In Vitro Methods for Estimating Unbound Drug Concentrations in the Brain Interstitial and Intracellular Fluids

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ABSTRACT:
Concentrations of unbound drug in the interstitial fluid of the brain are not rapidly measured in vivo. Therefore, measurement of total drug levels, i.e., the amount of drug per gram of brain, has been a common but unhelpful practice in drug discovery programs relating to central drug effects. This study was designed to evaluate in vitro techniques for faster estimation of unbound drug concentrations. The parameter that relates the total drug level and the unbound interstitial fluid concentration is the unbound volume of distribution in the brain (V_{u,brain}). It was measured in vitro for 15 drugs using brain slice uptake and brain homogenate binding methods. The results were validated in vivo by comparison with V_{u,brain} microdialysis results. The slice method results were within a 3-fold range of the in vivo results for all but one compound, suggesting that this method could be used in combination with total drug levels to estimate unbound interstitial fluid concentrations within reasonable limits. Although successful in 10 of 15 cases, the brain homogenate binding method failed to estimate the V_{u,brain} of drugs that reside predominantly in the interstitial space or compounds that are accumulated intracellularly. Use of the simple methods described in this article will 1) allow quantification of active transport at the blood-brain barrier in vivo, 2) facilitate the establishment of a relationship between in vitro potency and in vivo activity for compounds acting on central nervous system targets, and 3) provide information on intracellular concentrations of unbound drug.

Determination of drug levels in the brain tissue of experimental animals is routinely undertaken in drug discovery programs for various purposes, including studies on blood-brain barrier (BBB) transport and equilibration. Drug levels are also studied in conjunction with pharmacodynamic experiments to link in vivo effects with in vitro potency, or to elucidate the mechanism and site of action. The practical approach to routinely investigating large numbers of new compounds has been to measure the amount of drug in brain (A_{brain}), which is given in amount per gram of brain and therefore is commonly referred to as the total brain concentration.

Because assessment of A_{brain} has historically been the most common method of measuring CNS exposure in drug discovery, medicinal chemistry programs have favored compounds and classes displaying high total CNS-to-plasma concentration ratios. This type of data, expressed as “log BB”, has resulted in the establishment of general criteria for physicochemical properties of compounds with potentially high or low CNS exposure (Kelder et al., 1999). Although this method has the advantage of experimental simplicity, the use of total tissue levels (A_{brain}) is also associated with limitations. It is generally accepted that it is the unbound drug that exerts the effect on the receptor. Large amounts of drug in the brain do not necessarily mean high concentrations available to the receptor, since the drug may bind to or dissolve in tissue components. Measurements of A_{brain} alone can thus be very misleading. Similarly, a high brain-to-plasma ratio based on measurements of A_{brain} may be reflective of extensive binding to brain tissue rather than of unrestrained transport across the BBB.

According to the free drug hypothesis, the unbound drug concentration in tissue is equal to the unbound drug concentration in plasma at equilibrium. This may not be the case for brain tissue since there are active efflux and influx processes at the BBB. Cerebrospinal fluid (CSF) drug concentrations are potentially more closely related to the concentrations of unbound drug in brain interstitial fluid (C_{u,brainISF}), because of the separation from blood by the blood-CSF barrier. Also, the ependymal lining of the ventricles allows diffusional and convectional exchange with the brain interstitium (Abbott, 2004; Liu et al., 2006). However, the CSF represents a different compartment and the turnover of CSF is different from that of brain ISF (Abbott, 2004). Investigations have demonstrated that drug concentrations in the CSF are not necessarily equal to those in brain ISF (de Lange and Danhof, 2006).
2002; Shen et al., 2004). The only method of directly measuring $C_{u,brain}$ is microdialysis. Unfortunately, the utility of this method in drug discovery programs is limited by the time requirements and by specific technical difficulties with lipophilic drugs.

Wang and Welty (1996) introduced the unbound volume of distribution in the brain ($V_{u,brain}$) to relate $C_{u,brain}$ to $A_{brain}$ where $V_{blood} \times C_{blood}$ is the amount of drug present in the blood vessels of the brain:

$$V_{u,brain} (MD) = \frac{A_{brain} - V_{blood} \times C_{blood}}{C_{u,brain}}$$

Thus, $V_{u,brain}$ describes the distribution of drug inside the brain regardless of brain-to-plasma distribution. A low value for $V_{u,brain}$ close to the volume of the interstitial space, would thus describe predominantly extracellular distribution, whereas a high value would indicate that the drug enters brain cells and binds to tissue components (Gupta et al., 2006).

If the value of $V_{u,brain}$ can be reliably obtained for a compound, $C_{u,brain}$ can be calculated from available total drug levels, thus circumventing the need for microdialysis. Methods other than microdialysis that have been used for estimating $V_{u,brain}$ include the brain slice uptake technique (Kakee et al., 1996) and the brain homogenate binding method (Kalvass and Maurer, 2002; Mano et al., 2002). Recent workers have used the fraction unbound in brain, $f_{u,brain}$, to describe much the same property (Becker and Liu, 2002; Liu et al., 2006). The fraction unbound in brain, like the fraction unbound in plasma, is an easily understood concept. However, it shares the limitation of the homogenate method from which it originates; there is no distinction made between intra- and extracellular distribution.

The present study aims at evaluating methods for $C_{u,brain}$ estimation to guide industrial drug discovery programs or academic research related to CNS drug exposure. The $V_{u,brain}$ concept is used as a link between the total brain concentration ($A_{brain}$) and the pharmacologically active unbound brain ISF concentration ($C_{u,brain}$). Along with a characterization of the methods, we present the first comprehensive comparison of in vitro $V_{u,brain}$ data and in vivo microdialysis measurements. We also discuss how the integrative use of these $V_{u,brain}$ methods paves the way for estimation of intracellular unbound drug concentrations.

### Materials and Methods

#### Compound Selection

The literature was searched for microdialysis reports containing both unbound and total brain drug concentrations, i.e., the data needed to calculate $V_{u,brain}$. Studies that were performed using probe calibration in vivo by retrodialysis were favored. Nearly every compound that fulfilled the criteria was included in the study. The set of 14 compounds listed in Table 1 is pharmacologically diverse, including opioids and their metabolites (morphine, codeine, oxycodone, morphine-3-glucuronide, morphine-6-glucuronide), anti-infectives (aludovine, norfloxacin), anticholinergics (R- and S-cetirizine), dopamine agonists (R- and S-apomorphine), an anxiolytic (diazepam), an anticonvulsant (gabapentin), and an anesthetic agent (thiopental).

The set is also chemically diverse in terms of ionization state at pH 7.4 and lipophilicity (Table 1). However, it was recognized that most of the included drugs were less lipophilic than the majority of compounds in contemporary drug discovery programs. To balance this, additional microdialysis experiments were performed with a lipophilic base (CP-122721; [cis-R-[2-methoxy-5-(trifluoromethyl)phenyl][methyl]-2-phenyl-3-piperidinamine]) and included in the study.

#### Chemicals

Alovudine (3’-fluorothymidine), R,S-apomorphine, codeine, diazepam, R,- and S-cetirizine, and an internal standard (morphine, morphine-3-glucuronide, morphine-6-glucuronide, and oxycodone were obtained from Sigma-Aldrich (St. Louis, MO). Morphine, morphine-3-glucuronide, morphine-6-glucuronide, and oxycodone were obtained from Lipomed (Arlesheim, Switzerland). Tritiated GABA (3H-GABA) was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Gabapentin was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada).

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Classification</th>
<th>ACDLogD7.4</th>
<th>Log $D_{1.4}$</th>
<th>ACDpK$_u$</th>
<th>pK$_u$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alovudine</td>
<td>Neutral</td>
<td>-0.52</td>
<td>1.5$^b$</td>
<td>9.21</td>
<td>2.93, 8.00$^a$</td>
</tr>
<tr>
<td>Apomorphine</td>
<td>Base</td>
<td>2.51</td>
<td>0.22$^b$</td>
<td>7.88</td>
<td>8.22$^b$</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>Zwitherion</td>
<td>-1.25</td>
<td>-0.07$^b$</td>
<td>3.4</td>
<td>8.25</td>
</tr>
<tr>
<td>Codeine</td>
<td>Base</td>
<td>0.30</td>
<td>-0.12$^b$</td>
<td>9.85</td>
<td>8.21$^b$</td>
</tr>
<tr>
<td>CP-122721</td>
<td>Base</td>
<td>1.88</td>
<td>-0.79$^b$</td>
<td>3.4</td>
<td>8.18$^b$</td>
</tr>
<tr>
<td>Diazepam</td>
<td>Neutral</td>
<td>2.96</td>
<td>-0.98</td>
<td>8.22$^b$</td>
<td>7.57</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Zwitherion</td>
<td>-1.31</td>
<td>4.72, 10.27</td>
<td>3.4</td>
<td>7.95</td>
</tr>
<tr>
<td>Morphine</td>
<td>Base</td>
<td>-0.36</td>
<td>8.25</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>M3G</td>
<td>Zwitherion</td>
<td>-4.81</td>
<td>2.79, 9.75</td>
<td>2.97</td>
<td>8.18$^b$</td>
</tr>
<tr>
<td>M6G</td>
<td>Zwitherion</td>
<td>-4.39</td>
<td>2.79, 9.73</td>
<td>2.97</td>
<td>8.22$^b$</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>Zwitherion</td>
<td>-0.98</td>
<td>0.18, 8.34</td>
<td>8.25</td>
<td></td>
</tr>
<tr>
<td>Oxycodone</td>
<td>Base</td>
<td>1.27</td>
<td>7.57</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>Thiopental</td>
<td>Neutral</td>
<td>2.99</td>
<td>7.95</td>
<td>7.57</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Plember van Balen et al., 2001.
$^b$ Avdeef et al., 1996.

### Intraparenchymal Microdialysis of CP-122721

Adsorption to the FEP (fluorinated ethylene propylene) tubing (CMA Microdialysis, Solna, Sweden) and CMA/12 probe necessitated the inclusion of 0.5% BSA in the perfusion fluid, as described previously (Gupta et al., 2006). Probe recovery and delivery of CP-122721 were found in vitro to be equal, supporting the use of the in vivo retrodialysis calibration method. In vivo experiments in rats were performed as described previously (Bostrom et al., 2006). After retrodialysis and washout with blank perfusate, the drug was administered intravenously as a bolus dose plus a 4-h constant rate infusion to obtain steady state. The size of the bolus
Concentration (9–14 out for the investigated compounds by quantification of the buffer protein

into the slice, the concentration of drug in ECF buffer, and the adherent

and the slice homogenates were stored at 70°C until analysis.

Brain Homogenate Binding. Drug-naive animals were sacrificed under
dosing protocol to approach the lowest possible value in vivo, which is the


dose and the infusion rate were adjusted according to the plasma pharmacokinetics. CP-12727 was dissolved in saline. Microdialysis samples were collected at 20-min intervals from 2 h post-bolus dose until the termination of the experiment at 4 h, when brain tissue was sampled. V_u,brain was calculated using eq. 1, assuming a blood volume of 3% of brain weight (Shockley and LaManna, 1988) and a blood/plasma distribution ratio of unity.

Brain Slice Uptake Experiments. The brain slice uptake experiments were performed as described previously (Kakee et al., 1996), with minor modificaitons. Drug-naive animals were sacrificed under isoflurane anesthesia, and the brain was removed and immersed in ice-cold oxygenated pH 7.4 buffer (122

The chemical instability of apomorphine enantiomers necessitated the use of

and brain homogenate experiments were added to a 96-deepwell plate (Nalge Nunc International, Rochester, NY) and diluted with a volume of 0.2% formic acid. Samples containing apomorphine were stored at −70°C until analysis.

Analytical Procedures. The amount of drug in the various sample matrices was quantified with reversed phase liquid chromatography and multiple reaction monitoring mass spectrometry (liquid chromatography-tandem mass spectrometry) detection using a Micromass Quattro Ultima instrument (Waters, Manchester, UK) equipped with electrospray run in positive mode for all

TABLE 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass Transition</th>
<th>Column</th>
<th>Initial Conditionsa</th>
<th>Final Conditionsa</th>
<th>Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alovudine</td>
<td>245.1→126.9</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.04</td>
</tr>
<tr>
<td>R,S-Apomorphine</td>
<td>268.3→191.1</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.17</td>
</tr>
<tr>
<td>R,S-Cetirizine</td>
<td>389.2→200.9</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.70</td>
</tr>
<tr>
<td>Codeine</td>
<td>300.0→151.9</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>2.05</td>
</tr>
<tr>
<td>CP-12272</td>
<td>381.4→160.1</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.51</td>
</tr>
<tr>
<td>Diazepam</td>
<td>285.2→154.1</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.86</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>172.2→137.1</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>0.88</td>
</tr>
<tr>
<td>Morphine</td>
<td>286.0→152.0</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.45</td>
</tr>
<tr>
<td>M3G</td>
<td>462.0→285.9</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>1.25</td>
</tr>
<tr>
<td>M6G</td>
<td>462.3→286.1</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>1.41</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>320.3→276.2</td>
<td>Polaris C18 5×2.5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.07</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>316.0→240.9</td>
<td>Atlantis C18 5×2.1, 5 μl</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>2.01</td>
</tr>
<tr>
<td>Thiopepental</td>
<td>241.0→100.9</td>
<td>Hypurity C18 5×2.1, 5 μl</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.64</td>
</tr>
</tbody>
</table>

\[ V_{u,brain} = \frac{A_{slice} - V_i \times C_{buffer}}{C_{buffer} \times (1 - V_i)} \]

The equation describes a relationship where the lowest possible \( V_{u,brain} \) is 0.8 ml·g brain−1 if there is no binding and the drug occupies only the brain water space. This is an inherent limitation of the technique for \( V_{u,brain} \) predictions of compounds that approach the lowest possible value in vivo, which is the volume of the brain interstitial fluid (0.2 ml·g brain−1).

The chemical instability of apomorphine enantiomers necessitated the use of 50 mM ascorbic acid in the buffer for brain homogenization and equilibrium dialysis. Samples containing apomorphine were stored at −70°C until analysis.

\[ V_{u,brain}(k) = 1 + D \left( \frac{1}{D_{obs}} - 1 \right) \]

A simple \( V_{u,brain} \) prediction model was established using linear regression analysis of in vivo log \( V_{u,brain} \) and log \( D_{obs} \) of the studied compounds. The in vivo \( V_{u,brain} \) for each compound was predicted from its log \( D_{obs} \) using the regression line of the other compounds, not including itself. Calculated ACDLogD7.4 values (Table 1) were used for all compounds except morphine glucuronides and cetirizine, for which experimentally obtained values from the literature were considered more reliable.

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\[ V_{u,brain}(k) = 1 + D \left( \frac{1}{D_{obs}} - 1 \right) \]
acid containing an appropriate amount of acetonicitrile. Fifty-microliter samples of brain homogenates were protein-purified with 150 μl of ice-cold acetonicitrile containing 0.2% formic acid. After 1 min of vortexing and 20 min of centrifugation at 4000 rpm (Rotanta/TR; Hettich, Tuttlingen, Germany) at 4°C, the supernatant was transferred to a new plate and appropriately diluted with 0.2% formic acid. Microdialysis samples containing 0.5% BSA were protein-removed by definition. Drug molecules that are associated with the cells are either unbound in the intracellular fluid or bound intra- or extracellularly.

Data Presentation and Statistical Analysis. Values of V_u,brain are expressed as means ± standard deviation. Data were log-transformed for the statistical analysis, and the in vivo values of V_u,brain were taken as accurate. Agreement in vivo V_u,brain data were assessed according to the method of Altman and Bland (Altman and Bland, 1983; Bland and Altman, 1999). For each in vivo method, the significance of the mean bias was tested with Student’s t test. The agreement is expressed as the 90% confidence interval ratio (CIR) around the mean, which was calculated using the t distribution. The 90% CIR indicates the likely difference for a future single compound mean across seven slices or three dialysis cells. The 90% confidence interval is the mean difference (bias) divided by the CIR to the mean difference multiplied by the CIR. The in vivo agreement of the Log D_{7.4}-based prediction model was also assessed using 90% CIR.

Definitions and Relationships. The basic assumption of this study was that the intrabrain distribution of a drug can be described by a distributional model in which the drug is unbound in the brain ISF. Distribution occurs by permeation into brain cells and by binding to membranes or proteins located intra- or extracellularly (Fig. 1). Specifically, we assumed that C_u,brainISF measured with a microdialysis probe was representative of the whole brain, i.e., that there would be only limited regional variations in the brain-to-plasma unbound drug concentration ratio. Morphine has been studied in rats and pigs, indicating some spatial differences (Matos et al., 1992; Tunblad et al., 2004), whereas carbamazepine showed no differences in the rat (Van Belle et al., 1995).

The V_u,brain value, in ml · g brain⁻¹ with brain ISF as the reference fluid, reflects the distribution of the drug inside the brain, as distinct from the brain-to-plasma concentration ratio. The amount of drug present in whole brain tissue versus the unbound concentration in brain ISF depends on cell membrane permeability and the affinity of the drug for tissue components. V_u,brain is unrelated to the brain volume of distribution term, V_D, which is synonymous with the brain-to-plasma concentration ratio and has blood or plasma as a reference. The V_D term is commonly used with respect to the intravenous injection technique (Patatik et al., 1983), the in situ brain perfusion method (Dagenais et al., 2000), and positron emission tomography (Koeppe, 2002).

Equations were derived from the definition of V_u,brain (eq. 1) and the distributional model (Fig. 1) to describe how the components influence its numerical value. Accounting for the amount of intravascular drug simplifies the expression for V_u,brain:

\[ A_{\text{brain}} = \frac{A_{\text{brainISF}}}{C_{u,\text{brainISF}}} \]  

which comprises the amount of unbound drug in the ISF plus the amount of drug associated with the cells:

\[ A_{\text{brain}} = V_{\text{brainISF}} \times C_{u,\text{brainISF}} + V_{\text{cell}} \times A_{\text{cell}} \]  

V_{\text{brainISF}} and V_{\text{cell}} are the physiologic fractional volumes of the brain ISF and brain cells, respectively (ml · g brain⁻¹), and A_{\text{cell}} is the amount of drug associated with the cells (μmol · ml cell⁻¹). The distribution volume of unbound drug in the cell, V_{\text{cell}} (ml ISF · ml cell⁻¹) is also introduced, as this relates A_{\text{cell}} to the intracellular concentration of unbound drug, C_{u,\text{cell}} (μmol · ml ICF⁻¹):

\[ A_{\text{cell}} = V_{\text{cell}} \times C_{u,\text{cell}} \]  

Replacing eq. 6 into eq. 5 and dividing by C_{u,\text{brainISF}} gives:

\[ V_{u,\text{brain}} = V_{\text{brainISF}} + V_{\text{cell}} \times C_{u,\text{cell}} \times \frac{C_{u,\text{cell}}}{C_{u,\text{brainISF}}} \]  

It can be seen from eq. 7 that if no drug enters the brain parenchymal cells, C_{u,\text{cell}} is zero and V_{u,\text{brain}} becomes equal to the volume of ISF, typically a value around 0.2 ml · g brain⁻¹ (Nicholson and Sykova, 1998). This is, from a physiologic perspective, the smallest V_{u,\text{brain}} possible. A value close to the brain water volume (0.8 ml · g brain⁻¹) (Reinoso et al., 1997) may indicate even distribution in the whole brain tissue. Likewise, a V_{u,\text{brain}} larger than 0.8 suggests that the drug has affinity for brain tissue.

It cannot be directly assumed that the concentration of unbound drug in brain ISF is equal to the concentration of unbound drug in brain ISF. Apart from the effects of active transport mechanisms, the lower intracellular pH could cause basic drugs to be trapped intracellularly, as they are not able to permeate the cell membrane in their ionized form. Assuming for acidic and basic drugs that passive diffusion of the un-ionized species dominates permeation of the membrane, the distribution of unbound drug at equilibrium is determined by the drug pK_a and the pH in the extra- and intracellular compartments, pH_{ISF} and pH_{cell} respectively (eq. 8 and 9).

\[ V_{u,\text{brain}} = V_{\text{brainISF}} + V_{\text{cell}} \times V_{\text{cell}} \times C_{u,\text{cell}} \times \frac{10^{\text{pK}_{\text{a}} - \text{pH}_{\text{ISF}}}}{10^{\text{pK}_{\text{a}} - \text{pH}_{\text{cell}}}} \]  

V_{\text{cell}}, which describes the affinity of the drug for physical binding inside the cells, was estimated using the brain homogenate binding experiment and taking V_{\text{cell}} into account in the dilution factor:

\[ V_{\text{cell}} = D_{\text{cell}} \left( \frac{1}{V_{\text{cell}}} - 1 \right) \]  

Accordingly, the cells in the homogenate are diluted not only with the added buffer but also in a small volume of brain ISF devoid of plasma proteins. It is assumed in eq. 5 to 10 that drug binding to the outside of the cell is negligible compared with binding inside the cells. This is a reasonable approximation since for a typical human cell, the outside surface area of the cell membrane represents less than 0.5% of the total membrane surface area (Freitas, 1999). Furthermore, no single type of organelle would have a sufficiently large fractional volume to substantially influence V_{\text{cell}} in the case of a moderate concentration difference of unbound drug between the cytosol and the organelle. These approximations are not valid for molecules that are entirely confined to the extracellular domain and significantly bound. Macromolecules with specific protein interactions may possess such a combination of properties. It is, however, most unlikely for low molecular weight molecules for which binding and membrane permeation are largely determined by lipophility. Finally, it should be noted that C_{cell} represents the overall concentration of unbound drug in the ICF, although variations may exist among different cell types.

Supplemental Data Appendix I describes suggested procedures for experimental estimation of unbound drug concentrations in brain ISF and ICF as well.
### Results

The in vivo $V_{u,\text{brain}}$ of the compounds in this study spanned 3 orders of magnitude, from 0.2 ml · g brain$^{-1}$ for the morphine glucuronides, indicating exclusive distribution outside brain cells and minimal binding to proteins or membranes, to 210 ml · g brain$^{-1}$ for CP-122721, revealing extensive tissue binding and distribution to the intracellular space (Table 3). Values for $V_{u,\text{brain}}$ obtained with the investigated methods are also illustrated in Figs. 2 and 3.

#### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>In Vivo Microdialysis</th>
<th>In Vivo Brain Slice Uptake (n = 5–7)</th>
<th>In Vivo Brain Homogenate Binding (n = 3)</th>
<th>Predicted from Log D$_{7.4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allovalnine (A)</td>
<td>(Linden et al., 2003)</td>
<td>0.52 ± 0.04</td>
<td>1.07 ± 0.091</td>
<td>1.05 ± 0.30</td>
</tr>
<tr>
<td>$R$-Apomorphine (R-A)</td>
<td>(Sam et al., 1997)</td>
<td>15s</td>
<td>18.1 ± 1.9</td>
<td>15.5 ± 0.38</td>
</tr>
<tr>
<td>$S$-Apomorphine (S-A)</td>
<td>(Sam et al., 1997)</td>
<td>26s</td>
<td>14.0 ± 1.4</td>
<td>14.4 ± 0.76</td>
</tr>
<tr>
<td>$R$-Cetirizine (R-C)</td>
<td>(Gupta et al., 2006)</td>
<td>2.39</td>
<td>6.62 ± 0.56</td>
<td>11.8 ± 0.80</td>
</tr>
<tr>
<td>$S$-Cetirizine (S-C)</td>
<td>(Gupta et al., 2006)</td>
<td>2.86</td>
<td>6.56 ± 0.61</td>
<td>11.8 ± 0.80</td>
</tr>
<tr>
<td>Codeine (C)</td>
<td>(Xie and Hammarlund-Udenaes, 1998)</td>
<td>3.6 ± 1.2b</td>
<td>2.77 ± 0.22</td>
<td>1.46 ± 0.18</td>
</tr>
<tr>
<td>CP-122721 (CP)</td>
<td>(Dubey et al., 1989)</td>
<td>207 ± 14</td>
<td>210 ± 17</td>
<td>152 ± 19</td>
</tr>
<tr>
<td>Diazepam (D)</td>
<td>(Dubey et al., 1989)</td>
<td>22s</td>
<td>17.7 ± 0.80</td>
<td>24.6 ± 1.2</td>
</tr>
<tr>
<td>Gabapentin (G)</td>
<td>(Wang and Welty, 1996)</td>
<td>5.5 ± 2.0b</td>
<td>3.97 ± 0.72w</td>
<td>1.04 ± 0.14</td>
</tr>
<tr>
<td>Morphine (M)</td>
<td>(Tenblad et al., 2003)</td>
<td>1.7c</td>
<td>2.69 ± 0.43</td>
<td>2.00 ± 0.20</td>
</tr>
<tr>
<td>Morphine-3-glucuronide (M3G)</td>
<td>(Xie et al., 2000)</td>
<td>0.25 ± 0.02c</td>
<td>0.528 ± 0.059</td>
<td>1.27 ± 0.18</td>
</tr>
<tr>
<td>Morphine-6-glucuronide (M6G)</td>
<td>(Boe et al., 2001)</td>
<td>0.20 ± 0.02c</td>
<td>0.731 ± 0.078</td>
<td>1.24 ± 0.10</td>
</tr>
<tr>
<td>Norfloxacin (N)</td>
<td>(Ooie et al., 1997)</td>
<td>0.98 ± 0.59c</td>
<td>2.01 ± 0.10</td>
<td>1.72 ± 0.37</td>
</tr>
<tr>
<td>Oxycodone (O)</td>
<td>(Bostrom et al., 2006)</td>
<td>2.20 ± 0.53b</td>
<td>3.60 ± 0.35</td>
<td>2.95 ± 0.33</td>
</tr>
<tr>
<td>Thiopental (T)</td>
<td>(Mathet et al., 2000)</td>
<td>10f</td>
<td>5.07 ± 0.29</td>
<td>6.51 ± 0.89</td>
</tr>
</tbody>
</table>

*a* Drug administration by subcutaneous bolus dose.

*b* Estimation of $V_{u,\text{brain}}$ during non-steady-state conditions.

*c* Probe calibration by in vivo retrodialysis by drug.

*d* $V_{u,\text{brain}}$ calculated from data in original literature report.

*e* Drug administration by continuous subcutaneous infusion.

*f* Experiments performed in guinea pigs.

*g* Drug administration by short intravenous infusion.

*h* $V_{u,\text{brain}}$ reported in original literature.

*i* Probe calibration by in vivo retrodialysis by calibrator.

*j* $V_{u,\text{brain}}$ experimentally obtained from original data in this study.

*k* Probe calibration in vitro.

*l* Probe placement in cortex.

*m* $V_{u,\text{brain}}$ calculated from slices incubated for 1 h.

*n* Probe calibration in vivo by reference compound.

*o* Probe placement in hippocampus.

*p* Probe calibration by in vivo retrodialysis by drug and correction.

as the unbound brain-to-plasma and ICF-to-ISF distribution ratios $K_{p,\text{un}}$ and $K_{p,\text{un,cell}}$.

The in vivo $V_{u,\text{brain}}$ of the compounds in this study spanned 3 orders of magnitude, from 0.2 ml · g brain$^{-1}$ for the morphine glucuronides, indicating exclusive distribution outside brain cells and minimal binding to proteins or membranes, to 210 ml · g brain$^{-1}$ for CP-122721, revealing extensive tissue binding and distribution to the intracellular space (Table 3). Values for $V_{u,\text{brain}}$ obtained with the investigated methods are also illustrated in Figs. 2 and 3.

The distribution volume of the extracellular marker $^{14}$C-inulin after 4 h of incubation was 0.36 ± 0.037 ml · g brain$^{-1}$. Because the cell viability from a slice preparation is difficult to assess absolutely or quantitatively, the levels of ATP were monitored during the incubation period. There was little change in ATP concentration from the time of preparation of the brain slice to the end of the 4-h incubation (~8 nmol · mg protein$^{-1}$). The $V_{u,\text{brain}}$ of $^{14}$GABA, used to demonstrate functionality in terms of cellular transport, reached a maximum (17 ± 4.7 ml · g brain$^{-1}$) after 60 min and then slowly declined.

The time course of drug uptake in the brain slices was studied by terminating the incubations at various prespecified times (Fig. 2). The extent of uptake of these compounds clearly varied, but they also differed in the time required to reach equilibrium. Since all the compounds had reached equilibrium at 240 min, this time point was used in the calculations. Variability of $V_{u,\text{brain}}$ in slices from different rats was not greater than variability in slices from the same rat (data not shown).

Characterization of the brain homogenate binding method included time course studies using diazepam and gabapentin. Because these studies indicated that 8 h of incubation was necessary to achieve equilibrium between the dialysis cells, overnight incubation for 16 h was assumed sufficient for all compounds.

Agreement between the methods is illustrated in Fig. 3, in which in vitro $V_{u,\text{brain}}$ determined by the slice or homogenate method was plotted against in vivo $V_{u,\text{brain}}$ determined by microdialysis. The brain slice method predicted $V_{u,\text{brain}}$ within a 3-fold range for all but 1 of the 15 compounds, whereas the brain homogenate binding technique predicted $V_{u,\text{brain}}$ within a 3-fold range for 10 of the 15 compounds. There was no statistically significant bias for the in vitro methods in relation to the in vivo data (Table 4). The 90% CIs expressing the likely (-fold) difference compared with the in vivo data were 3.0 and 6.0 for the slice and homogenate methods, respectively (Table 4).

There were instances of deviations from agreement between the methods. For example, the homogenate $V_{u,\text{brain}}$ for morphine-3-glucuronide (1.3 ml · g brain$^{-1}$) was higher than the in vivo value (0.25 ml · g brain$^{-1}$). The value for this drug using the slice method (0.53 ml · g brain$^{-1}$) was closer to the in vivo value. The extracellular slice distribution volume of $^{14}$C-inulin was 0.36 ml · g brain$^{-1}$. Furthermore, the gabapentin $V_{u,\text{brain}}$ from the homogenate experiment (1.04 ml · g brain$^{-1}$) indicated that this drug was not significantly bound to brain tissue. In contrast, in vivo microdialysis and the brain slice method gave values of 5.5 and 4.0 ml · g brain$^{-1}$, respectively, indicating that the total amount in brain was much higher than ISF concentrations of unbound gabapentin. Conversely, the reverse situation was seen with both cetirizine enantiomers: the brain homogenate method indicated considerable binding to brain tissue ($V_{u,\text{brain}}$ 12 ml · g brain$^{-1}$) that was indicated to a lesser extent in the slice method (6.5 ml · g brain$^{-1}$) compared with in vivo microdialysis (2.5 ml · g brain$^{-1}$).

Linear regression analysis of all data points of a plot of in vivo $V_{u,\text{brain}}$ versus log $D_{7.4}$ (Fig. 4) indicated a correlation between the
lipophilicity of the compound and the in vivo $V_{u,brain}$ value. The $V_{u,brain}$ values that were predicted from log $D_{7.4}$ using the regression line are presented in Table 3. All $V_{u,brain}$ predictions were made without gabapentin in the model, since the $V_{u,brain}$ value for gabapentin was known to reflect active transport mechanisms. The $V_{u,brain}$ predictions from log $D_{7.4}$ were not as accurate as those using experimental methods. Excluding the $V_{u,brain}$ prediction of gabapentin gave a 90% CIR of the log $D_{7.4}$ model of 6.0. Including gabapentin gave a 90% CIR of 9.0. Excluding the in vitro estimates of gabapentin $V_{u,brain}$ reduced the 90% CIR of the homogenate method to 4.9 but had no effect on the CIR of the slice method (Table 4).

$K_{pu,cell}$, the ratio of intracellular to extracellular unbound drug concentrations was calculated for each compound using $V_{u,brain}$ from the slice method and $V_{u,cell}$ from the homogenate method (Supplemental Data Appendix I, eq. A2). Five of the six basic compounds had a $K_{pu,cell}$ greater than 1. Neutral compounds had ratios close to or slightly below 1. The hydrophilic morphine-glucuronides had the lowest ratios, followed by the zwitterionic cetirizine enantiomers. Gabapentin had a $K_{pu,cell}$ of 4.5 (Fig. 5).

Discussion

Since only the unbound drug is available to occupy extracellular receptors, estimation of $C_{u,brain}$ could explain why some compounds fail to demonstrate in vivo activity despite in vitro potency and reasonable amounts of drug in brain ($A_{brain}$). Estimation of $C_{u,brain}$ also allows quantification of the extent of BBB drug transport and investigation of the function of active transporters in vivo without confounding by nonspecific brain tissue binding. Since methods for routine measurement of unbound drug concentrations are lacking or have not yet been sufficiently evaluated in vivo, much research still relies on the easily measured $A_{brain}$. As an attractive alternative to microdialysis, which directly measures $C_{u,brain}$ in vivo, we propose the combined use of in vivo $A_{brain}$ and in vitro estimates of $V_{u,brain}$ to calculate $C_{u,brain}$. This approach is less labor-intensive than microdialysis and likely to be more successful with lipophilic drugs.

The slice method estimated $V_{u,brain}$ within a 3-fold range of in vivo results for 14 of the 15 compounds investigated; $V_{u,brain}$ for morphine-6-glucuronide was slightly more than 3 times greater than the in vivo result. This indicates that the slice method has potential for accurately estimating the brain distribution of compounds with diverse properties. The brain homogenate binding method did not provide the same level of agreement with in vivo results; $V_{u,brain}$ fell within the 3-fold range for only 10 of the 15 compounds. For example, $V_{u,brain}$ for the morphine-glucuronides, which are known to reside in the interstitial space in vivo (Xie et al., 2000; Bouw et al., 2001), was around 1 ml · g brain$^{-1}$ in the homogenate method. This discrepancy can be explained by the inherent inability of the homogenate method to differentiate between intra- and extracellular distribution due to disruption of cell membranes in the homogenate. Thus, the homogenate method measures the physical binding to brain constituents, which does not determine $V_{u,brain}$ alone. This was clearly demonstrated for gabapentin, which is actively transported into brain cells by the system L $\alpha$-amino acid transporter (Su et al., 1995). Because this process cannot be captured in the homogenate method, the $V_{u,brain}$ value of close to 1 contrasted with the higher in vivo value of 5.5 and the slice $V_{u,brain}$ value of 4 ml · g brain$^{-1}$.
As suggested by the gabapentin result, the $V_{u,brain}$ value for any transporter substrate at the level of brain parenchymal cells will vary according to which of these methods is used. Multidrug resistance-associated proteins have been located beyond the BBB in microglia, astrocytes, neurons, and oligodendrocytes (Dallas et al., 2006). Whenever $V_{u,brain}$ is influenced by active transport mechanisms in the brain parenchyma, the slice method can be expected to provide more accurate estimates. Furthermore, intracellular accumulation of basic drugs will also occur as the ionized species is trapped by the lower pH of the intracellular fluid. $C_{u,cell}$ could potentially be 2-fold higher than $C_{u,brain}$, depending on the $pK_a$ of the drug and assuming a difference of 0.3 pH unit between intra- and extracellular compartments (Davson and Segal, 1996). This phenomenon was observed for the basic model compounds in our study.

Whereas the cells are entirely disrupted in the brain homogenate method, the cellular integrity of incubated brain slices could also be compromised near the cut surfaces, thus affecting discrimination between intra- and extracellular compartments. In fact, the measured slice distribution volume of the extracellular marker $^{14}$C-inulin (0.36 ml·g brain$^{-1}$) was higher than in vivo values (Nicholson and Sykova, 1998). Slice viability, measured as the ATP levels, was stable; ATP levels were similar to those in previous reports (12–14 nmol·mg protein$^{-1}$) (Lipton and Whittingham, 1984). Factors potentially affecting the in vivo characteristics of the brain slice include the choice of slicing technique, thickness of slice, oxygen supply, composition and pH of the medium, incubation time, and degree of medium convection at the surface of the slice (Lipton and Whittingham, 1984).

The attainment of equilibrium is essential for any method that measures $V_{u,brain}$ including in vivo microdialysis. In vivo experiments have established that equilibration of gabapentin concentrations between the intra- and extracellular compartments is rapid compared with BBB transport (Wang and Welty, 1996), but this finding cannot be generally

![Fig. 3. Relationship between in vivo $V_{u,brain}$ values and in vitro slice values (A), in vitro brain homogenate values (B), and $V_{u,brain}$ values (C) predicted from log $D_{7.4}$. The solid line represents perfect agreement. The dashed lines represent a 3-fold over- or underestimation compared with in vivo $V_{u,brain}$ values. Symbols for drugs are defined in Table 3.](image)

![Fig. 4. Relationship between in vivo $V_{u,brain}$ values and lipophilicity estimated as log $D_{7.4}$. The equation and solid line show the best fit of the linear regression analysis. Gabapentin (open circle) was excluded from the analysis based on information of active uptake.](image)

### Table 4: Statistics of in vitro-in vivo agreement for $V_{u,brain}$ estimations

<table>
<thead>
<tr>
<th></th>
<th>Slice</th>
<th>Homogenate</th>
<th>Log $D_{7.4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bias (ratio of in vitro to in vivo estimation)</td>
<td>1.34 (N.S.)</td>
<td>1.31 (N.S.)</td>
<td>0.807 (N.S.)</td>
</tr>
<tr>
<td>Bias excluding gabapentin</td>
<td>1.40 (N.S.)</td>
<td>1.50 (N.S.)</td>
<td>0.985 (N.S.)</td>
</tr>
<tr>
<td>90% CIR</td>
<td>2.95</td>
<td>6.00</td>
<td>8.97</td>
</tr>
<tr>
<td>90% CIR excluding gabapentin</td>
<td>2.96</td>
<td>4.91</td>
<td>6.02</td>
</tr>
</tbody>
</table>

N.S., not significantly different from 1 ($P > 0.05$).
extrapolated. In the in vitro systems, the equilibration time is dependent on the permeability of the brain slice or the dialysis membrane in the homogenate method. The initial distance to reach equilibrium should also be considered. At the start of the slice incubations, all the drug is in the medium, potentially far from equilibrium. Although this is not a problem if sufficient incubation time is allowed, equilibrium time could theoretically reach impractical levels as $V_{u,brain}$ values increase. It was concluded that 4 h of incubation was sufficient for compounds with a $V_{u,brain}$ not exceeding 200 ml g brain$^{-1}$.

The level of uncertainty in in vivo estimates of $V_{u,brain}$ should also be considered. Even the most careful probe implantation causes a transient loss of BBB integrity. If leakage of drug occurs, it can potentially reach impractical levels as $V_{u,brain}$ values increase. Although this is not a problem if sufficient incubation time is allowed, equilibrium time could theoretically reach impractical levels as $V_{u,brain}$ values increase. It was concluded that 4 h of incubation was sufficient for compounds with a $V_{u,brain}$ not exceeding 200 ml g brain$^{-1}$.

In conclusion, there is a recognized need in drug discovery programs for methods of estimating unbound drug concentrations in the brain in an efficient, reliable manner. Estimations of $V_{u,brain}$, using the slice method agreed well with in vivo microdialysis measurements. Deviation from in vivo results was greater with the homogenate method. It is therefore suggested that total brain concentrations from in vivo experiments be combined with results from brain slice studies. This will allow quantification of active transport at the BBB in vivo. 2) provide a better understanding of the relationship between in vitro potency and in vivo activity for compounds acting on CNS targets. 3) in combination with the homogenate method, provide additional information on intracellular concentrations of unbound drug.

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References


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