharmacokinetics in plasma and brain tissues. The definition of pharmacokinetic parameters is listed in the Materials and Methods.

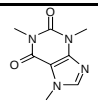
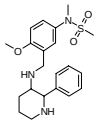
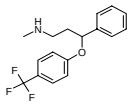
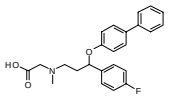
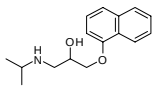
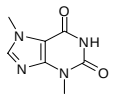
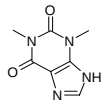
Figure 2. The plasma and brain concentration-time profiles and brain-plasma-concentration ratio (B/P) time profiles of seven model compounds after subcutaneous administration. The concentration was normalized to the dose. Triangle and circle symbols represent observed plasma and brain concentrations (mean ± SD, n = 3-4), respectively. The lines are the best fit of the PBPK model to the data. The pharmacokinetic parameters are presented in Table 2.

Figure 3. The relationship of in vivo BBB permeability (in vivo PS) calculated using the PBPK model from plasma and brain concentration data in Figure 2 and in situ BBB permeability (in situ PS) determined using the in situ brain perfusion method (A), in vivo unbound fraction in brain tissue (in vivo $f_{u,brain}$) and determined unbound fraction in brain homogenate (in vitro $f_{u,brain}$) (B), and unbound fraction in plasma (in vitro $f_{u,plasma}$) and the unbound fraction in brain homogenate (in vitro $f_{u,brain}$) (C). The solid lines indicate the results of least-squares regression.

Figure 4. The relationship of intrinsic brain equilibrium half-life ($t_{1/2eq,in}$) and the product of in situ BBB permeability and the unbound fraction in brain homogenate ($PS \bullet f_{u,brain}$) (A) and the intrinsic brain equilibrium half-life and the in situ BBB permeability (in situ PS) (B). The solid lines indicate the results of least-squares regression.

Figure 5. Simulated time courses of brain-plasma-concentration ratio for different BBB permeability (Condition 1 versus 2) and brain tissue binding (Condition 1 versus 3). The pharmacokinetic parameters used for the simulation are: Dose = 1 mg/kg, Q = 312 mL/h/kg, k_a = 1 h^{-1}, V_p = mL/kg, Cl_p = 4000 mL/h/kg. For Condition 1: PS = 4000 mL/h/kg, $f_{u,plasma}$ = 0.1, $f_{u,brain}$ = 0.01; for Condition 2: PS = 400 mL/h/kg, $f_{u,plasma}$ = 0.1, $f_{u,brain}$ = 0.01; for Condition 3: PS = 4000 mL/h/kg, $f_{u,plasma}$ = 0.1, $f_{u,brain}$ = 0.001.

Table 1. PS values and unbound fraction of seven model compounds in rat plasma and brain tissue (Mean ± SD, n = 4).

Compound	Structure	BP_{ko}/BP_{wt} [a]	Class	Molecular Weight	cLogD [b]	TPSA [b]	In Situ PS [b] ($mL \cdot h^{-1} \cdot kg^{-1}$)	$f_{u,plasma}$	In Vitro $f_{u,brain}$ [c]
Caffeine		1.1 [d]	Neutral	194.2	-0.0800	58.4	223	0.96 ± 0.03	1.1 ± 0.2
CP-141938		50 [e]	Base	403.6	-1.26	70.7	6.4	0.56 ± 0.01	0.22 ± 0.03
Fluoxetine		1.5 [d]	Base	309.3	1.69	21.3	619	0.060 ± 0.001	0.00094 ± 0.00050
NFPS		NA	Zwitterion	393.5	2.84	49.8	31	0.041 ± 0.005	0.0017 ± 0.0002
Propranolol		NA	Base	259.4	1.34	41.5	1043	0.15 ± 0.02	0.036 ± 0.009
Theobromine		NA	Neutral	180.2	-0.790	67.2	23	0.92 ± 0.06	0.61 ± 0.09
Theophylline		1.3 [f]	Neutral	180.2	0.0200	69.3	31	0.29 ± 0.01	0.39 ± 0.06

[a] BP_{ko} and BP_{ko} represents the brain-to-plasma concentration ratio in mdr1a/1b gene knockout and FVB mice, respectively.

[b] Liu et al (2004)

[c] Unbound fraction in brain tissue determined using brain homogenate

[d] Doran et al (2005)

[e] Smith et al (2001)

[f] Unpublished data

Table 2. Pharmacokinetic parameters[a] associated with the hybrid brain PBPK model.

Compound	K_a (hr^{-1})	V_p ($mL \cdot kg^{-1}$)	V_{sp} ($mL \cdot kg^{-1}$)	Cl_p ($mL \cdot hr^{-1} \cdot kg^{-1}$)	Cl_d ($mL \cdot hr^{-1} \cdot kg^{-1}$)	PS ($mL \cdot hr^{-1} \cdot kg^{-1}$)	K_p	In vivo $f_{u,brain}$[b]
Caffeine	0.74 ± 0.04	440 ± 30	NA	330 ± 30	NA	82 ± 80	0.70 ± 0.07	1.4
CP-141938	3.1 ± 1.0	9700 ± 1500	NA	3200 ± 380	NA	9.3 ± 2.9	0.30 ± 0.04	1.9
Fluoxetine	NA	7900 ± 900	9200 ± 2900	5000 ± 500	6200 ± 2000	2000 ± 110	26 ± 1	0.0023
NFPS	4.2 ± 0.6	3700 ± 200	NA	780 ± 40	NA	11 ± 1	0.48 ± 0.06	0.085
Propranolol	NA	6500 ± 100	NA	4800 ± 100	NA	4000 ± 2600	18 ± 4	0.0083
Theobromine	1.4 ± 0.7	1800 ± 500	NA	190 ± 80	NA	33 ± 30	0.47 ± 0.06	2.0
Theophylline	9.9 ± 1.8	760 ± 30	NA	150 ± 10	NA	110 ± 30	0.28 ± 0.01	1.0

[a]Parameters (mean ± SE) were obtained by fitting Equations 1-7 to the data in Figure 2 with nonlinear least squares regression analysis.

[b]$f_{u,brain} = f_{u,plasma}/K_p$

Table 3. Derived pharmacokinetic parameters of the seven model compounds and the observed brain half-lives.

Compound	k_{out}[a] (hr^{-1})	k_{el}[b] (hr^{-1})	$t_{1/2eq}$[c] (hr)	$t_{1/2eq,in}$[d] (hr)	Obs.$t_{1/2eq}$[e] (hr)
Caffeine	18	0.75	0.040	0.039	0.1
CP-141938	2.7	0.33	0.29	0.26	1
Fluoxetine	0.70	0.23	1.5	1.0	1
NFPS	0.14	0.21	NA[f]	5.0	>24
Propranolol	5.1	0.74	0.16	0.14	0.1
Theobromine	10	0.11	0.071	0.070	0.1
Theophylline	17	0.20	0.041	0.040	0.1

[a] $k_{out} = PS \bullet f_{u,brain}/V_b$

[b] $k_{el} = Cl_p/V_p$ except for fluoxetine. The k_{el} of fluoxetine was calculated from Cl_p, Cl_d, V_p, and V_{sp} using Equation 8.

[c] $t_{1/2eq} = \ln 2/(k_{out}-k_{el})$

[d] $t_{1/2eq,in} = \ln 2/k_{out}$

[e] Estimated visually from the BP-time course in Figure 2.

[f] Not applicable due to $k_{out} < k_{el}$

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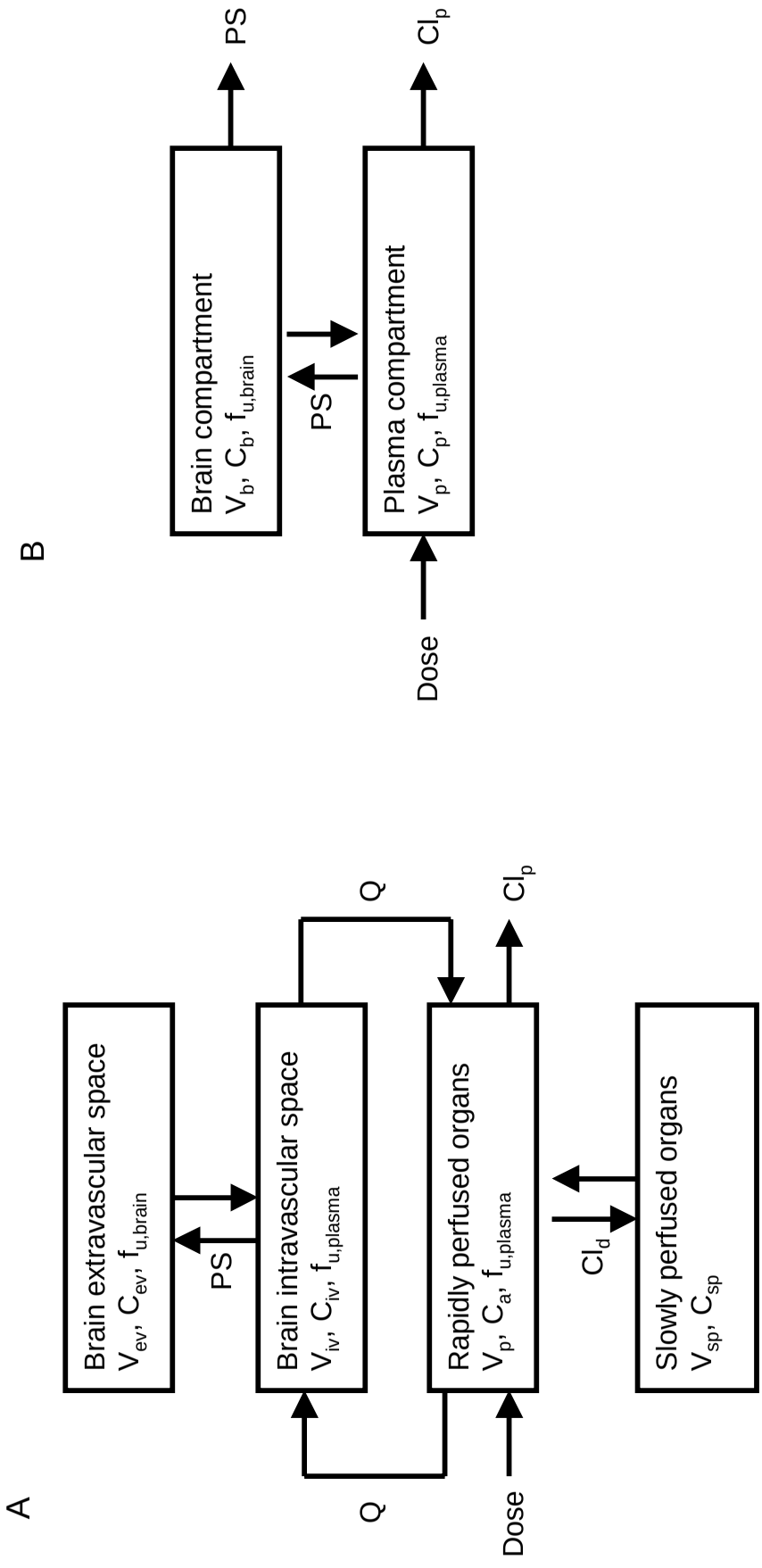

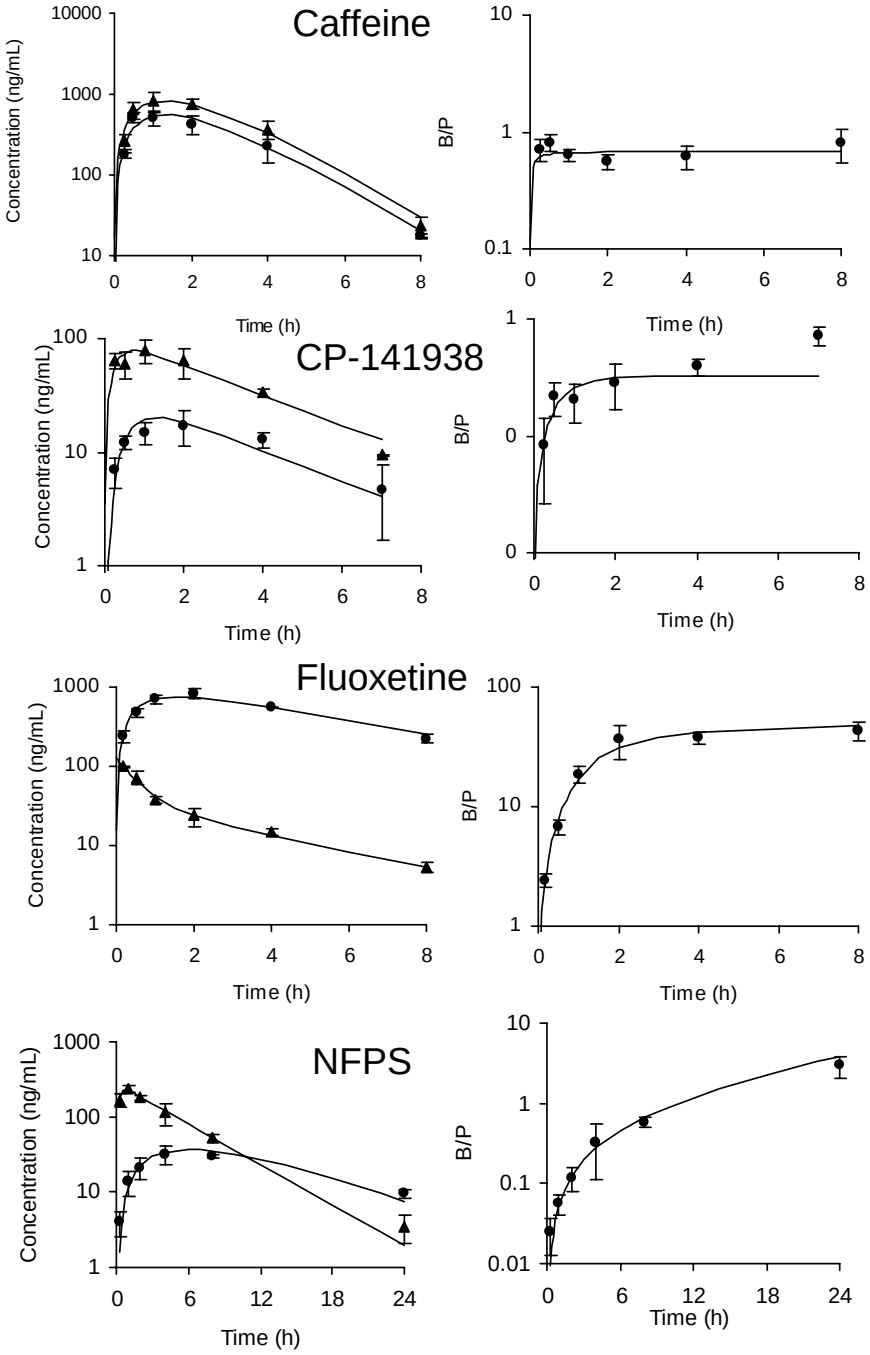

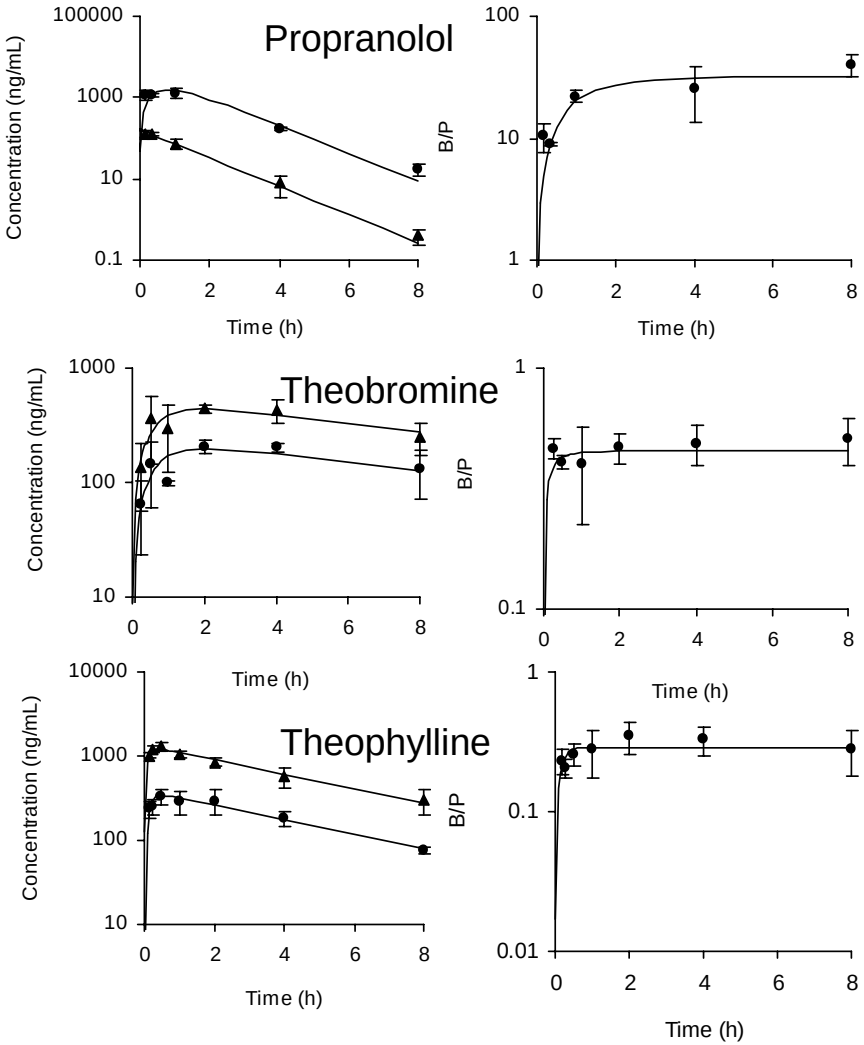

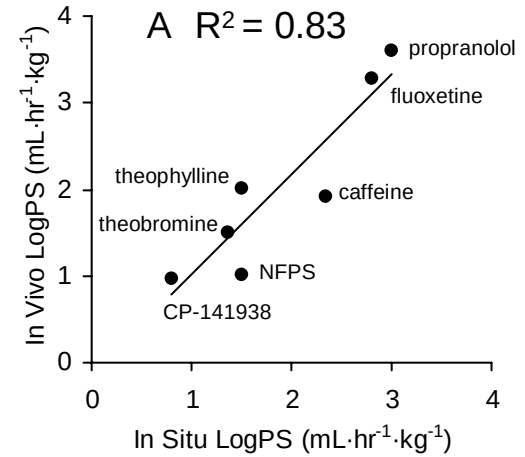

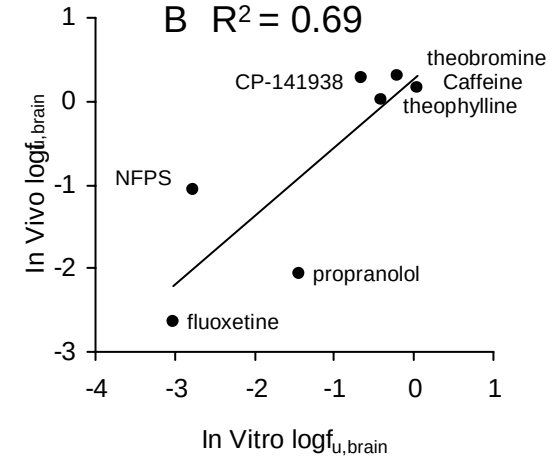

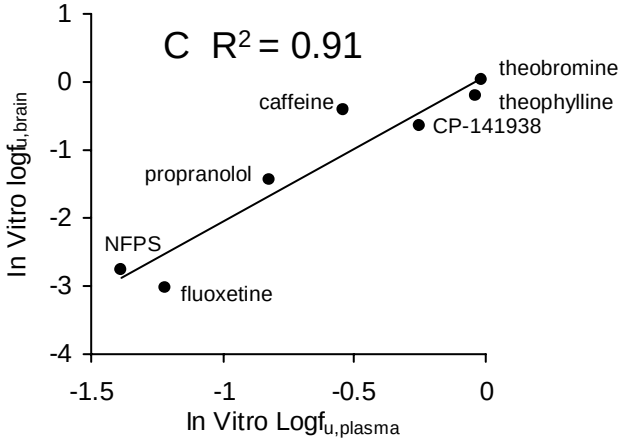

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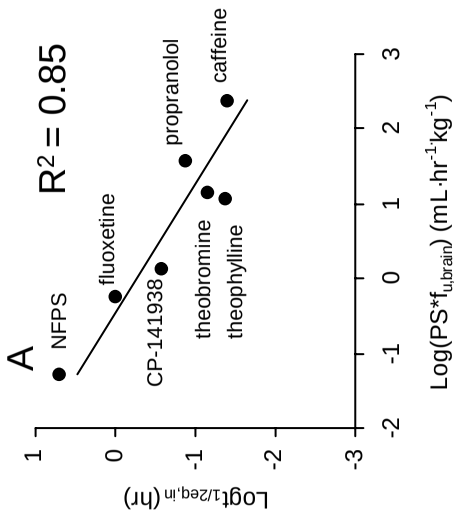

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