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Evaluation of the Utility of Brain Slice Methods to Study Brain Penetration

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Running title: Brain Slices

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Abbreviations used are: P-gp, P-glycoprotein; BBB, blood-brain barrier; CNS, central nervous system; BCSFB, blood-cerebrospinal fluid barrier; AUC, area under the curve; KO, knockout; WT, wild type; DMSO, di-methyl sulfoxide ; NFPS, N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy) propyl]sarcosine; CP-141938, methoxy-3-[(2-phenyl-piperadinyl-3-amino)-methyl]-phenyl-N-methyl-methane-sulfonamide

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ABSTRACT

The objective of this study was to evaluate the utility of brain tissue slices to determine the effect of plasma and brain tissue nonspecific binding on the brain-to-plasma ratio (K_p). Mouse or rat brain slices (400 µm) were prepared using a McIlwain tissue chopper and incubated with 1 µg/mL of compound at 37^{o}C either in a physiological buffer to determine the buffer-to-slice concentration ratio, i.e. unbound fraction in brain tissue ($f_{u,slice}$) or in plasma to determine the slice-to-plasma concentration ratio (C_{slice}/C_{plasma}). The unbound fraction in plasma, $f_{u,plasma}$ was determined using equilibrium dialysis. *In vitro-in vivo* correlation of the brain-to-plasma ratio was examined for thirteen and eight model compounds in mice and rats, respectively. C_{slice}/C_{plasma} and $f_{u,plasma}/f_{u,slice}$ predicted the K_p in rats and C_{slice}/C_{plasma} predicted the K_p in FVB mice for non-P-glycoprotein substrates within 3-fold but over predicted K_p for P-glycoprotein substrates by more than 3-fold. However, C_{slice}/C_{plasma} predicted the K_p in mdr1a/1b knockout mice for both non-P-glycoprotein and P-glycoprotein substrates. Our present study demonstrates that a brain slice method can be used to differentiate whether a compound having a low K_p is due to the effect of low nonspecific binding to brain tissue relative to plasma proteins or due to efflux transport at the blood-brain barrier.

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INTRODUCTION

Brain is separated from the systemic circulation by two barriers: a blood-brain barrier (BBB) and a blood-cerebrospinal-fluid barrier (BCSFB). The BBB and BCSFB represent physical and enzymatic barriers to restrict and regulate the penetration of compounds into and out of the brain and maintain the homeostasis of the brain microenvironment (Davson and Segal, 1995). Brain penetration is essential for compounds where the site of action is within the central nervous system (CNS). For targets outside the CNS, BBB penetration may need to be minimized to reduce CNS-related side effects (Chen et al., 2003).

The brain-to-plasma concentration ratio (K_p) is the most commonly used parameter for measuring brain penetration in a drug discovery setting. A large K_p is considered a favorable property of a good CNS compound. This approach implies that a compound having a high K_p penetrates into brain tissue better than one having a low K_p. However, according to the definition of K_p ($K_p = C_{brain}/C_{plasma}$) and unbound fraction in plasma ($f_{u,plasma}=C_{u,plasma}/C_{plasma}$) and brain ($f_{u,brain}= C_{u,brain}/C_{brain}$) the following equation can be obtained:

$$K_p = \frac{f_{u,plasma}}{f_{u,brain}} \bullet \frac{C_{u,brain}}{C_{u,plasma}} = K_{p,in} \bullet K_{p,free} \qquad \text{Equation 1}$$

$K_{p,in}$, defined as the ratio of $f_{u,plasma}$ over $f_{u,brain}$, can be considered the "intrinsic" partition coefficient between brain and plasma. It is determined by nonspecific binding in brain and plasma and is not related to BBB properties. $K_{p,free}$, defined as the ratio of the free brain concentration ($C_{u,brain}$) over the free plasma concentration ($C_{u,plasma}$), delineates BBB properties and governs the relationship between free brain and plasma concentrations. $K_{p,free}$ represents a better parameter than K_p to assess brain penetration or brain bioavailability for CNS compounds (Liu and Chen, 2005). However, it is difficult to determine $K_{p,free}$ experimentally. As a surrogate approach in drug discovery, K_p is often used to select compounds with good brain penetration. However, using K_p introduces the caveat that a high K_p can be due to a high $K_{p,in}$ or $K_{p,free}$ and a low K_p can be due to a low $K_{p,in}$ or $K_{p,free}$. Therefore, when K_p is used to select CNS compounds, it is critical to ensure that a low K_p is due to a low $K_{p,free}$ but not due to a low $K_{p,in}$. In order to overcome the limitation of K_p, we propose determining $K_{p,in}$ and then indirectly evaluating $K_{p,free}$ using Equation 1.

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$K_{p,in}$ may be calculated from $f_{u,plasma}$ and $f_{u,brain}$, which can be determined using *in vitro* approaches, such as equilibrium dialysis, ultrafiltration and ultracentrifugation from plasma and brain tissue homogenate (Fichtl et al., 1991; Kalvass and Maurer, 2002; Maurer et al., 2005). Recently, Maurer et al. (2005) demonstrated that nonspecific binding in brain homogenate and plasma can be used to predict *in vivo* K_p for 23 of 33 tested compounds within a 3-fold range. The concern of estimating $f_{u,brain}$ from brain homogenate is that brain homogenization may change brain binding properties by unmasking binding sites that are not normally accessible to a drug *in vivo*. In addition, the unbound fraction in brain tissue may not be directly extrapolated from the unbound fraction determined from diluted brain tissue homogenate.

Brain slices have been used to study neural physiology for almost half a century (Collinridge, 1994) Cellular structure is maintained in slices but the blood-brain barrier is not functional since a compound can directly penetrate into the brain slices from the incubation medium (Newman et al., 1988). The use of brain slices to estimate the $f_{u,brain}$ has been reported in the literature (Van Peer et al., 1981). In that study, 1000 μm brain slices were incubated for one hour and the buffer-to-slice ratio was assumed to be equal to the *in vivo* $f_{u,brain}$. Brain slices have also been used to study the partition coefficient between slice and incubation buffer (Ooie et al., 1997; Gredell et al., 2004). Based on the same concept, Kakee et al. (1996) used brain slices to estimate the apparent distribution volume in brain. Our main concern was that diffusion into brain tissue may be time dependent and whether one hour was sufficient time for the 1000 μm brain slices to reach equilibrium. No extensive validation study was reported for the brain slice method. In the present study, the experimental conditions for a brain slice uptake study and the utility of brain slices to determine $K_{p,in}$ are evaluated.

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MATERIALS AND METHODS

Chemicals. Supplies of midazolam, 9-hydroxyrisperidone and metocloperamide were obtained from Pfizer Global Material Management (Groton, CT). Caffeine, fluoxetine, propranolol, theobromine, sulpiride, thiopental, quinidine, zolpidem and theophylline were obtained from Sigma-Aldrich (St. Louis, MO). Propoxyphene was obtained from US Pharmacopeia (Rockville, MD). 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 a purity greater than 98%. All other chemicals used in the experiments were of the highest available grade.

Animal Experiments. Male FVB (wild type) and mdr1a/1b (-/-,-/-) mice (20-30 g) were obtained from Taconic (Germantown, NY). All animals were housed in a controlled temperature and humidity environment with an alternating 12-hour light and dark cycle with free access to food and water.

Mice received a 10 mg/kg subcutaneous dose of NFPS, propranolol, theobromine, or theophylline. The doses were prepared in 0.9% saline and delivered in a volume of 2 mL/kg. Animals were sacrificed in a CO_2 chamber. Blood samples were collected in heparin treated tubes at designated times between 10 minutes and 24 hours 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, 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 adjusted to pH 7.4 and then spiked with compound (500 ng/mL) and 150 µL of the spiked matrix was added to individual wells of the dialysis apparatus. The receiver side contained 150 µL of phosphate buffer.

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The 96-well equilibrium dialysis apparatus was maintained on a rotator in a Thermo Forma Stericult incubator (Marietta, OH) at 37°C for 5 hours. Ten µL of either plasma or brain homogenate and 50 µL of buffer were taken from the apparatus and added to silanized glass vials in a 96-well block containing 100 µL of acetonitrile fortified with internal standard. The samples were then vortexed, centrifuged and stored at –20°C prior to analysis. The unbound fractions determined from diluted brain tissue homogenates were corrected to yield an estimate of unbound fraction in the intact brain tissue using a previously published method (Kalvass and Maurer, 2002).

Brain Slices. After rats and mice were sacrificed in a CO_2 chamber, whole brain was obtained and immediately stored in ice-cold physiological buffer (122 mM NaCL, 25 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.4 mM $CaCl_2$ and 1.2 mM $MgSO_4$ pH 7.4, Ooie et al. 1997). Cerebral cortex was dissected from whole brain and the hemispheres were separated. Coronal brain slices of the cortex (400 µm) were prepared using a McIlwain Tissue Chopper (Surrey, UK). The slices were immediately transferred to ice-cold buffer using a paintbrush to prevent injury to the slices. One rat brain slice or 2 mouse brain slices were added to a silanized 25 mL scintillation vial containing either buffer or plasma spiked with 1 µg/mL of a test compound. The incubation was conducted on a rotator inside a Stericult incubator with 95%/5% O_2/CO_2 and relative humidity of 75% at 37°C. At designated time points, 100 µL of incubation buffer or plasma was transferred to an eppendorf tube. The buffer eppendorf contained 100 µL of acetonitrile to wash the pipette tip for nonspecifically bound drug. The brain slices were removed and rinsed in a silanized vial with the buffer. Rat or mouse slices were then transferred to a test tube containing 975 µL or 488 µL of phosphate buffer. Samples were stored at -20^{o}C until analysis.

Sample Analyses. Whole brain tissues were homogenized in 4 volumes (w/v) of water. Twenty µL of plasma or brain homogenate, 20 µL of DMSO and 200 µL of acetonitrile containing an internal standard were mixed in silanized 96-well glass tubes. The samples were then centrifuged at 3000 rpm for ten minutes and the supernatant was used for HPLC/MS/MS analysis.

For the protein binding samples a mixed matrix method was utilized. Plasma and brain donor samples (containing 10 µL of plasma or brain homogenate and 100 µL of acetonitrile in silanized 96-well glass vials) were mixed with 50 µL of buffer and 50 µL of 50% methanol/water and vortexed. Similarly, 150 µL of receiver sample (containing 50 µL of receiver buffer and 100 µL of acetonitrile in silanized 96-well glass) were mixed with 10 µL of plasma or brain homogenate and 50 µL of 50% methanol/water and vortexed. Standards were made in a like manner. One hundred and sixty µL of mixed matrix (100 µL acetonitrile, 50 µL buffer, 10 µL plasma or brain homogenate) and 50 µL of standard stock solutions were mixed and vortexed. Twenty µL of internal standard in acetonitrile was then added to standards and samples and vortexed. The 96-well plate was then centrifuged at 3000 rpm for 10 minutes and the supernatant was analyzed by HPLC/MS/MS.

Brain slices were homogenized in phosphate buffer at an approximately 40-fold dilution where the estimated brain slice weights were 25 mg and 12.5 mg for rat and mouse, respectively. All incubation buffer, plasma and brain homogenate samples were diluted to the linear range of the standard curves and then precipitated with acetonitrile containing an internal standard followed by centrifugation at 3000 rpm for 10 minutes. The supernatant was then analyzed by HPLC/MS/MS for all except caffeine, theobromine and theophylline. The supernatant of these three compounds was dried down with N_2 at room temperature. The residual was reconstituted with 30-50 µL of acetonitrile, vortexed and underwent HPLC/MS/MS analysis.

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). Sample injection volume was 10 µl. HPLC/MS/MS conditions for the 13 compounds can be found in Table 1. For all of the assays, the concentration of samples was within the linear range of quantitation. The relative accuracy was between 80% and 120%.

Protein normalization was performed to correct for dilution of brain homogenate. The Pierce BCA protein assay (Rockford, IL) was conducted on all of the brain homogenate samples.

The weights of brain slices were calculated by comparing the protein concentration of the brain homogenate to a control brain homogenate containing a known weight of brain (Newman et al., 1995).

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RESULTS

Optimization of brain slice uptake conditions. The effect of brain slice thickness on C_{slice}/C_{plasma} was evaluated in the present study. Using fluoxetine as a model substrate, the C_{slice}/C_{plasma} for slices with a thickness of 200, 300, 400, and 500 µm was similar after incubation for 6 hours. As some of the 200 µm and 300 µm slices appeared slightly damaged at the end of 6-hour incubation, 400 µm slices were used in the present study.

The time to reach equilibrium between the slices and incubation buffer and the slices and plasma was determined for eight model compounds; caffeine, CP-141938, fluoxetine, NFPS, propranolol, quinidine, theobromine, and theophylline. $F_{u,slice}$ decreased over time before reaching equilibrium between 1 and 6 hours. As representative examples, Figure 1A and 1C show the time course of $f_{u,slice}$ for caffeine and propranolol, respectively. Similarly, C_{slice}/C_{plasma} increased over time and reached equilibrium between 1 and 6 hours. Figure 1B and 1D show the time course of C_{slice}/C_{plasma} for caffeine and propranolol, respectively. For those compounds not extensively binding to brain tissue, such as caffeine, the equilibrium of brain slices and incubation medium can be achieved at one hour after incubation. However, for those compounds extensively binding to brain tissue, such as propranolol two to six hours were needed to achieve equilibrium.

The effect of fresh and previously frozen plasma on C_{slice}/C_{plasma} was examined using caffeine and fluoxetine as model compounds. The C_{slice}/C_{plasma} of caffeine was 0.364 ± 0.05 and 0.382 ± 0.03 in fresh plasma and previously frozen rat plasma, respectively. The C_{slice}/C_{plasma} of fluoxetine was 20.1 ± 2.9 and 20.6 ± 6.9 for fresh plasma and previously frozen plasma, respectively. No statistical difference was observed for the C_{slice}/C_{plasma} values of caffeine ($p = 0.65$) or fluoxetine ($p = 0.91$) between fresh and previously frozen plasma, indicating previously frozen plasma can be used for the brain slice uptake study.

Correlation of predicted $K_{p,in}$ from brain slices and *in vivo* observed K_p. Eight compounds, caffeine, CP-141938, fluoxetine, NFPS, propranolol, quinidine, theobromine, and theophylline were selected to evaluate the utilities of a brain slicemethod. The selection of these model compounds was based on their physicochemical properties, BBB permeability, in vivo K_p values, and P-gp transporter activity. All the rat related and mouse related parameters are

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presented in Table 2 and 3, respectively. All *in vivo* rat K_p values calculated from the area under the curve (AUC) ratio of brain concentration and plasma concentration were obtained from previous studies (Liu et al., 2005). K_p data for quinidine was from the literature (Kusuhara et al., 1997). *In vivo* mouse K_p values for NFPS, propranolol, theobromine, and theophylline were determined in the present study. All other mouse K_p values were obtained from the literature (Smith et al., 2001; Doran et al., 2005). All *in vivo* mouse K_p values were calculated from the AUC ratio of brain concentration and plasma concentration.

Direct and indirect brain slice methods were evaluated. The *in vitro* and *in vivo* values were considered to be consistent if the values were within 3-fold. This criterion was chosen to allow for differences due to experimental error and for actual differences that would be considered to be of little pharmacologic consequence (Maurer et al., 2005). The direct brain slice method uses C_{slice}/C_{plasma} to estimate $K_{p,in}$. The relationship between rat K_p and C_{slice}/C_{plasma} for the eight model compounds are exhibited in Figure 2A. Six of the eight model compounds, caffeine, fluoxetine, NFPS, propranolol, theobromine, and theophylline were non-P-gp substrates. The C_{slice}/C_{plasma} values for the 6 non-P-gp substrates were within 3-fold of the observed *in vivo* K_p. CP-141938 and quinidine were P-gp substrates. The C_{slice}/C_{plasma} values of CP-141938 and quinidine were 9 and 5 fold greater than their *in vivo* K_p, respectively. The relationship between K_p and C_{slice}/C_{plasma} in FVB mice and mdr1a/1b gene knockout mice for the eight model compounds are presented in Figure 3A and 3B, respectively. For the six non-P-gp substrates, the C_{slice}/C_{plasma} values were within 3-fold of the observed *in vivo* K_p in FVB and mdr1a/1b knockout mice. The C_{slice}/C_{plasma} for the two P-gp substrates, quinidine and CP-141938, were 17- and 26-fold greater than the observed K_p in FVB mice, respectively, but were within 3-fold of the observed K_p in mdr1a/1b knockout mice. These results demonstrate that in rats and wild type mice, C_{slice}/C_{plasma} was consistent with the *in vivo* K_p for non-P-gp substrates but over predicted the *in vivo* K_p for P-gp substrates. In P-gp knockout mice, however, C_{slice}/C_{plasma} was consistent with K_p for both non-P-gp and P-gp substrates.

The indirect slice method uses $f_{u,plasma}/f_{u,slice}$ to predict $K_{p,in}$. The relationship between rat K_p and $f_{u,plasma}/f_{u,slice}$ for the 8 model compounds is shown in Figure 2B. The $f_{u,plasma}/f_{u,slice}$ values

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for 5 of the 6 non-P-gp substrates were within 3-fold of the observed K_p. Only NFPS showed 4-fold of difference between $f_{u,plasma}/f_{u,slice}$ and K_p. The $f_{u,plasma}/f_{u,slice}$ for the two P-gp substrates, CP-141938 and quinidine, were 16- and 12-fold, respectively, greater than the K_p. Therefore, both the direct and indirect brain slice methods were able to predict *in vivo* K_p for non-P-gp substrates but over predicted P-gp substrates in P-gp competent animals.

Comparison of brain slices method and brain homogenate method. In order to compare the brain slice and brain homogenate methods, we used the brain homogenate method to examine the eight model compounds in rats. The relationship between K_p and unbound fraction in plasma over the unbound fraction determined from brain homogenate ratio ($f_{u,plasma}/f_{u,homogenate}$) for the eight model compounds is shown in Figure 2C. The $f_{u,plasma}/f_{u,homogenate}$ for four of the six non-P-gp substrates(caffeine, fluoxetine, theobromine and theophylline) were within 3-fold of *in vivo* K_p. However, the $f_{u,plasma}/f_{u,homogenate}$ for 2 non-P-gp substrates, NFPS and propranolol, over predicted 27-fold and under predicted 4-fold the K_p, respectively. The $f_{u,plasma}/f_{u,homogenate}$ for the two P-gp substrates, CP-141938 and quinidine, were 8-fold and 9-fold, respectively, greater than the K_p.

Furthermore, we selected additional five compounds, midazolam, 9-hydroxyrisperidone, sulpiride, thiopental, and zolpidem for which the brain homogenate method appeared unable to predict K_p (Maurer et al., 2005), to examine whether the brain slice method was able to better predict $K_{p,in}$. The correlation between *in vivo* K_p and C_{slice}/C_{plasma} and the correlation between *in vivo* K_p and the $f_{u,plasma}/f_{u,homogenate}$ in FVB and mdr1a/1b knockout mice is presented in Figure 4A and 4B, respectively. Midazolam, sulpiride, thiopental, and zolpidem were not P-gp substrates while 9-hydroxyrisperidone was a P-gp substrate. For these five compounds, $f_{u,plasma}/f_{u,homogenate}$ over predicted the *in vivo* K_p in FVB and mdr1a/1b knockout mice greater than 3-fold. However, C_{slice}/C_{plasma} was able to predict within 3-fold of the observed *in vivo* K_p for thiopental in FVB and mdr1a/1b mice and for 9-hydroxyrisperidone in mdr1a/1b mice. For midazolam, zolpidem, and sulpiride, both methods were unable to predict *in vivo* K_p within 3-fold. These results indicate the brain slice method is comparable to or slightly better than the brain homogenate method in predicting K_p.

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DISCUSSION

The objective of the present study was to assess the utility of brain slices to determine $K_{p,in}$, a parameter dependent on nonspecific binding in brain tissue and plasma proteins. The main conclusions of this work are: 1) for 400 μm brain slices, the equilibrium can be achieved in six hours; 2) $K_{p,in}$ can be estimated from C_{slice}/C_{plasma} (direct brain slice method) or $f_{u,plasma}/f_{u,slice}$ (indirect brain slice method) with 3-fold of error; 3) similar accuracy was observed between brain slice and brain homogenate methods.

Our study indicates that the optimal conditions for brain slice thickness and incubation time in the tissue binding study are 400 μm and six hours, respectively. Brain slices have been used to assess brain tissue binding in the literature (Van Peer et al., 1981), where 1000-μm brain slices were incubated for one hour. The rate of diffusion and time to achieve equilibrium was a concern for that study. In order to address these issues, the effect of brain slice thickness and the duration of incubation were assessed.

Some of the brain slices with thickness less than 400 μm appeared to be damaged during the incubation process, therefore 400 μm brain slices were selected for all of the studies. For those compounds not extensively binding to brain tissue, such as caffeine the equilibrium of brain slices and the incubation medium was achieved at one hour after incubation. However, for those compounds extensively binding to brain tissue, such as propranolol, two to six hours were needed to achieve equilibrium. These observations are consistent with the literature. In a study of brain penetration for quinolone antimicrobial agents, the time to reach equilibrium for 300 μm brain slices was less than one hour (Ooie et al., 1997). Similar equilibrium time was observed for 2-deoxyglucose in 540 μm brain slices (Newman et al., 1988). In addition, for 300 μm brain slices, the equilibrium was achieved at two to six hours after incubation for propofol (Gredell et al., 2004). In order to guarantee that equilibrium was achieved, all of our incubations were conducted for six hours. Our study also demonstrated that similar $f_{u,slice}$ and C_{slice}/C_{plasma} are observed in fresh prepared plasma and previously frozen plasma. Therefore, previously frozen plasma was also used in this study.

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The present study indicates that brain slices can be used to determine the intrinsic brain-plasma partition, $K_{p,in}$. Direct and indirect brain slice methods have been examined to estimate $K_{p,in}$. The direct brain slice method measures $K_{p,in}$ using the slice-plasma concentration ratio (C_{slice}/C_{plasma}), which was obtained by incubation of brain slices in plasma. To our knowledge, this method represents a novel *in vitro* approach to estimate $K_{p,in}$. This method was validated by the observation that in rats and wild type mice C_{slice}/C_{plasma} was consistent with the *in vivo* K_p for non-P-gp substrates but over predicted the *in vivo* K_p for P-gp substrates. In P-gp knockout mice C_{slice}/C_{plasma} was consistent with both non-P-gp and P-gp substrates. These results are expected from Equation 1. In wild type animals, $K_{p,free}$ is equal to unity for non-P-gp substrates and $K_{p,free}$ is less than unity for P-gp substrates, resulting in a $K_{p,in}$ equal to K_p for non-P-gp substrates and a $K_{p,in}$ greater than K_p for P-gp substrates assuming no other transporter is playing a significant role in brain penetration (Liu and Chen, 2005). In P-gp knockout animals, $K_{p,free}$ is equal to unity for both non-P-gp and P-gp substrates, resulting in a $K_{p,in}$ equal to K_p for both non-P-gp and P-gp substrates.

The indirect brain slice method calculates $K_{p,in}$ from the ratio of $f_{u,plasma}/f_{u,slice}$, where $f_{u,plasma}$ and $f_{u,slice}$ are determined from equilibrium dialysis and the brain slice method, respectively. For non-P-gp substrates, $f_{u,plasma}/f_{u,slice}$ was consistent with the *in vivo* observed K_p but for P-gp substrates, $f_{u,plasma}/f_{u,slice}$ was greater than the *in vivo* observed K_p in rats. The direct and indirect brain slice methods appear to do equally well in estimating $K_{p,in}$, but the direct method does not require the determination of $f_{u,plasma}$.

Our study indicates there is an agreement between the brain slice and brain homogenate methods for $K_{p,in}$ prediction. The brain homogenate method has been reported in the literature to predict *in vivo* K_p (Fichtl et al., 1991; Kalvass and Maurer, 2002; Maurer et al., 2005). The homogenization process is of concern since it may alter binding properties by unmasking binding sites that are not accessible to a drug in intact brain tissue. Consequently, the reliability of calculating the unbound fraction in the intact brain tissue from the unbound fraction determined from a diluted brain tissue homogenate remains uncertain.

In addition, many lipophilic compounds in current CNS drug discovery programs often

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have low plasma unbound fractions and brain unbound fractions. Nonspecific adsorption to the dialysis apparatus and poor recovery frequently hinders the accurate measurement of the unbound fraction (Ward and Azzarano, 2004). The indirect brain slice method offers an alternative and possibly more accurate approach to assess the unbound fraction in brain from intact brain tissue, as $f_{u,slice}$. A brain slice maintains brain cellular structure but has no barrier between the incubation media and the tissues. Early studies demonstrated that BBB non-permeable compounds, such as inulin, can penetrate into interstitial space in brain slice (Newman et al., 1988). Consistent with this assertion, we have observed that there was no significant difference for brain uptake of P-gp substrates, CP-141938 and quinidine, in P-gp knockout and wild type mice brain slices although 50- and 36-fold differences were observed in their K_p value in P-gp knockout and wild type mice. A brain slice incubation can be conducted in silanized glass vials and no dialysis membrane is involved, which further reduces the nonspecific adsorption for lipophilic compounds. The direct brain slice method further reduces the interference of adsorption and improves accuracy of the prediction by omitting the need to determine $f_{u,plasma}$.

In rats, there was a good correlation between the in vivo K_p and predicted using brain slice and brain homogenate methods for all model compounds except NFPS, whose $f_{u,plasma}/f_{u,homogenate}$ was 27-fold lower than the observed K_p while the direct and indirect brain slice method were within 3-fold and 4-fold of error, respectively. This discrepancy was due to the brain slice unbound fraction being approximately seven-fold greater than the value measured using brain homogenate. Whether the lower brain unbound fraction measured using the brain homogenate method was caused by greater access to binding sites normally inaccessible in intact brain tissue or non-specific binding to the dialysis apparatus remains to be determined.

In mice, the prediction using the direct brain slice method is consistent with that using the brain homogenate method. Both methods over-predicted the in vivo K_p for midazolam, sulpiride, and zolpidem, indicating a mechanism other than P-gp mediated efflux transport may cause the lower brain penetration for this group of compounds. For thiopental and 9-hydroxyrisperidone the brain slice method appears to be in better agreement with K_p. These results indicate the brain slice method is comparable to or probably slightly better than the brain homogenate method to

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predict K_p. Although brain slices offer advantages in theory, more studies are needed to examine if the brain slice method is superior to the brain homogenate method in a drug discovery setting.

There are at least two utilities of a brain slice technique to study brain penetration in CNS drug discovery setting. One is to determine if a compound having a low K_p is due to low nonspecific binding in brain tissue relative to plasma proteins, i.e., low $K_{p,in}$ or due to an efflux transporter at the BBB, i.e., low $K_{p,free}$. If the estimated $K_{p,in}$ of a compound is within 3-fold of the observed *in vivo* K_p, the low *in vivo* K_p is likely due to low binding in brain tissue relative to the plasma proteins indicating that brain penetration is not impaired. If the estimated $K_{p,in}$ of a compound is greater than 3-fold of the *in vivo* K_p, the low K_p is likely due to an efflux transporter at the BBB and the compound should be considered to have impaired brain penetration. In contrast to using *in vitro* techniques or transgenic animal models to assess if a specific transporter causes a low K_p, the brain slice approach is a mechanism independent method. Therefore, it is useful in a CNS drug discovery program to screen out efflux transporter substrates. To elucidate the mechanism of impaired brain penetration, other *in vitro* and *in vivo* methods are needed to investigate the underlying mechanism of low BBB penetration.

The other utility is to use $f_{u,slice}$ and total *in vivo* brain concentration to estimate the brain interstitial fluid drug concentration or free brain concentration. This may be used as a surrogate approach in place of resource intensive brain microdialysis studies to estimate free brain concentration. The data from Ooie et al. (Ooie et al., 1997) supports this approach. The slice-to-medium concentration ratios for norfloxacin, ofloxacin, fleroxacin and pefloxacin were 1.9, 1.34, 1.3, and 1.3, respectively. The calculated $f_{u,slice}$, the reciprocal of slice-to-medium ratios, are 0.5, 0.73, 0.8 and 0.75, respectively. They are within 3-fold of the observed *in vivo* $f_{u,brain}$, 1.3, 0.57, 0.37, and 0.47, which were determined using brain microdialysis for each compound. Brain slices may represent a particularly useful approach in drug discovery to evaluate whether the lack of efficacy in an *in vivo* pharmacological model is due to insufficient free drug concentration in the brain. More studies are needed to assess the utilities of this application.

In conclusion, the present study demonstrates that the brain slice technique can be used to determine intrinsic brain to plasma partitioning determined solely by nonspecific binding to

assess brain penetration issues in a drug discovery setting. A direct and an indirect brain slice method have been developed and validated. Brain slices represent a mechanism independent approach to assess if a low brain to plasma ratio is due to nonspecific binding in plasma and brain or due to efflux transport at the BBB. It may also be used to estimate brain free concentrations.

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References

Chen C, Hanson E, Watson J and Lee JS (2003) P-glycoprotein limits the brain penetration of nonsedating but not sedating H1-antagonists. *Drug Metabolism & Disposition.* **31:**312-318.

Davson H and Segal MB (1995) *Physiology of the CSF and blood-brain barriers*. CRC Press, Inc., Boca Raton.

Doran A, Obach RS, Smith BJ, Hosea NA, Becker S, Callegari E, Chen C, Chen X, Choo E, Cianfrogna J, Cox LM, Gibbs JP, Gibbs MA, Hatch H, Hop CECA, Kasman IN, LaPerle J, Liu J, Liu X, Logman M, Maclin D, Nedza FM, Nelson F, Olson E, Rahematpura S, Raunig D, Rogers S, Schmidt K, Spracklin DK, Szewc M, Troutman M, Tseng E, Tu M, Van Deusen JW, Venkatakrishnan K, Walens G, Wang EQ, Wong D, Yasgar AS and Zhang C (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: Evaluation using the Mdr1a/1b knockout mouse model. *Drug Metab Dispos* **33:**165-174.

Fichtl B, Nieciecki AV and Walter K (1991) Tissue binding versus plasma binding of drugs: general principles and pharmacokinetic eonsequences, in: *Advances in drug research* (Fleisch H ed), pp 117-166.

Gredell JA, Turnquist PA, MacIver MB and Pearce RA (2004) Determination of diffusion and partition coefficients of propofol in rat brain tissue: implications for studies of drug action in vitro. *Br. J. Anaesth.* **93:**810-817.

Kakee A, Terasaki T and Sugiyama Y (1996) Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier. *J Pharmacol Exp Ther* **277:**1550-1559.

Kalvass JC and Maurer TS (2002) Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. *Biopharmaceutical Drug Disposition.* **23:**327-338.

Kusuhara H, Suzuki H, Terasaki T, Kakee A, Lemaire M and Sugiyama Y (1997) P-Glycoprotein Mediates the Efflux of Quinidine across the Blood-Brain Barrier. *J Pharmacol Exp Ther* **283:**574-580.

Liu X and Chen C (2005) Strategies to optimize brain penetration in drug discovery. *Curr Opin Drug Discov Dev* **8:**505-512.

Liu X, Smith BJ, Chen C, Callegari E, Becker SL, Chen X, Cianfrogna J, Doran AC, Doran SD, Gibbs JP, Hosea N, Liu J, Nelson FR, Szewc MA and Van Deusen J (2005) 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. *J Pharmacol Exp Ther* **313:**1254-1262.

Maurer TS, DeBartolo DB, Tess DA and Scott DO (2005) Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. *Drug Metab Dispos* **33:**175-181.

Newman GC, Hospod FE and Wu P (1988) Thick brain slices model the ischemic penumbra. *J Cereb Blood Flow Metab* **8:**586-597.

Ooie T, Terasaki T, Suzuki H and Sugiyama Y (1997) Quantitative brain microdialysis study on the mechanism of quinolones distribution in the central nervous system. *Drug Metab Dispos* **25:**784-789.

Smith BJ, Doran AC, McLean S, Tingley FDr, O'Neil BT and Kajiji SM (2001) P-glycoprotein efflux at the blood-brain barrier mediates differences in brain disposition and pharmacodynamics between two structurally related neurokinin-1 receptor antagonists. *J Pharmacol Exp Ther* **298:**1252-1259.

Van Peer AP, Belpaire FM and Bogaert MG (1981) Binding of drugs in serum, blood cells and tissues of rabbits with experimental acute renal failure. *Pharmacology* **22:**146-152.

Ward KW and Azzarano LM (2004) Preclinical Pharmacokinetic Properties of the P-Glycoprotein Inhibitor GF120918A (HCl salt of GF120918, 9,10-Dihydro-5-methoxy-9-oxo-N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]phenyl]-4-acridine-carboxamide) in the Mouse, Rat, Dog, and Monkey. *J Pharmacol Exp Ther* **310:**703-709.

FIGURE LEGENDS

Figure 1. Time-course of unbound brain slice fraction (A) or brain slice to plasma ratio (B) for caffeine. Time-course of unbound brain slice fraction (C) or brain slice to plasma ratio (D) for propranolol.

Figure 2. Relationship between *in vivo* K_p and C_{slice}/C_{plasma} (A), $f_{u,plasma}/f_{u,slice}$ ratio (B) and $f_{u,plasma}/f_{u,homogenate}$ ratio (C) in rats. Solid and open symbols represent non-P-gp substrates (caffeine, fluoxetine, NFPS, propranolol, theobromine and theophylline) and P-gp substrates (CP-141938 and quinidine), respectively. Solid and dashed lines represent unity and 3-fold boundaries, respectively.

Figure 3. Relationship between *in vivo* K_p and brain slice to plasma ratio in FVB mice (A) and mdr1a/1b knockout mice (B), respectively. Solid and open symbols represent non-P-gp substrates (caffeine, fluoxetine, NFPS, propranolol, theobromine and theophylline) and P-gp substrates (CP-141938 and quinidine), respectively. Solid and dashed lines represent unity and 3-fold boundaries, respectively.

Figure 4. Relationship between K_p and C_{slice}/C_{plasma} ratio (circles) and between K_p and $f_{u,plasma}/f_{u,homogenate}$ (triangles) for 9-hydroxyrisperidone, midazolam, sulpiride, thiopental, and zolpidem in FVB mice (A) and mdr1a/1b knockout mice (B), respectively. Solid and dashed lines represent unity and 3-fold boundaries, respectively.

Table 1. HPLC/MS/MS Conditions for all 13 compounds

Compound	Retention Time (min.)	MRM Transition	Initial Condition	Final Condition	Column
Caffeine	1.03	195.2:138.1	70% A:30% B	70% A:30% B	Luna Phenyl Hexyl 50X4.6, 5µ
CP-141938	1.40	404.3:160.2	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6, 4µ
Fluoxetine	2.80	310.1:148.1	90%A:10%B	10%A:90%B	Primesphere C18 30X2, 5µ
Midazolam	1.48	326.2:291.1	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6 4µ
NFPS	3.66	394.0:102.1	90%A:10%B	10%A:90%B	Primesphere C18 30X2, 5µ
Propranolol	3.48	260.1:116.2	90%A:10%B	10%A:90%B	Primesphere C18 30X2, 5µ
Quinidine	2.83	325.3:307.4	90%A:10%B	10%A:90%B	Ace C18 50X4.6, 5µ
Theobromine	1.10	181.1:138.2	25% A: 50% B: 25 % C	25% A: 50% B: 25 % C	Ace C18 50X4.6, 5µ
Theophylline	1.25	181.1:124.1	25%A:75% B	25%A:75% B	Ace C18 50X4.6, 5µ
Thiopental	1.16	241.4:101.0	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6, 4µ
Sulpiride	1.61	342.0:112.1	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6, 4µ
Zolpidem	1.46	308.2:235.2	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6, 4µ
9-0H Risperidone	1.15	427.2:207.2	25%A:75% B	25%A:75% B	Synergi Polar RP 50X4.6, 4µ

A: ammonium acetate buffer at pH 7.0, B: acetonitrile, C: methanol

Table 2. The parameters of eight model compounds in rats.

Compound	Class	*In Vivo* K_p	$f_{u,plasma}$	$f_{u,homogenate}$	$f_{u,slice}$	C_{slice}/ C_{plasma}	$f_{u,plasma} / f_{u,slice}$	$f_{u,plasma} / f_{u,homogenate}$
Caffeine	Neutral	0.70[a]	0.96 ± 0.03	1.1 ± 0.2	0.71	0.36	1.3	0.87
CP-141938	Base	0.31[a]	0.56 ± 0.01	0.22 ± 0.03	0.11	2.8	5.0	2.5
Fluoxetine	Base	25[a]	0.060 ± 0.001	0.00094 ± 0.00050	0.0027	20	22	60
NFPS	Zwitterion	0.90[a]	0.041 ± 0.005	0.0017 ± 0.0002	0.011	2.7	3.7	24
Propranolol	Base	15[a]	0.15 ± 0.02	0.036 ± 0.009	0.019	8.4	7.9	4.2
Quinidine	Base	0.5[b]	0.184 ± 0.023	0.0392 ± 0.0035	0.032	6.1	5.8	4.7
Theobromine	Neutral	0.50[a]	0.92 ± 0.06	0.61 ± 0.09	0.62	1.6	1.5	1.5
Theophylline	Neutral	0.31[a]	0.29 ± 0.01	0.39 ± 0.06	0.31	0.60	0.94	0.74

[a] (Liu et al., 2005)
[b] (Kusuhara et al., 1997)

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Table 3. The parameters of thirteen model compounds in mice

Compound	$K_{p,wt}$	$K_{p,ko}$	$K_{p,ko}$ /$K_{p,wt}$	C_{slice} / C_{plasma}	$f_{u,plasma}$/ $f_{u,homogenate}$
Caffeine	1.0[a]	1.1[a]	1.1	0.78	2.2 [c]
CP-141938	0.10[b]	5.0[b]	50	2.6	NA
Fluoxetine	12[a]	18[a]	1.5	10	14
NFPS	0.84	1.6	2.0	2.2	NA
Propranolol	17	26	1.5	13	NA
Quinidine	0.30[a]	10[a]	36	5.2	NA
Theobromine	0.60	0.84	1.5	1.8	NA
Theophylline	0.73	0.92	1.3	1.3	NA
Midazolam	0.20[a]	0.20 [a]	1.0	1.8	1.7[c]
9-OH Risperidone	0.10[a]	1.0[a]	17	1.7	3.8[c]
Sulpiride	0.078[a]	0.20[a]	1.9	1.4	1.2[c]
Thiopental	0.40[a]	0.40[a]	1.2	1.0	2.1[c]
Zolpidem	0.29[a]	0.40[a]	1.4	2.9	1.2[c]

[a] (Doran et al., 2005)
[b] (Smith et al., 2001)
[c] (Maurer et al., 2005)

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Figure 1.

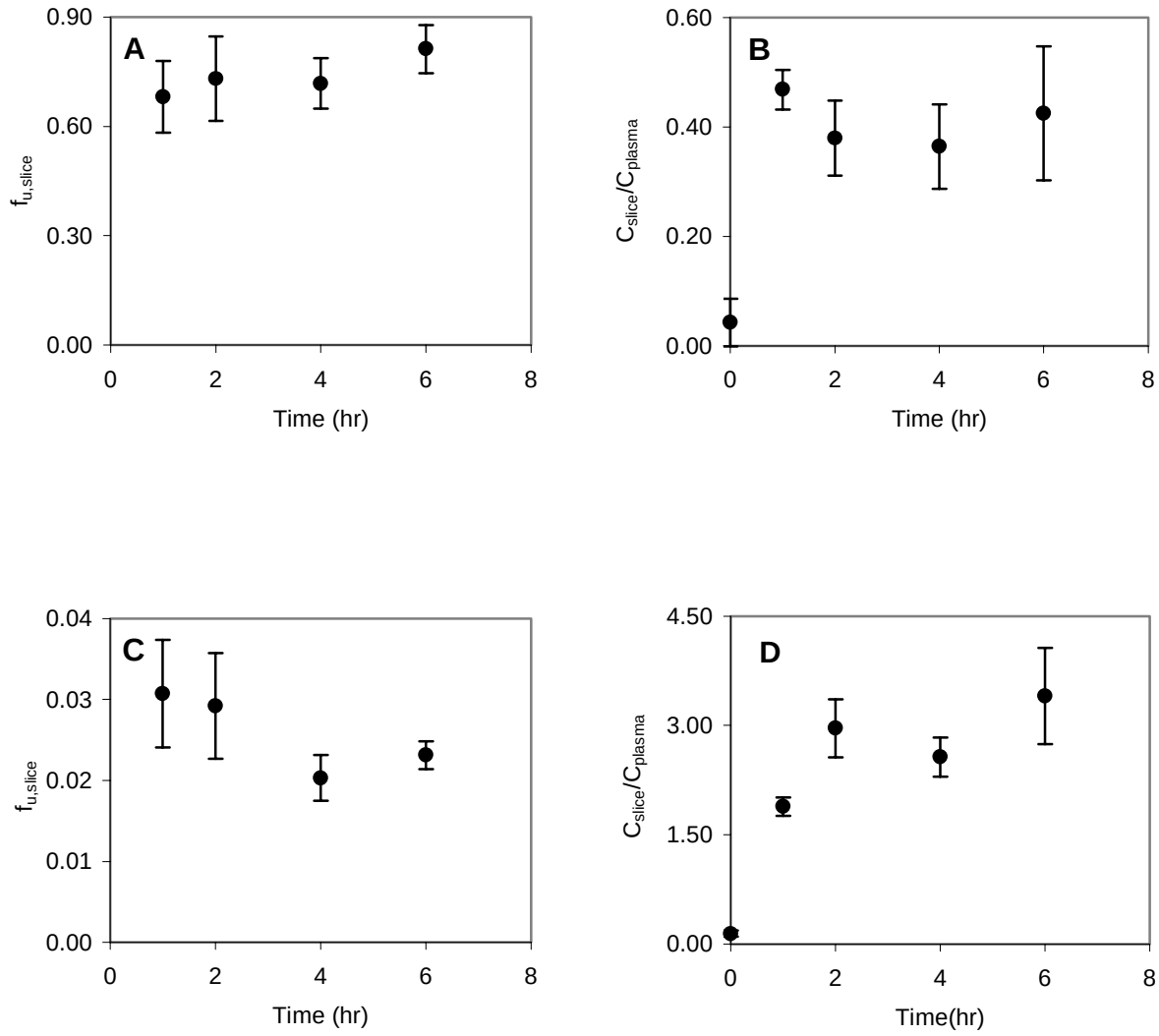

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Figure 2.

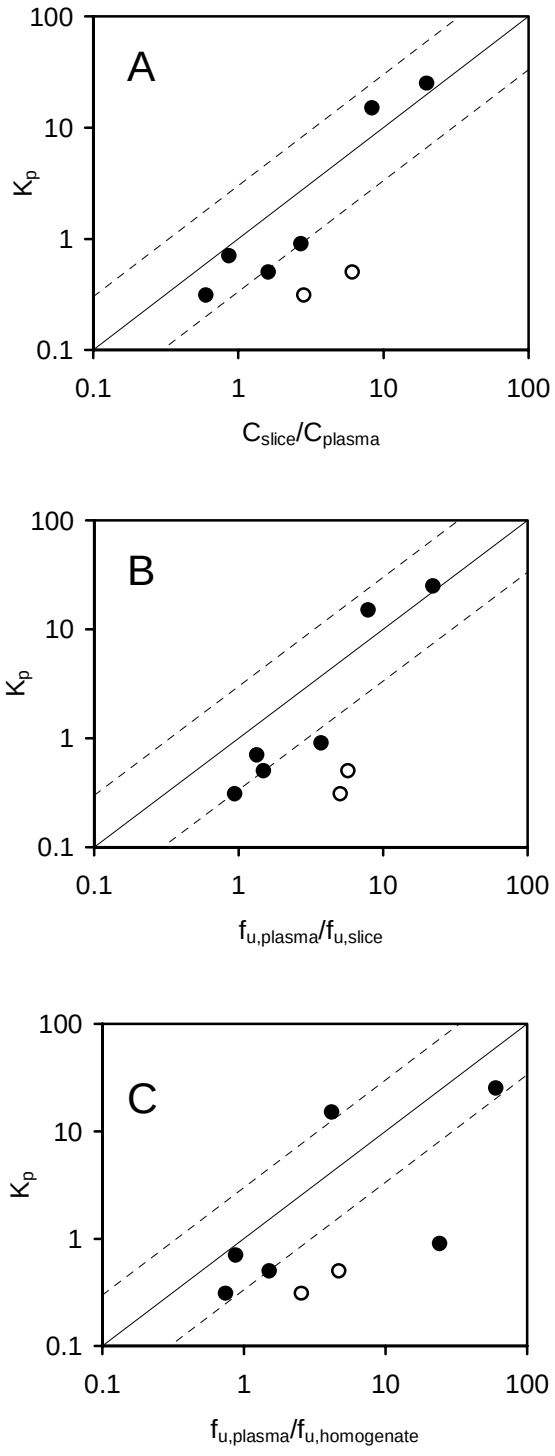

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Figure 3.

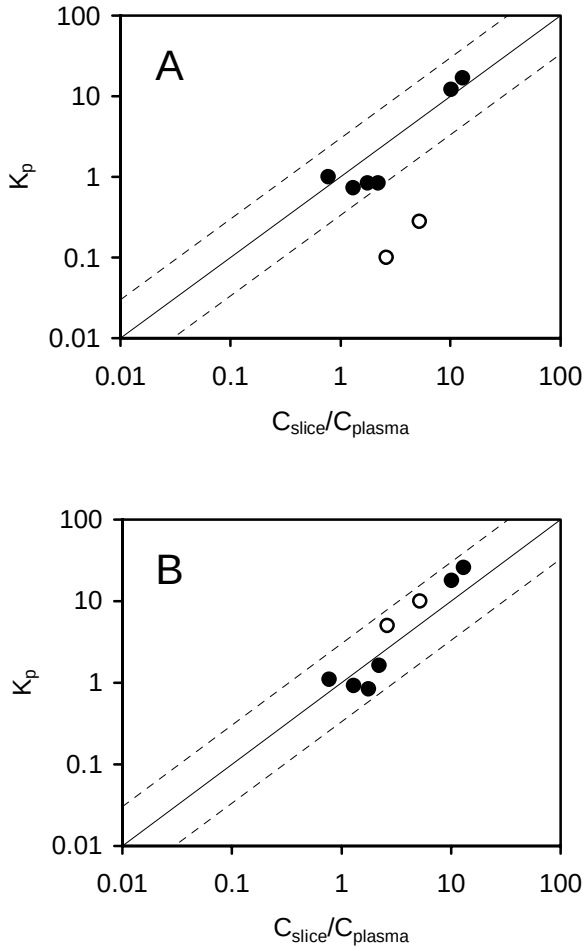

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Figure 4.