Measurement of Unbound Drug Exposure in Brain: Modeling of pH Partitioning Explains Diverging Results between the Brain Slice and Brain Homogenate Methods

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Received August 20, 2010; accepted December 13, 2010

ABSTRACT:
Currently used methodology for determining unbound drug exposure in brain combines measurement of the total drug concentration in the whole brain in vivo with estimation of brain tissue binding from one of two available in vitro methods: equilibrium dialysis of brain homogenate and the brain slice uptake method. This study of 56 compounds compares the fraction of unbound drug in brain (f_u,brain), determined using the brain homogenate method, with the unbound volume of distribution in brain (V_u,brain), determined using the brain slice method. Discrepancies were frequent and were primarily related to drug pH partitioning, attributable to the preservation of cellular structures in the slice that are absent in the homogenate. A mathematical model for pH partitioning into acidic intracellular compartments was derived to predict the slice V_u,brain from measurements of f_u,brain and drug pK_a.

This model allowed prediction of V_u,brain from f_u,brain within a 2.2-fold error range for 95% of the drugs compared with a 4.5-fold error range using the brain homogenate f_u,brain method alone. The greatest discrepancies between the methods occurred with compounds that are actively transported into brain cells, including gabapentin, metformin, and prototypic organic cation transporter substrates. It was concluded that intrabrain drug distribution is governed by several diverse mechanisms in addition to nonspecific binding and that the slice method is therefore more reliable than the homogenate method. An alternative, predictions of V_u,brain can be made from homogenate f_u,brain using the pH partition model presented, although this model does not take into consideration possible active brain cell uptake.

Introduction
Measurement of exposure of the brain to drugs is frequently undertaken in the drug discovery process to evaluate the influence of the blood-brain barrier (BBB) on the uptake of drugs and to evaluate central drug effects. The drug molecules that are not bound to brain tissue are the pharmacologically active entities, and the concentration of unbound drug in the brain interstitial fluid (C_u,brainISF) is the most important parameter for estimating brain exposure; the ratio of C_u,brainISF to plasma unbound drug concentrations is particularly useful. The brain/plasma ratio of unbound drug concentrations, known as K_p,free, provides an explicit value for the extent of BBB transport, i.e., brain exposure normalized to systemic exposure. Until recently, microdialysis has been the only method of measuring C_u,brainISF and K_p,free, but the associated technical challenges have precluded broad implementation of this method in the drug discovery process. In some instances, drug concentrations in cerebrospinal fluid have been measured as an alternative, but the validity of such measurements has been questioned. Instead, methods based on whole-brain concentrations have been used routinely, shaping much of the current information on the relationship between chemical structure and brain exposure and resulting in questionable predictions of drug effects. The brain slice (Kakee et al., 1996) and brain homogenate (Kalvass and Maurer, 2002; Mano et al., 2002) methods have recently been validated for measurement of the distribution or binding of drug within brain tissue (Fridén et al., 2007, 2009a; Liu et al., 2009). C_u,brainISF can be calculated by combining either in vitro method with conventionally measured total drug concentrations in whole brain in vivo. The implementation of these more high-throughput methodologies has now opened the possibility of guiding the design of drugs based on data relevant to brain exposure.

The brain slice method estimates the unbound volume of distribution in brain (V_u,brain milliliters · gram brain⁻¹), which quantifies the overall cellular uptake of drug; the value for V_u,brain will increase with
increases in the uptake and/or binding of the drug. The brain homog-
enate method, on the other hand, takes only nonspecific binding into
account and is used to determine the fraction of unbound drug in a
sample of homogenized tissue \( f_{u,\text{brain}} \). Although \( V_{u,\text{brain}} \) and \( f_{u,\text{brain}} \)
are inversely related and can be used for the same purpose, the brain
homogenate method is far more the commonly used method in the
drug industry. This is probably because of the ease of setting up the
assay and conducting the experiment. The slice method can also be
used in high-throughput processes (Fridén et al., 2009a), but it is
associated with higher initial costs and effort.

Although nonspecific binding is expected to dominate intrabrain
distribution, other processes are also involved. For example, one
important process involves the partitioning of weak acids and bases
along the pH gradient across cell membranes, which occurs because
uncharged species are more likely to passively diffuse through
membranes. This leads to an accumulation of basic drugs in cells
because the intracellular pH is lower than the extracellular pH. Per-
haps more important, basic drugs can be trapped in intracellular
subcompartments such as lysosomes, which can have a pH as low as
5 (de Duve et al., 1974; Daniel et al., 1995). Another important
process involves the carrier-mediated transport of drugs across mem-
branes. Although the role of drug transporters at the BBB is firmly
established, there is as yet little known about the impact of uptake and
efflux transporters on the intrabrain distribution of drugs. Cellular
uptake of, for example, gabapentin (Su et al., 1995) and 1-methyl-4-
phenylpyridinium (MPP) (Gorboulev et al., 1997; Gründemann et al.,
1998), is mediated by members of the solute carrier family of trans-
porters. Furthermore, efflux transporters belonging to the ABC super-
family such as P-glycoprotein (P-gp), breast cancer resistance-associated
protein (BCRP), and multidrug resistance protein 1 (MRP1) are ex-
pressed in brain parenchyma and may limit the cellular uptake of drugs
(Bleasby et al., 2006; Dallas et al., 2006). All in all, drug distribution
within brain tissue is far more complex than nonspecific binding.

The present study was undertaken to systematically investigate the
quantitative relationship between \( f_{u,\text{brain}} \) and \( V_{u,\text{brain}} \) obtained using the
two methods and to explain the differences between the methods
in terms of drug \( pK_a \) values, cellular pH gradients, and carrier-
mediated transport.

**Materials and Methods**

**Compound Selection.** The 56-compound set used in this study was an
extension of a previously used set of 43 physicochemically diverse drugs
belonging to five different therapeutic areas (Fridén et al., 2009b). The
additional 13 compounds were included mainly to address carrier-mediated
transport. The added compounds were basic and cationic compounds associated
with carrier-mediated cation transport; they included MPP, tetraethylenamo-
nium (TEA), neostigmine, metformin, amantadine, clonidine, three additional
substrates of P-gp (N-methylpyridinium, quinidine, and quinidine), two acidic
molecules (diclofenac and fluorescein), and two neutral drugs (alprazolam and
midazolam).

**Chemicals.** 2-Ethyl-2-phenylmalonamide was purchased from Acros Or-
ganics (Geel, Belgium), amitriptyline and thioridazine were purchased from ICN
Biomedicals (Eschwege, Germany), and delavirdine and gabapentin were purchased
from Toronto Research Chemicals Inc. (North York, ON, Canada). Morphin, morphine-3-glucuronide, morphine-6-glucuronide, oxycodone, and
oxyomorphine were obtained from Lipomed (Arlesheim, Switzerland). Sa-
cyclic acid and tramadol were obtained from Fluka BioChemika (Poole, UK),
omalactam and oxepenol were purchased from MP Biomedical (Illkirch,
France), fenspiride mesylate was purchased from Apin Chemicals (Abingdon,
UK), and \(^{14} \text{C} \)dimethylxalolazolinedione was purchased from American Ra-
diolarabeled Chemicals (St. Louis, MO). All other drugs and compounds were
purchased from Sigma-Aldrich (St. Louis, MO).

**Animals.** Male Sprague-Dawley rats (Harlan, Horst, The Netherlands,
weighing 300 to 400 g, were used for the in vitro brain slice experiments and
preparation of the brain homogenate. All animals were housed in groups at 18
to 22°C under a 12-h light/dark cycle with free access to food and water for at
least 5 days before the experiment. The study was approved by the Animal
Ethics Committee of Gothenburg (221-2008).

**Protocol for Brain Slice Experiments.** \( V_{u,\text{brain}} \) for the 56 compounds was
determined in fresh brain slices using a previously published protocol without
modifications (Fridén et al., 2009a). In brief, freshly prepared 300-μm brain
slices from drug-naive rats were incubated for 5 h in a buffer containing up to
10 drugs at very low concentrations (100 nM). Propranolol was included as a
quality control in each incubation. The unbound drug concentration in the slice
interstitial fluid was taken to be equal to the drug concentration in the buffer.

\[ V_{u,\text{brain}} = \frac{A_{\text{brain}}}{C_{\text{brain,dil}}} = \frac{A_{\text{brain}}}{C_{\text{buffer}}} \]  

**Protocol for Brain Homogenate Experiments.** Equilibrium dialysis of brain homogenate was performed as previously described (Wan et al., 2007) to
determine \( f_{u,\text{brain}} \) for the 56 compounds. The brain homogenate was prepared
in 3 volumes of 180 mM phosphate buffer (pH 7.4) using the same part of
the brain as that used for preparing brain slices. Equilibrium dialysis of 0.80 ml of
homogenate and buffer was performed in at least triplicate for 16 h at 37°C in
1-ml Plexiglas cells mounted with a 5-kDa cutoff cellulose membrane (Di-
anorm GmbH, München, Germany). Compounds were pooled at concentra-
tions of 5 μM with propranolol as a quality control. An aliquot of homogenate was
sampled before and after incubation to assess the compound stability.
The average fraction of unbound drug in diluted brain homogenate \( (f_{u,\text{brain}}) \), i.e.,
the buffer/homogenate concentration ratio, was calculated for the replicates
(eq. 2).

\[ f_{u,\text{brain}} = \frac{C_{\text{buffer}}}{C_{\text{homogenate}}} \]  

Next, \( f_{u,\text{brain}} \) was scaled to undiluted brain homogenate \( (f_{u,\text{brain}}) \) using eq. 3,

\[ f_{u,\text{brain}} = \frac{1}{1 + D \left( \frac{1}{f_{u,\text{brain}}} - 1 \right)} \]  

where \( D \) represents the dilution factor associated with preparation of the
homogenate.

**Brain Slice Experiments to Determine Distribution Mechanisms.** The contribution of lysosomal trapping to the \( V_{u,\text{brain}} \) of a prototypic weak base (propranolol) and a prototypic weak acid (indomethacin) was studied by incubation with various concentrations of monensin, which is an antibiotic
protonophore known to raise the lysosomal pH by facilitating the movement of
protons across membranes (Lake et al., 1987; Siebert et al., 2004). The extracellular pH, taken as the buffer pH, was measured using a pH electrode at the end of the incubation. The overall intracellular pH (\( \text{pH}_{i,\text{brain}} \)) was measured in each slice using the \(^{14} \text{C} \)dimethylxalolazolinedione method (Waddell and
Butler, 1959). The same experiment was also conducted for metformin and the
permanently cationic compounds, MPP, neostigmine, N-methylpyridinium, and
TEA. The contribution of carrier-mediated transport of the same cationic drugs
was assessed using decynium-22 (D22), which is a known inhibitor of organic
cation transporters (OCT). Propranolol was used as a control. Various con-
centrations of D22 were tested to ensure that results were obtained from
nontoxic conditions. The effect of drug efflux by P-gp on \( V_{u,\text{brain}} \) was assessed
for the P-gp substrates loperamide, saquinavir, nelfinavir, and
cyclosporine as an inhibitor (Wang et al., 2008). Fumit-

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were therefore conducted using more potent inhibitors and also shorter incubation times (3 h) to limit unspecific effects of the inhibitors.

**pK_a Measurements and Ion Classification.** Data on pK_a were obtained using a novel high-throughput assay based on capillary electrophoresis coupled to mass spectrometry (MS) (Wan et al., 2003; Wan and Ulander, 2006). In brief, the effective mobility of the compound was measured in a set of buffered pH solutions by capillary electrophoresis-MS, and the pK_a was obtained by nonlinear fit of the effective mobility as a function of pH using automated data analysis. Reference compounds were used as on-line quality controls, which provided accurate pK_a screening within 0.2 pK_a unit in most cases. Compounds were classified as acids, bases, zwitterions, neutrals, or permanent cations based on the predominant ionization state at pH 7.4. Hence, acids carry a net negative charge, bases carry a net positive charge (permanent cations disre- garded), neutrals may or may not have acidic or basic functions but are predominantly neutral at pH 7.4, and zwitterions carry at least one acidic and one basic function, which are ionized at pH 7.4.

**Analytical Procedures.** The drugs were quantified by reverse-phase liquid chromatography and multiple reaction monitoring mass spectrometry (liquid chromatography-tandem mass spectrometry) detection using Micromass (Waters, Milford, MA) triple-quadrupole instruments equipped with electrospray. As an alternative, a Waters Micromass LCT Premier TOF mass spectrometer (run with electrospray ionization and W-mode with extended dynamic range) was used for detection with the software MassLynx 4.0 and QuanLynx 4.0 for data acquisition and quantification, respectively. Gradient elution over 2 to 5 min with acetonitrile and 0.2% formic acid was used with various C18 columns. Sample preparation was adapted for any compound-specific requirements but followed a general procedure: samples of buffer and brain homog- enate were added in aliquots of 50 μl to 96-deepwell plates (Nalge Nunc International, Rochester, NY). Protein was precipitated by addition of 150 μl of cold acetonitrile containing 0.2% formic acid. After 1 min of vortexing and 20 min of centrifugation at 4000 rpm (Rotanta/TR; Hettich, Tuttlingen, Ger- many) at 4°C, the supernatant was transferred to a new plate and appropriately diluted with 0.2% formic acid. Samples of 5 to 20 μl from this plate were injected into the liquid chromatography-MS system.

Because absolute quantification of drug is not required in the homogenate (Wan et al., 2007) or slice (Fridén et al., 2009a) methods, chromatographic peak areas were used directly to calculate f_u,brain and V_u,brain, respectively. To ensure that the responses used in the calculations were within the linear range of the mass spectrometer, protein-precipitated samples were also diluted (10- and 100-fold) in 37.5% acetonitrile in 0.2% formic acid. These dilutions were analyzed with the undiluted samples. Any effects of nonlinearity in response were minimized by choosing an appropriate dilution (1-, 10-, or 100-fold) such that the peak areas for, for example, buffer and homogenate were of similar size for each compound. Before performing the calculations, the peak areas were scaled back to undiluted buffer and homog- enate by multiplying by the dilution factors 1, 10, and 100 as appropriate.

Radioactive [14C]dimethylxaloxalindione was quantified using a Wallac WinSpectral 1414 liquid scintillation counter (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland) and an OptiPhase HiSafe 3 scintillation cocktail (Fisher Scientific, Loughborough, UK). Homogenates of brain slices were solubilized with 1 ml of Soluene-350 (PerkinElmer Life and Analytical Sciences, Waltham, MA) and decolorized with 100 μl of hydrogen peroxide.

**Construction of pH Partition Models.** Models were derived to describe drug distribution in brain tissue compartments by pH partitioning. The primary aim was to obtain a predictive model for the unbound drug partitioning coefficient of the cell (K_p,u,cell), which describes the intracellular to extracel- lular unbound drug concentration ratio, where the intracellular unbound drug concentration (C_u,cell) is the average concentration of unbound drug in various intracellular compartments (eq. 4) (Fridén et al., 2007):

\[
K_{p,u,cell} = C_{u,cell} / C_{u,brain\text{ISF}} = f_{u,brain} \times V_{u,brain} \quad (4)
\]

If C_u,cell is equal to C_u,brainISF, then K_p,u,cell = 1. For such cases, binding in tissue is the only distribution mechanism, and it can be expected that f_{u,brain} is equal to 1/V_{u,brain}. However, because of pH partitioning or active transport, C_u,cell can be greater or smaller than C_u,brainISF, resulting in values for K_p,u,cell that differ from unity. Accordingly, K_p,u,cell not only describes intracellular exposure to unbound drug but also has a potential use as a conversion factor between the brain homogenate f_{u,brain} and brain slice V_{u,brain} methods (eq. 4).

The models were derived from the theory of pH partitioning (Shore et al., 1957), which assumes that membrane permeation is dominated by the nonion- ized species of the drug. The nonionized fraction of the drug is pH-dependent and can be predicted from the drug pK_a values using the Henderson-Hassel- balch equation. The concentration of nonionized drug is equal on both sides of a biological membrane at equilibrium. However, the gross drug concentration, including that of the ionized species, can differ widely according to the pH on each side.

The tissue compartments considered were the extracellular ISF, the cytosol of the intracellular space, and the acidic intracellular subcompartments, collectively denoted lysosomes (Fig. 1). Because it was not possible to obtain information on differential nonspecific binding in the various tissue compartments, it was necessary to assume a similar level of binding in all compartments.

The pH partition model for K_p,u,cell was constructed from its definition (eq. 4) by combining eq. 4 with the expression for V_u,brain (eq. 1) to get eq. 5:

\[
K_{p,u,cell} = f_{u,brain} \times \frac{A_{brain}}{C_{u,brain\text{ISF}}} \quad (5)
\]

A_{brain} is the sum of the amounts (A) of drug in each compartment, calculated as the physiological volume (V, milliliters · gram brain⁻¹) multiplied by the concentrations of unbound drug (C_u,brain millimolar concentration) divided by f_{u,brain} in each subcompartment, with subscripts brainISF, cyto, and lyso for brain ISF, the cell cytosol, and the lysosomes, respectively (eq. 6). The tissue density was assumed to be 1 g/ml:

\[
A_{brain} = A_{brainISF} + A_{braincyto} + A_{brainlyso} = V_{ISF} \times C_{u,brain\text{ISF}} + V_{cyto} \times C_{u,cyto} + V_{lyso} \times C_{u,lyso} / f_{u,brain} \quad (6)
\]

Combining eqs. 5 and 6 results in a three-compartment pH partition model (eq. 7; Fig. 1) in which f_{u,brain} is cancelled:

![Fig. 1. Schematic representation of the pH partitioning of acidic and basic drugs between the ISF of the extracellular space, the intracellular cytosol, and the acidic intracellular subcompartments (denoted lysosomes). The concentrations of the non- ionized species of acidic (HA) and basic (B) drugs are equal in all compartments at equilibrium and governed by the concentrations in ISF. HA and B are also in equilibrium with their ionized counterparts A⁻ and HB⁺, respectively, in each compartment. This equilibrium is shifted in acidic intracellular compartments (rich in protons, H⁺) toward the protonated forms HA and HB⁺, respectively, such that A⁻ is depleted and HB⁺ is elevated.](image-url)
\[ K_{p_{\text{um.cell}}} = V_{\text{ISF}} + K_{p_{\text{lyso}}} \times (V_{\text{cyto}} + V_{\text{hD}} \times K_{p_{\text{lyso}}}) \]  
where (eqs. 8 and 9)

\[ K_{p_{\text{um.lyso}}} = \frac{C_{u,\text{lyso}}}{C_{u,\text{brain}}/V_{\text{ISF}}} \]  

and

\[ K_{p_{\text{um.cyt}}} = \frac{C_{u,\text{cyto}}}{C_{u,\text{brain}}/V_{\text{ISF}}} \]  

As an alternative, a simpler two-compartment pH model was obtained by ignoring lysosome partitioning (eq. 10):

\[ K_{p_{\text{um.cyt}}} = V_{\text{ISF}} + K_{p_{\text{um.lyso}}} \times V_{\text{cyto}} \]  

\[ K_{p_{\text{um.cyt}}} \text{ and } K_{p_{\text{um.lyso}}} \] for weak acids or bases are predicted from the drug pKa using the pH partitioning theory, where pH_{\text{ISF}}, pH_{\text{cyto}}, and pH_{\text{lyso}} are the pH values for brain ISF, intracellular cytosol, and intracellular lysosomes (eqs. 11–14), respectively:

\[ K_{p_{\text{um.cyt}}} = 10^{p_{\text{Ka}} - p_{\text{u,lyso}}} + 1 \]  

\[ K_{p_{\text{um.lyso}}} = 10^{p_{\text{Ka}} - p_{\text{u,brain}}} + 1 \]  

\[ K_{p_{\text{um.cyt}}} = 10^{p_{\text{Ka}} - p_{\text{u,brain}}} + 1 \]  

\[ K_{p_{\text{um.lyso}}} = 10^{p_{\text{Ka}} - p_{\text{u,brain}}} + 1 \]  

\[ K_{p_{\text{um.lyso}}} = 10^{p_{\text{Ka}} - p_{\text{u,brain}}} + 1 \]  

\[ K_{p_{\text{um.cyt}}} = 10^{p_{\text{Ka}} - p_{\text{u,brain}}} + 1 \]  

Twisternionic drugs have both acidic and basic functions. K_{p_{\text{um.cyt}}} and K_{p_{\text{um.lyso}}} were calculated as the product of the K_{p_{\text{um}}} values associated with the respective acidic/basic function. Values for K_{p_{\text{um}}} were similarly calculated for drugs with several acidic or basic functions (MacIntyre and Cutler, 1988). Accordingly, there is a separate model equation for each class of drugs. These equations are not given here because of space restrictions. However, a combined model equation that accommodates up to two acidic and two basic functions is provided as an Excel template spreadsheet (Supplemental Table 1).

Modeling of K_{p_{\text{um.cell}}}—By assigning literature values to the parameters of the pH partition models, it is in principle possible to use the models directly to predict K_{p_{\text{um.cell}}} for conversion of f_{u,brain} to V_{u,brain}. However, the strategy chosen was to “fine-tune” the model to provide the optimal prediction of K_{p_{\text{um.cell}}}. This was done by fitting the models to experimental data on K_{p_{\text{um.cell}}}—by including pH as a parameter in the model and thus estimating pH in the regression analysis. Modeling of the data also enabled an investigation of whether it is necessary to take into account partitioning into subcellular compartments (three-compartment model, eq. 7) or whether a simpler model with a single intracellular compartment is sufficient (two-compartment model, eq. 10). Although pH_{\text{cyto}} and pH_{\text{hD}} were the estimated parameters, fixed values were used for all other model parameters. The pH_{\text{ISF}} was set as 7.30, which is reported to be the physiological value (Davson and Segal, 1996) and is the same as the buffer pH in the slice method. V_{\text{ISF}} was taken as the physiological value 0.20 ml g \text{ brain}^{-1} (Nicholson and Sykova, 1998) and 0.01 ml g \text{ brain}^{-1} was used for V_{\text{hD}} rounded up from a value of 0.0068 reported for the liver (Weibel et al., 1969) because values were not available for brain tissue and because the cell contains acidic compartments in addition to lysosomes. Values of 0.79 and 0.80 ml g \text{ brain}^{-1} were used for V_{\text{cyto}} in the three-compartment and two-compartment models, respectively. Physiological values were used as initial estimates for pH_{\text{cyto}} (7.02) (Friden et al., 2009a) and for pH_{\text{hD}} (5.0) (Ohkuma and Poole, 1978).

Experimental data for K_{p_{\text{um.cell}}} were included for drugs with a single acidic or basic function and for one drug with two acidic functions (moxalactam). This was to ensure that the pH partition model was developed based only on compounds for which K_{p_{\text{um.cell}}} is primarily determined by pH partitioning. Some compounds were not included in the model. The pK_{a} of the basic function of metformin was too high to quantify with the methods used (see measurement of pK_{a}). Carrier-mediated transport of metformin was also suspected, based on prior information (Kimura et al., 2005) and the deviating value of its K_{p_{\text{um.cell}}}. Sulfasalazine had the lowest pK_{a} (2.2) among the acids. It was considered an outlier because of the experimental value for K_{p_{\text{um.cell}}}. Permanently cationic compounds and neutral drugs were not included because these are thought not to be influenced by pH partitioning. Twisternionic drugs were similarly excluded, although minor effects are predicted depending on the pK_{a} values. Compounds that were not used in the modeling are indicated by footnotes in Table 1.

For each of the two general models (the two- and three-compartment models), three functions were included in the construction of the regression model (WinNonlin version 3.5) representing the pH partitioning of 1) monoprotic acids, 2) monoprotic bases, and 3) the diproic acid moxalactam. Simultaneous fitting of the three functions to the data results in a single set of parameter estimates for pH_{\text{cyto}} and pH_{\text{hD}} (three-compartment model) or a single value for pH_{\text{hD}} (two-compartment model). Goodness of fit was evaluated using the WinNonlin estimated precision of parameter estimates (coefficient of variation) and residual plot analysis. The two- and three-compartment models were compared by additionally calculating the Akaike information criterion (Akaike, 1974).

Data Presentation and Statistical Analysis—Aggregated K_{p_{\text{um.cell}}} values for compound classes are presented as means ± S.D. Values for V_{u,\text{brain}} and f_{u,\text{brain}} of single compounds are presented as means (S.E.). The S.E. for f_{u,\text{brain}} was calculated for the six replicate slices. Because f_{u,\text{brain}} is a single value that is scaled from the three to five replicate measurements of f_{u,\text{hD}}, it was necessary to use the propagation of error method (Kendall et al., 1987) to estimate S.E. for f_{u,\text{brain}}. This statistical approach estimates the S.E. of f_{u,\text{brain}} as a function of f_{u,\text{hD}} (eq. 15), using

\[ \text{S.E.} f_{u,\text{brain}} = \sqrt{\frac{(\text{S.E.} f_{u,\text{hD}})^2}{f_{u,\text{hD}}}} \]  

where S.E.f_{u,\text{hD}} and S.E.f_{u,\text{hD}} are the S.E.s of the function f_{u,\text{brain}} and the measurement f_{u,\text{hD}}. \text{S.E.} f_{u,\text{hD}} is the (partial) derivative of f_{u,\text{brain}} with regard to f_{u,\text{hD}}; i.e., \text{S.E.} f_{u,\text{hD}} = \frac{\partial f_{u,\text{brain}}}{\partial f_{u,\text{hD}}} = D'(D - (1 - D) \cdot f_{u,\text{brain}}^2), where D is the homogenous dilution factor.

The agreement of observed brain slice V_{u,\text{brain}} values with predictions from f_{u,\text{brain}} with or without using the pH partition model was evaluated according to the method of Altman and Bland (Bland and Altman, 1999). Because experimental variability appeared to be proportional to V_{u,\text{brain}}, i.e., the coefficient of variation was similar for compounds with very different V_{u,\text{brain}} values, log transformation of data was performed. The significance of a mean difference (bias) was tested with Student’s t test. The agreement between predicted and observed V_{u,\text{brain}} values was expressed as the 95% confidence interval ratio (CIR) around the observed mean difference (bias), which was calculated using the t distribution. Owing to the log transformation of data, the 95% confidence interval ranges from the mean difference divided by the 95% CIR to the mean difference multiplied by the 95% CIR.

Results—Overall Comparison of Brain Slice V_{u,\text{brain}} and Homogonate f_{u,\text{brain}}—Brain slice V_{u,\text{brain}} and brain homogonate f_{u,\text{brain}} were inversely correlated (r^2 = 0.78) and 1/f_{u,\text{brain}} was within a 3-fold range of V_{u,\text{brain}} for 41 of the 56 compounds (Fig. 2A; Table 1). However, there were several compounds, including those subject to carrier-mediated transport, for which V_{u,\text{brain}} was greatly underpredicted by 1/f_{u,\text{brain}}. The K_{p_{\text{um.cell}}} factor, which provides the differences between the slice and homogonate methods for a drug (eq. 4), was very high for MPP (77), metformin (9.5), TEA (9.0), paclitaxel (9.0), mitoxantrone (8.0), and gabapentin (4.6) (Fig. 2B). The K_{p_{\text{um.cell}}} results were clustered around 2.7 ± 0.6 for basic drugs carrying a net positive charge at pH 7.4 (metformin excepted). Neutral drugs (paclitaxel excepted) and basic drugs that are predominantly neutral at pH 7.4 showed the smallest discrepancies between the methods (K_{p_{\text{um.cell}}} = 0.99 ± 0.45). K_{p_{\text{um.cell}}} values for acidic drugs carrying a net negative charge at pH 7.4 were lower than unity (0.57 ± 0.18). After the acidic
sulfsalazine ($K_{pu,cell} = 0.27$), moxalactam with two acidic functions had the lowest $K_{pu,cell}$ (0.46) in our dataset. $K_{pu,cell}$ values for zwitterions (gabapentin and mitoxantrone excluded) were close to unity (1.1 ± 0.47). Apart from MPP and TEA, as mentioned above, $K_{pu,cell}$ values for the permanently cationic compounds neostigmine and N-methylquinidine were 3.1 and 1.1, respectively.

The reproducibility of the results for the substantially bound quality control drug, propranolol, was slightly better for the slice than for the homogenate method, as determined by the coefficient of variation (8 and 12%, respectively). For the whole dataset, it was observed that the slice method was consistently more precise than the homogenate method, irrespective of the degree of binding. In addition, the homog-
enate binding method was less precise at lower or no degrees of binding than at higher degrees of binding. The lower precision at low binding levels was shown statistically to result from the need to correct for brain homogenate dilution (eqs. 3 and 15) (Fig. 3B).

Modeling of pH Partitioning. The clustering of \( K_{p,\text{uu,cell}} \) values for drugs belonging to the same ion class, e.g., basic drugs, strongly suggested the involvement of pH partitioning in intrabrain distribution. The two pH partition models were fitted to the values of \( K_{p,\text{uu,cell}} \) and \( K_p \) by estimation of intracellular pHcyto alone (two-compartment model) or both pHcyto and pHlyso (three-compartment model). The three-compartment model described the data better than the two-compartment model as judged by the Akaike information criterion (5.06–5.29). Hence, it was necessary and appropriate to include the lysosomal subcompartment. The fit of the three-compartment model is shown in Fig. 4. The estimates (95% confidence intervals) for pHcyto and pHlyso were 7.06 (6.97–7.15) and 5.18 (5.06–5.29).

With use of the three-compartment model for \( K_{p,\text{uu,cell}} \), \( V_u,\text{brain} \) was predicted from \( f_u,\text{brain} \) for all except the anticancer drugs (paclitaxel and mitoxantrone) and the permanently cationic drugs (Fig. 5). The 95% CIR for the agreement between observed and predicted \( V_u,\text{brain} \) was 2.2 with no bias. This is to say that 95% of future predictions will fall within a 2.2-fold range of the observed values. Prediction of \( V_u,\text{brain} \) by assuming that it equalled \( 1/f_u,\text{brain} \) resulted in a bias of 0.64-fold and a 4.5-fold 95% CIR. Hence, prediction of brain slice \( V_u,\text{brain} \) using the homogenate \( 1/f_u,\text{brain} \) is associated with a 95% confidence interval between a 7.1-fold underprediction and a 2.9-fold overprediction.

Effect of Lysosomal Inhibition. To challenge the pH partition model, indomethacin (acid) and propranolol (base) were studied in brain slices, whereas the lysosomal pH gradient was inhibited using increasing concentrations of monensin. There was slight acidification of the incubation buffer at the two higher monensin concentrations (pH 7.16 and 7.02 for 5 and 50 nM monensin, respectively, versus pH 7.28 for lower monensin concentrations and the control). There was no effect on the intra- to extracellular pH gradient (~0.2 pH unit) except at 50 nM monensin, for which the gradient was reduced to 0.08 pH units. \( K_{p,\text{uu,cell}} \) for propranolol was reduced in a concentration-dependent manner across the whole range of monensin concentrations (50 pM–50 nM), and it was almost completely reduced to unity at the highest monensin concentrations (5 and 50 nM) (Fig. 6). To assess the extent of lysosomal inhibition, the observed values for \( K_{p,\text{uu,cell}} \) were compared with values predicted from the three-compartment model, with measured values for pHcyto and pHlyso incorporated under the assumption of complete lysosomal inhibition (pHcyto = pHlyso). The agreement of predicted and observed propranolol \( K_{p,\text{uu,cell}} \) at 5 and 50 nM monensin (Fig. 6) suggests that lysosomal trapping is nearly abolished at these concentrations. The effect of monensin on indomethacin was different; there was no observed effect at any concentrations except the highest (50 nM). These results were consistent with the three-compartment pH partition model, which predicts that lysosomal inhibition will have no effect on the \( K_{p,\text{uu,cell}} \) values of acidic drugs unless the overall pH gradient is also altered.

Inhibition of Carrier-Mediated Organic Cation Transport. Inhibition of potential carrier-mediated uptake was studied for metformin and all permanently cationic drugs (MPP, TEA, neostigmine, and N-methylquinidine) using D22 concentrations ranging from 4 nM to 1 μM. Acidification of buffer and the intracellular space was seen at higher concentrations, and the slices were significantly more swollen than control slices at 1 μM D22. There was little or no indication that the intracellular to extracellular pH gradient was altered by D22. \( K_{p,\text{uu,cell}} \) was reduced for all compounds at 1 μM D22 (Fig. 7A). The effect was greatest for MPP (18-fold) and TEA (7-fold) followed by 2-fold reductions for metformin, neostigmine, and N-methylquinidine. The transport of propranolol, which is supposedly not carrier-mediated and was thus acting as a control, was reduced by a factor of 1.5. MPP behaved differently from the other compounds in that its much

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**Figure 2.** Relationship between (A) \( 1/f_u,\text{brain} \) determined using the brain homogenate method and \( V_u,\text{brain} \) determined using the slice method and (B) \( K_{p,\text{umu,cell}} \) plotted against \( 1/f_u,\text{brain} \). In A, the solid and dashed lines represent agreement and a 3-fold difference. In B, the solid and dashed lines represent \( K_{p,\text{umu,cell}} = 1, 3, \) and 1/3, respectively. Compounds are classified as acids, bases, zwitterions, neutrals, and permanent cations on the basis of the predominant ionization state at pH 7.4.

**Figure 3.** Plot of the relative standard error (RSE) for (A) \( f_u,\text{brain} \) (homogenate method) and \( V_u,\text{brain} \) (slice method) versus \( f_u,\text{brain} \) and (B) simulated RSE for \( f_u,\text{brain} \), given an arbitrary 5% RSE for \( f_u,\text{brain} \), showing an estimated increase in RSE of the homogenate method with increasing \( f_u,\text{brain} \).
higher $K_{p,uu,cell}$ was also substantially reduced even at 0.2 µM D22 and that $K_{p,uu,cell}$ was slightly elevated at the lowest concentrations.

Although the steady-state value for $V_{u,brain}$ and hence for $K_{p,uu,cell}$ is generally expected to be reached after 5 h of incubation (Fridén et al., 2009a), it was seen for MPP, neostigmine, and N-methylquinidine (TEA was not studied) that $V_{u,brain}$ increased almost linearly from 1 to 5 h (data not shown). Hence, values of $K_{p,uu,cell}$ for cationic drugs are not necessarily seen as steady-state values in this study.

**Effect of Lysosomal Inhibition for Permanent Cations.** There was no apparent effect of lysosomal inhibition by monensin on the uptake of metformin, N-methylquinidine, neostigmine, or TEA. In contrast, the uptake of MPP was inhibited by monensin at concentrations similar to those required for inhibition of lysosomal trapping of propranolol (Fig. 7B). Inhibition of MPP uptake by monensin was more potent than that by D22.

**Effect of Active Efflux Inhibition.** The $V_{u,brain}$ values of the P-gp substrates loperamide, saquinavir, nelfinavir, and N-methylquinidine were not increased by the presence of 10 µM cyclosperine (Fig. 8). Rather, cellular uptake of nelfinavir was reduced by cyclosperine. The $V_{u,brain}$ values of the BCRP substrate mitoxantrone and the dual BCRP/MRP substrate sulfasalazine were also not increased in the presence of 10 µM fumitremorgin C, 20 µM MK-571, or both 10 µM fumitremorgin C and 20 µM MK-571.

**Discussion**

The distribution of drugs by cellular uptake within the brain can be studied in brain slices or in homogenized brain tissue. The primary interest in estimates of intrabrain distribution is their utility for converting measured total brain concentrations in vivo to pharmacologically active unbound drug concentrations. Because the brain slice and brain homogenate methods are currently being used interchangeably for this purpose, the present study sought to identify, quantify, and rationalize discrepancies between the methods in a dataset of 56 chemically diverse compounds exhibiting various mechanisms of distribution. Given the preserved cellular structure and viability of the brain slices, it is reasonable to assume that the brain slice $V_{u,brain}$ gives more information relevant to the in vivo situation than the homogenate method, which only measures nonspecific binding. This finding was previously indicated in a study comparing results from the two methods with in vivo microdialysis results (Fridén et al., 2007).

Nevertheless, the correlation ($r^2 = 0.78$) between $V_{u,brain}$ and $V_{f,brain}$ (Fig. 2) suggests that nonspecific binding contributes as much as 78% to the variations of $V_{u,brain}$ between drugs. The present study highlights another influential mechanism of drug distribution, namely pH partitioning. Much of the observed discrepancies between the methods were rationalized by the pH partition model, which accommodates the volumes and pH of the extra- and intracellular spaces and the intracellular lysosomal subcompartment. The model, which predicts $K_{p,uu,cell}$ clearly explains how basic and acidic drugs accumulate in cells to a greater and lesser extent, respectively, than would be expected from nonspecific binding. The model appears also to be applicable to compounds with more than a single basic or acidic function, because it was able to predict the very low value of $K_{p,uu,cell}$ for the diprotic acid oxalacacetam (Fig. 4).

Significant error is associated with using $V_{f,brain}$ and thereby assuming equivalence of the brain slice and homogenate methods ($V_{f,brain} = 1/V_{u,brain}$); the 95% confidence interval ranged from a 7.1-fold underprediction to a 2.9-fold overprediction. In contrast, using the pH partition model with drug $pK_a$ values, it is possible to predict $V_{u,brain}$ with a 95% confidence interval ranging between 2.2-fold under- and overprediction. Although it is preferable to measure the $pK_a$ values, it...
is recognized that the model is rather insensitive to \(pK_a\) in the expected ranges for common carboxylic acids (\(pK_a < 6\)) and amines (\(pK_a > 8\)) (Fig. 4). For many drugs, it is therefore sufficient to use default \(K_{puu,cell}\) values: \(\sim 3\) for amines, \(\sim 0.6\) for carboxylic acids, and \(1\) for neutral drugs or zwitterions.

The estimated pH values for lysosomes (\(pH_{lyso} = 5.2\)) and cytosol (\(pH_{cyto} = 7.1\)) were very similar to the physiological values and experimental measurements that were used as initial estimates. However, because all acidic subcompartments have been included in the lysosomal compartment, the estimated pH values should be seen primarily as parameters describing drug distribution. The impact of pH partitioning on overall distribution was greater for basic drugs than for acidic and, obviously, neutral drugs. Because of the large pH difference (\(\sim 2\) units) between the cytosol and the lysosomes, the concentration of basic drugs is approximately 100 times higher in lysosomes than in the cytosol. This means that lysosomes represent nearly 50% of the total intracellular drug content despite their very small volume (\(\sim 1%\) of the cell). In contrast, the lysosomal exclusion of acidic drugs has negligible impact on their overall distribution. The much smaller overall intracellular to extracellular pH gradient affects basic and acidic drugs to a similar extent but in opposite directions. Zwitterionic drugs are predicted to behave like neutral drugs, with small deviations depending on the exact \(pK_a\) of the acidic or basic function. Moreover, the effect of lysosomal trapping, which is predicted from the model, was found to be in line with results from experiments in which the lysosomal pH gradient was inhibited. The central role of pH partitioning in drug distribution, which has been demonstrated in this study in vitro, can be expected to translate to the in vivo situation. Early studies supporting this view showed that the in vivo tissue partition coefficients of various organs cannot be predicted for basic drugs using data from tissue homogenates (Harashima et al., 1984; Schuhmann et al., 1987).

The highest observed values for \(K_{puu,cell}\) were not predicted by the pH partition model. One of the compounds with a high \(K_{puu,cell}\) was the zwitterionic gabapentin (\(K_{puu,cell} = 4.5\)), which, although it was not bound nonspecifically at all \((f_{u,brain} = 1)\), had a \(V_{u,brain}\) of 4.5, indicating carrier-mediated active uptake. Gabapentin is known to be taken up into brain cells by the LAT1 transporter (Su et al., 1995). It is intriguing that the high \(K_{puu,cell}\) indicates active uptake rather than facilitated uptake, which is the mode of transport for LAT1. Whatever the mechanism for gabapentin uptake, the brain slice \(V_{u,brain}\) is very close to the value observed in vivo (5.5 ml · g brain\(^{-1}\)) (Wang and Welty, 1996).

The greatest discrepancy between the methods was seen for the permanently cationic prototypic OCT substrate MPP (\(K_{puu,cell} = 77\)), which is also known to be taken up by the dopamine transporter in nigrostriatal nerve end terminals. The slice uptake of MPP was potentially inhibited by the OCT inhibitor D22 but even more potently inhibited by the lysosomal inhibitor monensin. This finding could be explained by the involvement of facilitated transport by OCT, secondary active transport by a proton antiporter, or effects on the sodium-driven dopamine transporter. The \(K_{puu,cell}\) values for the other two model OCT substrates (metformin and TEA) were reduced by 1 \(\mu\)M D22 but not by monensin, suggesting no involvement of
proton-coupled transport. As for LAT1, there should be no net transport of drug against an electrochemical gradient by the facilitating mode of OCT transport. However, TEA and MPP are different from gabapentin in that they carry a permanent positive charge and are thus subject to the electric potential of the cell membrane. The Nernst equation predicts that a membrane potential of $-60 \text{ mV}$ results in an equilibrium chemical concentration gradient of $1 \times 10^5$. Compared with most basic drugs, metformin may be particularly influenced by the electric potential, because its exceptionally high basic $pK_a$ makes it practically permanently charged.

There was no indication in the data that drug efflux by ATP-binding cassette transporters in the parenchyma plays a significant role in limiting uptake into cells. The well characterized P-gp substrate loperamide seemed to be distributed intracellularly just like any other basic drug and $K_{p,\text{out,cell}}$ was not increased by inhibition of P-gp. $V_{u,\text{brain}}$ of nelfinavir was unexpectedly reduced by cyclosporine by an unidentified mechanism. Furthermore, there were no indications of drug efflux of the BCRP and MRP substrates mitoxantrone and sulfasalazine, there was no effect on mitoxantrone, and $V_{u,\text{brain}}$ of sulfasalazine was paradoxically reduced. It seems likely that the expression level of ATP-binding cassette transporters in brain parenchyma is insufficient to produce significant efflux.

One important mechanism of distribution that has not been considered so far involves specific binding to the target protein of the drug. The contribution of specific binding to the overall distribution can be anticipated to be highly dependent on the level of expression of the target protein. It is speculated that the very large $K_{p,\text{out,cell}}$ for the two anticancer drugs paclitaxel (9.0) and mitoxantrone (8.0) could be somehow related to specific binding to tubulin and DNA, respectively, which exist at much higher molar concentrations than most target receptors, enzymes, and transporters. In fact, accumulation of paclitaxel in platelets is known to be saturable (Wild et al., 1995).

The pharmacological significance of intrabrain and intracellular distribution is currently not as clear as that of BBB transport. However, the concepts discussed in the present article are relevant to intracellular drug targeting not only in brain but also in other organs; for the same unbound drug concentration in the extracellular fluid, the intracellular exposure will be slightly higher for basic drugs and lower for acidic drugs. Furthermore, by measuring both nonspecific binding (homogenate) and overall uptake (slice), the calculated $K_{p,\text{out,cell}}$ may provide insight into the intracellular exposure to unbound drug and the involvement of active uptake.

In conclusion, drug distribution within brain tissue occurs by a variety of processes including nonspecific and specific binding, pH partitioning across the cell membrane and the membranes of intracellular lysosomes, and carrier-mediated uptake driven by chemical gradients, membrane potential, and possibly pH gradients. It was also indirectly demonstrated that these physiological mechanisms are intact in the brain slices and that the slice method therefore has the potential to capture these mechanisms when intrabrain drug distribution is estimated. In addition to being closer to the in vivo situation and therefore providing more accurate values of intrabrain distribution, the data from the slice method were also associated with higher and more consistent precision for both extensively and poorly binding drugs. If the homogenate method is used as an alternative for estimation of intrabrain drug distribution, the bias associated with acids and bases should be corrected for by using the proposed pH partition model for $K_{p,\text{out,cell}}$. This correction is performed by dividing the calculated $K_{p,\text{out,cell}}$ by the measured value of $f_{u,\text{brain}}$, yielding an estimated value for $V_{u,\text{brain}}$ or equivalently, by dividing $f_{u,\text{brain}}$ by $K_{p,\text{out,cell}}$, yielding a value for $f_{u,\text{brain}}$ that is more relevant to the in vivo situation.
Authorship Contributions

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