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 quantitation 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 convective 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).

This work was supported by AstraZeneca R&D Mölndal. Nomenclature list and Appendix I are available as supplemental data. The online version of this article (available at http://dmd.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: BBB, blood-brain barrier; BSA, bovine serum albumin; CIR, confidence interval ratio; CNS, central nervous system; CSF, cerebrospinal fluid; ICF, intracellular fluid; ISF, interstitial fluid; CP-122721, [cis-n-[2-methoxy-5-(trifluoromethoxy)phenyl]methyl]-2-phenyl-3-piperidinamine.
2002; Shen et al., 2004). The only method of directly measuring $C_{u,\text{brain}}$ 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 ($V_{u,\text{brain}}$) to relate $C_{u,\text{brainISF}}$ to $A_{\text{brain}}$, where $V_{\text{blood}} \times C_{\text{blood}}$ is the amount of drug present in the blood vessels of the brain:

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

Thus, $V_{u,\text{brain}}$ describes the distribution of drug inside the brain regardless of brain-to-plasma distribution. A low value for $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ can be reliably obtained for a compound, $C_{u,\text{brainISF}}$ can be calculated from available total drug levels, thus circumventing the need for microdialysis. Methods other than microdialysis that have been used include $V_{u,\text{brain}}$ 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, $f_{u,\text{brain}}$, to describe much the same property (Becker and Liu, 2006; 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 $C_{u,\text{brainISF}}$ estimation to guide industrial drug discovery programs or academic research related to CNS drug exposure. The $V_{u,\text{brain}}$ concept is used as a link between the total brain concentration ($A_{\text{brain}}$) and the pharmacologically active unbound brain ISF concentration ($C_{u,\text{brainISF}}$). Along with a characterization of the methods, we present the first comprehensive comparison of in vitro $V_{u,\text{brain}}$ data and in vivo microdialysis measurements. We also discuss how the integrative use of these $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$. 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 (alovudine, norfloxacin), antihistamines (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-3R-(2-methoxy-5-(trifluoromethoxy)phenyl]methyl]-2-phenyl-1-piperidinamine) and included in the study.

#### Chemicals

Alovudine (3′-fluorothymidine), R,S-apomorphine, codeine, diazepam, 14C-inulin, norfloxacin, thiopental, and bovine serum albumin (BSA) (initial fractionation by cold alcohol precipitation, lot 40K0896) 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). Racemic cetirizine, the pure enantiomers S- and R-cetirizine, and an internal standard (uch20002), [2-[2-(4-benzhydrylidene-piperidin-1-yl)-ethoxy]-ethoxy]acetic acid chloride, were supplied by UCB Pharma (Braine l’Alleud, Belgium). CP-122721 was synthesized at AstraZeneca R&D (Mölndal, Sweden) with purity greater than 95%. All other chemicals were of analytical grade. All solvents were of high-performance liquid chromatography grade.

#### Animals

Male and female Sprague-Dawley rats (Harlan, Horst, the Netherlands) weighing 250 to 350 and 280 to 280 g were used for in vitro experiments and in vivo microdialysis, respectively. Male Dunkin Hartley guinea pigs weighing 450 to 500 g were purchased from Lidköpings Kaninfarm (Lidköping, Sweden). All animals were group-housed at 18–22°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 Göteborg (346-2002, 412-2005).

#### Intracerebral 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

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**Table 1**

Physicochemical description of drugs included in the study

<table>
<thead>
<tr>
<th>Compound</th>
<th>Classification</th>
<th>ACDLogD7.4</th>
<th>Log $D_{7.4}$</th>
<th>ACDpK_a</th>
<th>$pK_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alovudine</td>
<td>Neutral</td>
<td>-0.52</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apomorphine</td>
<td>Base</td>
<td>2.51</td>
<td>1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.22</td>
<td>2.93, 8.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cetirizine</td>
<td>Zwitterion</td>
<td>-1.25</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.23</td>
<td></td>
</tr>
<tr>
<td>Codeine</td>
<td>Base</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-122721</td>
<td>Base</td>
<td>1.88</td>
<td></td>
<td>9.85</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>Neutral</td>
<td>2.96</td>
<td></td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Zwitterion</td>
<td>-1.31</td>
<td></td>
<td>4.72, 10.27</td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>Base</td>
<td>-0.36</td>
<td>-0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.25</td>
<td>8.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M3G</td>
<td>Zwitterion</td>
<td>-4.81</td>
<td>-1.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.79, 9.75</td>
<td>8.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>M6G</td>
<td>Zwitterion</td>
<td>-4.39</td>
<td>-0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79, 9.73</td>
<td>8.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>Zwitterion</td>
<td>-0.98</td>
<td></td>
<td>0.18, 8.34</td>
<td></td>
</tr>
<tr>
<td>Oxycodone</td>
<td>Base</td>
<td>1.27</td>
<td></td>
<td>7.57</td>
<td></td>
</tr>
<tr>
<td>Thiopental</td>
<td>Neutral</td>
<td>2.99</td>
<td></td>
<td>7.95</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Plemer van Balen et al., 2001.<br>
<sup>b</sup>Avdeef et al., 1996.
dose and the infusion rate were adjusted according to the plasma pharmacokinetics. CP-122721 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. \( V_u,\text{brain} \) 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 modifications. Drug-naive animals were sacrificed under isoflurane anesthesia, and the brain was removed and immersed in ice-cold oxygenated pH 7.4 buffer (122 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 3 mM KCl; 1.4 mM CaCl₂, 1.2 mM MgSO₄, 0.4 mM K₂HPO₄, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). A 6-mm coronal section was cut with a razor and m coronal slices of striatal areas were cut. The slices were preincubated at 37°C for 5 min in 10 ml of ECF buffer before the drug, dissolved in ECF buffer, was added. The concentration of drug in the slice, the concentration of drug in ECF buffer, and the adherent brain homogenate and buffer was performed in triplicate for 16 h at 37°C in 1 ml of Plexiglas cells mounted with a 5-kDa cutoff Diachema cellulose membrane (Diaion GmbH, München, Germany). An aliquot of homogenate was sampled before and after coincubation to assess the compound stability. The fraction of unbound drug in diluted brain homogenate, \( f_{\text{buffer}} \) i.e., the buffer-to-homogenate concentration ratio, was used to calculate \( V_u,\text{brain} \), while taking into account the dilution, \( D \), associated with homogenate preparation (eq. 3) (Kalvass and Maurer, 2002).

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

The equation describes a relationship where the lowest possible \( V_{u,\text{brain}} \) 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 \( V_{u,\text{brain}} \) 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.

### 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 Quattro Ultima instrument (Waters, Manchester, UK) equipped with electrospray run in positive mode for all compounds except thiopental. Gradient elution over 2 min with acetonitrile and 0.2% formic acid with a flow rate of 0.6 ml/min was used. Mass transitions and detection using a Micromass Quattro Ultima instrument (Waters, Manchester, UK) accomplished in 3 min. The equation describes a relationship where the lowest possible \( V_{u,\text{brain}} \) 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 \( V_{u,\text{brain}} \) 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).

### Chemical Instability of Apomorphine Enantiomers

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.

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass Transition</th>
<th>Column</th>
<th>Initial Conditions</th>
<th>Final Conditions</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 μm</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 μm</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 μm</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 μm</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>2.05</td>
</tr>
<tr>
<td>CP-122721</td>
<td>381.4→160.1</td>
<td>Atlantis C18 5 × 2.1, 5 μm</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 μm</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 μm</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 μm</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 μm</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 μm</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, 5 μm</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.07</td>
</tr>
<tr>
<td>Oxycodeone</td>
<td>316.0→240.9</td>
<td>Atlantis C18 5 × 2.1, 5 μm</td>
<td>C 100%</td>
<td>C 80%, B 20%</td>
<td>2.01</td>
</tr>
<tr>
<td>Thiopeptol</td>
<td>241.0→100.9</td>
<td>Hypurin C18 5 × 2.1, 5 μm</td>
<td>A 95%, B 5%</td>
<td>A 10%, B 90%</td>
<td>1.64</td>
</tr>
</tbody>
</table>

\*M3G, morphine-3-glucuronide; M6G, morphine-6-glucuronide.
\*A, 2% acetonitrile in 0.2% formic acid; B, 0.2% formic acid in acetonitrile; C, 0.2% formic acid in deionized water.
\*Varian, Inc., Palo Alto, CA.
\*Waters Corporation, Manchester, UK
\*Chemotek, Hägersten, Sweden.

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.

### 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 Quattro Ultima instrument (Waters, Manchester, UK) equipped with electrospray run in positive mode for all compounds except thiopental. Gradient elution over 2 min with acetonitrile and 0.2% formic acid with a flow rate of 0.6 ml/min was used. Mass transitions and detection using a Micromass Quattro Quattro Ultima instrument (Waters, Manchester, UK) accomplished in 3 min. The equation describes a relationship where the lowest possible \( V_{u,\text{brain}} \) 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 \( V_{u,\text{brain}} \) 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).

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### Analytical Procedures

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Sample preparation was adapted for any compound-specific requirements but followed a general procedure: buffer (100 μl) from the brain slice 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.
acid containing an appropriate amount of acetonicrile. Fifty-microliter samples of brain homogenates were protein-preficitated with 150 μl of ice-cold acetonicrile containing 0.2% formic acid. After 1 min of vortexing and 20 min of centrifugation at 4000 rpm (Rotanta/TR; Hettich, Tuttinglen, Germany) at 4°C, the supernatan was transferred to a new plate and appropriately diluted with 0.2% formic acid. Microdialysis samples containing 0.5% BSA were protein-preficitated in a similar manner. External calibration curves with at least five different concentrations were made from a serial dilution in 50% acetonicrile and 0.2% formic acid by standard addition to the blank matrices in a 1:9 volume ratio. Enantioselective analysis of cetirizine was undertaken using a previously reported method (Gupta et al., 2005). The coefficient of correlation, $R^2$, for each calibration curve was 0.990 or higher.

Radioactive isotopes were quantified using a Wallac WinSpectral 1414 liquid scintillation counter (GE Healthcare) and an OptiPhase HiSafe 3 scintillation cocktail (Fisher Chemicals, Loughborough, UK). Brain slices were solubilized with 1 ml of Soluene-350 (PerkinElmer Life and Analytical Sciences, Boston, MA) and decolorized with 100 μl of hydrogen peroxide.

**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 with in vivo $V_{u,brain}$ data were assessed according to the method of Altman and Bland (Altman and Bland, 1993; Bland and Altman, 1999). For each in vitro 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 $D_{ac}$-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 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 plasma as

![Figure 1](image-url)  
**Fig. 1.** The compartment model describing intrabrain drug distribution. Drug molecules that reside in the brain interstitial fluid compartment (Brain ISF) are unbound by definition. Drug molecules that are associated with the cells are either unbound in the intracellular fluid or bound intra- or extracellularly.

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 $V_{u,brain}$:

$$V_{u,brain} = \frac{A_{brain}}{C_{u,brainISF}}$$  (4)

$A_{brain}$ (μmol ⋅ g brain$^{-1}$) comprises the amount of unbound drug in the ISF plus the amount of drug associated with the cells:

$$A_{brain} = V_{brainISF} \times C_{u,brainISF} + V_{cell} \times A_{cell}$$  (5)

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

$$A_{cell} = V_{cell} \times C_{u,cell}$$  (6)

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

$$V_{u,brain} = V_{brainISF} + V_{cell} \times C_{u,cell} \frac{C_{u,brainISF}}{C_{u,cell}}$$  (7)

It can be seen from eq. 7 that if no drug enters the brain parenchymal cells, $C_{u,cell}$ is zero and $V_{u,brain}$ 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 $V_{brain}$ possible. A value close to the brain water volume (0.8 ml ⋅ g brain$^{-1}$) (Reinoss et al., 1997) may indicate even distribution in the whole brain tissue. Likewise, a $V_{u,brain}$ 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).

$$V_{u,brain} = V_{brainISF} + V_{cell} \times C_{u,cell} \times 10^{\frac{-pK_a}{H1}} \text{ (bases)}$$  (8)

$$V_{u,brain} = V_{brainISF} + V_{cell} \times C_{u,cell} \times 10^{\frac{pK_a}{H1}} \text{ (acids)}$$  (9)

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

$$V_{cell} = 1 + \frac{D}{V_{cell}} \left(\frac{1}{T_{lab}} - 1\right)$$  (10)

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 $V_{cell}$ 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 $C_{u,cell}$ 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 as
The values are given in ml · g brain⁻¹ and are presented as means ± S.D. The "microdialysis" values are taken from the literature according to the references given. Unless otherwise indicated, continuous intravenous infusions of drug were administered to rats, and sampling of whole brain tissue and striatal microdialysate was undertaken at steady state.

The time course of drug uptake in the brain slices was studied by the slice uptake and homogenate binding methods, and by predictions from log D₇.₄. The uptake of these compounds clearly varied, but they also differed in the extent of uptake of these compounds. Since all the compounds had reached equilibrium at 240 min, this time point was used in the calculations. Variability of V_u,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 dizepam 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 the in vitro V_u,brain determined by the slice or homogenate method was plotted against in vivo V_u,brain determined by microdialysis. The brain slice method predicted V_u,brain within a 3-fold range for all but 1 of the 15 compounds, whereas the brain homogenate binding technique predicted V_u,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% CIRs 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,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 space distribution volume of ¹⁴C-inulin was 0.36 ml · g brain⁻¹. Furthermore, the gabapentin V_u,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,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,brain versus log D₇.₄ (Fig. 4) indicated a correlation between the
lipophilicity of the compound and the in vivo $V_{u,\text{brain}}$ value. The $V_{u,\text{brain}}$ values that were predicted from log $D_{7.4}$ using the regression line are presented in Table 3. All $V_{u,\text{brain}}$ predictions were made without gabapentin in the model, since the $V_{u,\text{brain}}$ value for gabapentin was known to reflect active transport mechanisms. The $V_{u,\text{brain}}$ predictions from log $D_{7.4}$ were not as accurate as those using experimental methods. Excluding the $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ reduced the 90% CIR of the homogenate method to 4.9 but had no effect on the CIR of the slice method (Table 4).

$K_{p,\text{out.cell}}$, the ratio of intracellular to extracellular unbound drug concentrations was calculated for each compound using $V_{u,\text{brain}}$ from the slice method and $V_{u,\text{cell}}$ from the homogenate method (Supplementary Data Appendix I, eq. A2). Five of the six basic compounds had a $K_{p,\text{out.cell}}$ 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 $K_{p,\text{out.cell}}$ of 4.5 (Fig. 5).

**Discussion**

Since only the unbound drug is available to occupy extracellular receptors, estimation of $C_{u,\text{brain-ISF}}$ could explain why some compounds fail to demonstrate in vivo activity despite in vitro potency and reasonable amounts of drug in brain ($A_{\text{brain}}$). Estimation of $C_{u,\text{brain-ISF}}$ 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 $A_{\text{brain}}$. As an attractive alternative to microdialysis, which directly measures $C_{u,\text{brain-ISF}}$ in vivo, we propose the combined use of in vivo $A_{\text{brain}}$ and in vitro estimates of $V_{u,\text{brain}}$ to calculate $C_{u,\text{brain-ISF}}$. This approach is less labor-intensive than microdialysis and likely to be more successful with lipophilic drugs.

The slice method estimated $V_{u,\text{brain}}$ within a 3-fold range of in vivo results for 14 of the 15 compounds investigated; $V_{u,\text{brain}}$ 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; $V_{u,\text{brain}}$ fell within the 3-fold range for only 10 of the 15 compounds. For example, $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ value of close to 1 contrasted with the higher in vivo value of 5.5 and the slice $V_{u,\text{brain}}$ value of 4 ml · g brain$^{-1}$. 

![FIG. 2. Time course of slice $V_{u,\text{brain}}$ estimations of the 15 model compounds. Error bars represent the standard deviations for five to seven slices.](image-url)
As suggested by the gabapentin result, the $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ 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. $C_{u,\text{cell}}$ could potentially be 2-fold higher than $C_{u,\text{brain}}$, 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) 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) (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 $V_{u,\text{brain}}$, 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

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**TABLE 4**

Statistics of in vitro-in vivo agreement for $V_{u,\text{brain}}$ estimations

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

N.S., not significantly different from 1 ($P > 0.05$).

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**Fig. 3.** Relationship between in vivo $V_{u,\text{brain}}$ values and in vitro slice values (A), in vitro brain homogenate values (B), and $V_{u,\text{brain}}$ 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 $V_{u,\text{brain}}$ values. Symbols for drugs are defined in Table 3.

**Fig. 4.** Relationship between in vivo $V_{u,\text{brain}}$ 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.
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 $V_{u,\text{brain}}$ values increase. It was concluded that 4 h of incubation was sufficient for compounds with a $V_{u,\text{brain}}$ not exceeding 200 ml · g brain$^{-1}$.

The level of uncertainty in in vivo estimates of $V_{u,\text{brain}}$ should also be considered. Even the most careful probe implantation causes a transient loss of BBB integrity. If leakage of drug occurs, it can produce artificially low values of $V_{u,\text{brain}}$ since elevated concentrations around the site of the probe are no longer representative of the overall $C_{u,\text{brainISF}}$, and the $A_{u,\text{brain}}$ is measured in whole brain. The invasiveness of microdialysis has been much discussed, but the large number of studies showing very low unbound drug brain-to-plasma ratios indicates that microdialysis measures the $C_{u,\text{brainISF}}$ reasonably accurately (de Lange et al., 1994; Xie et al., 2000; Gupta et al., 2006).

In the context of the methodological issues discussed above, and considering that the microdialysis experiments were performed at different laboratories, our findings demonstrated remarkable in vitro-in vivo agreement for $V_{u,\text{brain}}$ measurements. Whether the 3-fold range in agreement of this study provides enough accuracy for determination of intrabrain distribution patterns remains open for discussion. It is, however, our opinion that this would be acceptable for most situations in drug discovery programs. Our recommendation is to use the slice method when estimating the $C_{u,\text{brainISF}}$ of compounds that have not been previously characterized in this respect. The slightly easier homogenate method could be used for certain series of compounds after demonstrating agreement with slice or microdialysis $V_{u,\text{brain}}$ values. Using similar methods, Becker et al. (2006) concluded that the brain slice method was equal to or better than the homogenate method for predicting total brain-to-plasma ratios in P-glycoprotein-deficient mice. In silico predictions of $V_{u,\text{brain}}$ based on physicochemical properties may prove more useful than indicated in our study; incorporation of additional molecular descriptors and a larger training dataset of in vivo or slice $V_{u,\text{brain}}$ values would, however, be required.

Counterintuitively, brain tissue binding, as reflected by $V_{u,\text{brain}}$, does not affect exposure of the brain to unbound drug: the steady-state $C_{u,\text{brainISF}}$ is specifically determined by systemic exposure to unbound drug and the unbound drug brain-to-plasma concentration ratio (Hammarlund-Udenaes et al., 1997; Liu and Chen, 2005; Syvanen et al., 2006). Thus, the interest in estimating $V_{u,\text{brain}}$ is associated with the ability to convert $A_{u,\text{brain}}$ to $C_{u,\text{brainISF}}$. Suggested applications of $V_{u,\text{brain}}$ measurements are outlined in Supplemental Data Appendix I. It is doubtful that there will ever be a direct way of measuring $C_{u,\text{cell}}$. The difficulty is in knowing whether the cell-associated amount of drug reflects cellular binding or the uptake and efflux processes that determine $C_{u,\text{cell}}$. In this article, we have provided a theoretical framework and methodology for making that discrimination; the slice method gives the amount of drug associated with the cells ($A_{u,\text{cell}}$) which, in turn, converted to $C_{u,\text{cell}}$ with the homogenate estimate of intracellular binding ($V_{u,\text{cell}}$). This integrative use of the slice and homogenate methods allowed us to estimate the slice $C_{u,\text{cell}}$/ $C_{u,\text{brainISF}}$ ratio ($K_{u,\text{cell/brainISF}}$) for the 15 study compounds. In effect, the slice and homogenate methods may be used in parallel to provide insight into whether active transport systems are operating beyond the BBB. This was clearly observed for gabapentin in comparison with the other drugs. The framework of $C_{u,\text{cell}}$ estimations could also be adapted to tissues other than brain and used in a variety of research areas.

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 $V_{u,\text{brain}}$ 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 1) 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, and 3) in combination with the homogenate method, provide additional information on intracellular concentrations of unbound drug.

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MEASUREMENT OF UNBOUND DRUG IN BRAIN

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