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In vitro methods for estimating unbound drug concentrations in the brain interstitial and intracellular fluids

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D, Non standard abbreviations:

BBB – blood-brain barrier

BSA – bovine serum albumin

CIR – confidence interval ratio

CNS – central nervous system

CSF – cerebrospinal fluid

GABA – gamma-aminobutyric acid

ICF – intracellular fluid

ISF – interstitial fluid

LC-MS/MS – liquid chromatography tandem mass spectrometry

M3G – morphine-3-glucuronide

M6G – morphine-6-glucuronide

Nomenclature list and Appendix I are available as supplemental data.

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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 brain has been a common but unfortunate 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 paper will 1) allow quantification of active transport at the BBB *in vivo*, 2) facilitate the establishment of a relationship between *in vitro* potency and *in vivo* activity for compounds acting on CNS targets, and 3) provide information on intracellular concentrations of unbound drug.

Introduction

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 in order 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 brain and therefore commonly referred to as the total brain concentration.

Since 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 “logBB”, 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

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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, 2002; Shen et al., 2004). The only method of directly measuring $C_{u,brainISF}$ 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 (1995) introduced the unbound volume of distribution in the brain ($V_{u,brain}$) to relate $C_{u,brainISF}$ to A_{brain} , where $V_{blood} \times C_{blood}$ is the amount of drug present in the blood vessels of the brain:

$$V_{u,brain(MD)} = \frac{A_{brain} - V_{blood} \times C_{blood}}{C_{u,brainISF}} \quad 1$$

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, while 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, $C_{u,brainISF}$ 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

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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 in order 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_{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 $V_{u,brain}$. 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 fourteen 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) and included in the study.

Chemicals

Alovudine (3'-fluorothymidine), R,S-apomorphine, codeine, diazepam, ^{14}C - inulin, norfloxacin, thiopental and bovine serum albumin (BSA) (initial fractionation by cold alcohol precipitation, Lot 40K0896) were obtained from Sigma (St Louis, MO). Morphine, morphine-3-glucuronide, morphine-6-glucuronide and oxycodone were obtained from Lipomed (Arleshem, Switzerland). Tritiated γ -amino-butyric acid (^3H -GABA) was purchased from Amersham (Uppsala, Sweden). Gabapentin was purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Racemic cetirizine, the pure enantiomers S- and R-cetirizine, and an internal standard (IS, ucb20028), [2-[2-(4-benzhydrylidene-piperidin-1-yl)-ethoxy]-

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ethoxy]-acetic acid chlorhydrate, were supplied by UCB Pharma (Braine l'Alleud, Belgium). CP-122721 [cis-n-[[2-methoxy-5-(trifluoromethoxy)phenyl]m-ethyl]-2-phenyl-3-piperidinamine] was synthesized at AstraZeneca R&D (Mölndal, Sweden) with purity greater than 95%. All other chemicals were of analytical grade. All solvents were of HPLC grade.

Animals

Male and female Sprague-Dawley rats (Harlan, the Netherlands) weighing 250-350 and 230-280 grams were used for *in vitro* experiments and *in vivo* microdialysis, respectively. Male Dunkin Hartley guinea pigs weighing 450-500 grams 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 five 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 tubing (CMA microdialysis, Solna) 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 previously described (Bostrom et al., 2006). After retrodialysis and washout with blank perfusate, the drug was administered intravenously as a bolus dose plus a four-hour constant rate infusion to obtain steady state. The size of the bolus dose and the infusion rate were adjusted according to the plasma pharmacokinetics. CP-122721 was dissolved in saline. Microdialysate samples were collected at 20-minute intervals from two hours post bolus dose until the termination of the experiment at four hours when brain tissue was

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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 modifications. Drug-naïve animals were sacrificed under isoflurane anesthesia, and the brain was removed and immersed in ice-cold oxygenated pH 7.4 ECF buffer (NaCl, 122; NaHCO₃, 25; glucose, 10; KCl, 3; CaCl₂, 1.4; MgSO₄, 1.2; K₂HPO₄, 0.4; and 4-(2-hydroxyethyl)-n-piperazine ethanesulfonic acid, 10 mmol/L). A 6 mm coronal section was cut with a razor and mounted with cyanoacrylate glue onto the tray of a DTK-Zero1 Microslicer (Dosaka instruments, Japan). Eight 300 µm coronal slices of striatal areas were cut. The slices were preincubated at 37 °C for 5 minutes in 10 ml ECF buffer before the drug, dissolved in ECF buffer, was added. The concentration of drug in the buffer was chosen to match the brain unbound drug concentrations observed in the corresponding *in vivo* microdialysis experiment. The incubations were continuously supplied with a mixture of 5% carbon dioxide in oxygen, keeping the bubbles at some distance from the slice. At prespecified time points after addition of drug (15, 30, 60, 120 and 240 minutes), the slice (~40 mg) was removed from the solution, dried on filter paper and weighed. The slices were homogenized in 9 volumes (w/v) of de-ionized water with an ultrasonic probe (Branson Sonifier 250, Banbury, CT, USA). The ECF buffer and the slice homogenates were stored at -20°C until analysis. $V_{u,brain}$ was calculated according to eq. 2 where A_{slice} , C_{buffer} and V_i are the amount of drug in the slice, the concentration of drug in ECF buffer, and the adherent water volume, respectively.

$$V_{u,brain(s)} = \frac{A_{slice} - V_i \times C_{buffer}}{C_{buffer} (1 - V_i)} \quad 2$$

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V_i was estimated using ^{14}C -inulin in a separate experiment in which brain slices were incubated for 1, 2 and 4 minutes. V_i ($0.091 \text{ mL} \cdot \text{g slice}^{-1}$) was obtained from a plot of $A_{\text{slice}}/C_{\text{buffer}}$ using zero-time back-extrapolation. The possibility of significant drug binding to floating brain debris in the ECF buffer was ruled out for the investigated compounds by quantification of the buffer protein concentration ($9\text{--}14 \mu\text{g/mL}$) with a QuantiProTM BCA Assay Kit (Sigma, St Louis, MO). The ATP content of the slice was determined using an ATP Bioluminescence Assay Kit (CLS II) from Roche Diagnostics (Mannheim, Germany).

The chemical instability of apomorphine enantiomers necessitated the use of $300 \mu\text{mol/L}$ ascorbic acid in the ECF buffer as an antioxidant. At the end of the incubation, ascorbic acid was added to the buffer at a final concentration of 5 mmol/L . Brain slices from apomorphine incubations were homogenized in 5 mmol/L ascorbic acid. Samples containing apomorphine were stored at -70°C until analysis.

Brain homogenate binding

Drug-naïve animals were sacrificed under isoflurane anesthesia, the brain was removed and three volumes of a 180 mmol/L phosphate buffer ($\text{pH } 7.4$) were added. The brains were homogenized on ice with an ultrasonic probe and stored at -20°C until required, when the brain homogenate was thawed and the drug added. Equilibrium dialysis of 1 mL homogenate and buffer was performed in triplicate for 16 hours at 37°C in 1 mL plexi-glass cells mounted with a 5 kD cut-off Diachema cellulose membrane (Dianorm GmbH, München, Germany). An aliquot of homogenate was sampled before and after co-incubation to assess the compound stability. The fraction of unbound drug in diluted brain homogenate, $f_{u,\text{hD}}$, i.e. the buffer-to-homogenate concentration ratio, was used to calculate $V_{u,\text{brain}}$, while also taking into account the dilution, D , associated with homogenate preparation (eq. 3) (Kalvass and Maurer,

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2002).

$$V_{u,brain(h)} = 1 + D \left(\frac{1}{f_{u,hD}} - 1 \right) \quad 3$$

The equation describes a relationship where the lowest possible $V_{u,brain}$ is $0.8 \text{ mL} \cdot \text{g brain}^{-1}$ 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 ($0.2 \text{ mL} \cdot \text{g brain}^{-1}$). The chemical instability of apomorphine enantiomers necessitated the use of 50 mmol/L ascorbic acid in the buffer for brain homogenization and equilibrium dialysis. Samples containing apomorphine were stored at -70°C until analysis.

$V_{u,brain}$ predictions based on $\text{LogD}_{7.4}$

A simple $V_{u,brain}$ prediction model was established using linear regression analysis of *in vivo* $\text{Log } V_{u,brain}$ and $\text{LogD}_{7.4}$ of the studied compounds. The *in vivo* $V_{u,brain}$ for each compound was predicted from its $\text{LogD}_{7.4}$ using the regression line of the other compounds, not including itself. Calculated $\text{ACDLogD}_{7.4}$ 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.

Analytical procedures

The amount of drug in the various sample matrices was quantified with reversed phase liquid chromatography and multiple reaction monitoring mass spectrometry (LC-MS/MS) detection using a Micromass Quattro Ultima instrument (Waters, Manchester, UK) equipped with electrospray run in positive mode for all compounds except thiopental. Gradient elution over 2 minutes with acetonitrile and formic acid 0.2 % with a flow rate of 0.6 mL/min was used.

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Mass transitions and detailed chromatographic conditions for each compound are given in Table 2.

Sample preparation was adapted for any compound specific requirements but followed a general procedure: buffer samples (100 μ L) from the brain slice and brain homogenate experiments were added to a 96-deepwell plate (Nalgen Nunc International, NY) and diluted with a volume of 0.2 % formic acid containing an appropriate amount of acetonitrile. Fifty μ L samples of brain homogenates were protein precipitated with 150 μ L cold acetonitrile containing 0.2 % formic acid. After 1 min vortexing and 20 min 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 precipitated in a similar manner. External calibration curves with at least five different concentrations were made from a serial dilution in 50 % acetonitrile 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 (Turku, Finland) and an OptiPhase 'HiSafe 3' scintillation cocktail (Fisher Chemicals, Loughborough, England). Brain slices were solubilized with 1 mL Soluene-350 (Perkin Elmer, Boston, MO) and decolorized with 100 μ L hydrogen peroxide.

Data presentation and statistical analysis

Values of $V_{u,brain}$ are expressed as means \pm standard deviation. Data were log transformed for the statistical analysis, and the *in vivo* values of $V_{u,brain}$ were taken as accurate. Agreement

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with *in vivo* $V_{u,brain}$ data was assessed according to Altman and Bland (Altman and Bland, 1983; 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 7 slices or 3 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 $\text{LogD}_{7.4}$ -based prediction model was also assessed using 90 % CIR.

Definitions and relationships

The basic assumption of this study was that the intra-brain 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 $\text{mL} \cdot \text{g brain}^{-1}$ 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 vs 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

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synonymous with the brain-to-plasma concentration ratio and has blood or plasma as reference. The V_D term is commonly used with respect to the intravenous injection technique (Patlak et al., 1983), the *in situ* brain perfusion method (Dagenais et al., 2000) and positron emission tomography (PET) (Koeppe, 2002).

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

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

A_{brain} ($\mu\text{mol} \cdot \text{g brain}^{-1}$) comprises the amount of unbound drug in the ISF plus the amount of drug associated with the cells:

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

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

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

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

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

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 $0.2 \text{ mL} \cdot \text{g brain}^{-1}$ (Nicholson and Sykova, 1998). This is, from a physiologic perspective, the smallest $V_{u,brain}$

possible. A value close to the brain water volume ($0.8 \text{ mL} \cdot \text{g brain}^{-1}$) (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 ICF 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 pKa and the pH in the extra- and intracellular compartments, pH_{ISF} and pH_{cell} , respectively (eq. 8, 9).

$$V_{u,\text{brain}} = V_{\text{brainISF}} + V_{\text{cell}} \times V_{u,\text{cell}} \times \frac{10^{\text{pKa} - \text{pH}_{\text{cell}}} + 1}{10^{\text{pKa} - \text{pH}_{\text{ISF}}} + 1} \quad (\text{bases}) \quad 8$$

$$V_{u,\text{brain}} = V_{\text{brainISF}} + V_{\text{cell}} \times V_{u,\text{cell}} \times \frac{10^{\text{pH}_{\text{cell}} - \text{pKa}} + 1}{10^{\text{pH}_{\text{ISF}} - \text{pKa}} + 1} \quad (\text{acids}) \quad 9$$

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

$$V_{u,\text{cell}} = 1 + \frac{D}{V_{\text{cell}}} \left(\frac{1}{f_{u,\text{hD}}} - 1 \right) \quad 10$$

Accordingly the cells in the homogenate are not only diluted with the added buffer but also in a small volume of brain ISF devoid of plasma proteins. It is assumed in eq. 5-10 that drug binding to the outside of the cell is negligible compared to 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

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substantially influence $V_{u,cell}$ 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 lipophilicity. 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.

Appendix I describes suggested procedures for experimental estimation of unbound drug concentrations in brain ISF and ICF as well as the unbound brain-to-plasma and ICF-to-ISF distribution ratios $K_{p,uu}$ and $K_{p,uu,cell}$.

Results

The *in vivo* $V_{u,brain}$ of the compounds in this study spanned three 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 $V_{u,brain}$ obtained with the investigated methods are also illustrated in Figs 2-3.

The distribution volume of the extracellular marker ^{14}C -Inulin after 4 hours' incubation was $0.36 \pm 0.037\ mL*g\ brain^{-1}$. As 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-hour incubation ($\sim 8\ nmol*mg\ protein^{-1}$). The $V_{u,brain}$ of 3H -GABA, used to demonstrate functionality in terms of cellular transport, reached a maximum ($17 \pm 4.7\ mL*g\ brain^{-1}$) after 60 minutes 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 minutes, 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 diazepam and gabapentin. Because these indicated that 8 hours of incubation was necessary to achieve equilibrium between the dialysis cells, overnight incubation for 16 hours was

assumed sufficient for all compounds.

Agreement between the methods is illustrated in Fig. 3, in which *in vitro* $V_{u,brain}$ determined by the slice or homogenate methods 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, while 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 to 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 ($1.3 \text{ mL} \cdot \text{g brain}^{-1}$) was higher than the *in vivo* value ($0.25 \text{ mL} \cdot \text{g brain}^{-1}$). The value for this drug using the slice method ($0.53 \text{ mL} \cdot \text{g brain}^{-1}$) was closer to the *in vivo* value. The extracellular slice distribution volume of ^{14}C -inulin was $0.36 \text{ mL} \cdot \text{g brain}^{-1}$. Further, the gabapentin $V_{u,brain}$ from the homogenate experiment ($1.04 \text{ mL} \cdot \text{g brain}^{-1}$) 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 $\text{mL} \cdot \text{g brain}^{-1}$, 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 ($V_{u,brain} 12 \text{ mL} \cdot \text{g brain}^{-1}$) that was indicated to a lesser extent in the slice method ($6.5 \text{ mL} \cdot \text{g brain}^{-1}$) compared to *in vivo* with microdialysis ($2.5 \text{ mL} \cdot \text{g brain}^{-1}$).

Linear regression analysis of all data points of a plot of *in vivo* $V_{u,brain}$ vs. $\text{LogD}_{7.4}$ (Fig. 4)

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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 $LogD_{7.4}$ using the regression line are presented in Table 3. All $V_{u,brain}$ predictions were made without gabapentin in the model, as the $V_{u,brain}$ value for gabapentin was known to reflect active transport mechanisms. The $V_{u,brain}$ predictions from $LogD_{7.4}$ were not as accurate as those using experimental methods. Excluding the $V_{u,brain}$ prediction of gabapentin gave a 90% CIR of the $LogD_{7.4}$ 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).

$K_{p,uu,cell}$, 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 (Appendix I, eq. A2). Five of the six basic compounds had a $K_{p,uu,cell}$ 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 $K_{p,uu,cell}$ of 4.5 (Fig 5).

Discussion

Since only the unbound drug is available to occupy extracellular receptors, estimation of $C_{u, \text{brainISF}}$ 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 $C_{u, \text{brainISF}}$ also allows quantification of the extent of BBB drug transport and investigation of the function of active transporters *in vivo* without confounding by non-specific 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 $C_{u, \text{brainISF}}$ *in vivo*, we propose the combined use of *in vivo* A_{brain} and *in vitro* estimates of $V_{u, \text{brain}}$ to calculate $C_{u, \text{brainISF}}$. 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 three 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 α -amino acid transporter (Su et al., 1995). As 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 (MRPs) 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 $C_{u,brainISF}$, depending on the pKa of the drug and assuming a difference of 0.3 pH units 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 ¹⁴C-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,

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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 to BBB transport (Wang and Welty, 1996), but this finding cannot be generally 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. While 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 hours' 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 produce artefactually low values of $V_{u,brain}$, since elevated concentrations around the site of the probe are no longer representative of the overall $C_{u,brainISF}$, and the A_{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 indicate that microdialysis measures the $C_{u,brainISF}$ 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

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microdialysis experiments were performed at different laboratories, our findings demonstrated remarkable *in vitro* - *in vivo* agreement for $V_{u,brain}$ measurements. Whether the 3-fold range in agreement of this study provides enough accuracy for determination of intra-brain 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 $C_{u,brainISF}$ 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 $V_{u,brain}$ 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 Pgp-deficient mice. *In silico* predictions of $V_{u,brain}$ 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 $V_{u,brain}$ values would, however, be required.

Counter-intuitively, brain tissue binding, as reflected by $V_{u,brain}$, does not affect exposure of the brain to unbound drug: the steady-state $C_{u,brainISF}$ 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 $V_{u,brain}$ is associated with the ability to convert A_{brain} to $C_{u,brainISF}$. Suggested applications of $V_{u,brain}$ measurements are outlined in Appendix I.

It is doubtful if there will ever be a direct way of measuring $C_{u,cell}$. The difficulty is in knowing whether the cell-associated amount of drug reflects cellular binding or the uptake and efflux processes that determine $C_{u,cell}$. In this paper, we have provided a theoretical

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framework and methodology for making that discrimination; the slice method gives the amount of drug associated with the cells (A_{cell}) which is, in turn, converted to $C_{\text{u,cell}}$ with the homogenate estimate of intracellular binding ($V_{\text{u,cell}}$). This integrative use of the slice and homogenate methods allowed us to estimate the slice $C_{\text{u,cell}}/C_{\text{u,brainISF}}$ ratio ($K_{\text{p,uu,cell}}$) 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 $C_{\text{u,cell}}$ 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 $V_{\text{u,brain}}$ 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 are 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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Footnotes

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Legends for figures

Fig. 1

The compartment model describing intra-brain 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.

Fig. 2

Time course of slice $V_{u,brain}$ estimations of the 15 model compounds. Error bars represent the standard deviations for 5-7 slices.

Fig. 3

Relationship between *in vivo* $V_{u,brain}$ values and (A) *in vitro* slice values, (B) *in vitro* brain homogenate values, and (C) $V_{u,brain}$ values predicted from $\text{LogD}_{7.4}$. The solid line represents perfect agreement. The dashed lines represent a 3-fold over- or under-estimation compared with *in vivo* $V_{u,brain}$ values. Symbols for drugs are defined in Table 3.

Fig. 4

Relationship between *in vivo* $V_{u,brain}$ values and lipophilicity estimated as $\text{LogD}_{7.4}$. 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.

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Fig 5.

The $K_{p,uu,cell}$ of the model compounds represents the ratio of concentrations of unbound drug in the intracellular and interstitial brain fluids ($C_{u,cell}/C_{u,brainISF}$). Basic compounds (black bars) generally had higher ratios than neutral compounds (open bars). The zwitterionic compounds, gabapentin and morphine-3-glucuronide (gray bars) had the highest and lowest $C_{u,cell}/C_{u,brainISF}$ ratios, respectively.

Tables

Table 1.

Physicochemical description of drugs included in the study. Values of LogD_{7.4} (ACDLogD pH7.4) and pKa (ACDpKa) were calculated using ACDlabs databases version 9.03 (Advanced Chemistry Development Inc., Toronto, Canada). Experimentally determined LogD_{7.4} and pKa values were obtained from the literature.

Compound	Classification	ACDlogD7.4	logD _{7.4}	ACDpK _a	pK _a
Alovudine	Neutral	-0.52		9.21	
Apomorphine	Base	2.51		7.88	
Cetirizine	Zwitterion	-1.25	1.5 ^a	6.22	2.93, 8.00 ^a
Codeine	Base	0.30	0.22 ^b	8.23	8.22 ^b
CP-122721	Base	1.88		9.85	
Diazepam	Neutral	2.96		3.4	
Gabapentin	Zwitterion	-1.31		4.72, 10.27	
Morphine	Base	-0.36	-0.07 ^b	8.25	8.21 ^b
M3G	Zwitterion	-4.81	-1.12 ^b	2.79, 9.75	8.18 ^b
M6G	Zwitterion	-4.39	-0.79 ^b	2.79, 9.73	8.22 ^b
Norfloxacin	Zwitterion	-0.98		0.18, 8.34	
Oxycodone	Base	1.27		7.57	
Thiopental	Neutral	2.99		7.95	

^a (Plember van Balen et al., 2001)

^b (Avdeef et al., 1996)

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Table 2.

Conditions for HPLC/MS/MS analysis.

Compound	Mass Transition	Column	Initial Conditions	Final Conditions	Retention Time (min)
Alovudine	245.1→126.9	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.04
R,S-Apomorphine	268.3→191.1	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.17
R,S-Cetirizine	389.2→200.9	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.70
Codeine	300.0→151.9	Atlantis C18 5×2.1, 5 μ ^a	C 100%	C 80%, B 20 %	2.05
CP-122721	381.4→160.1	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.51
Diazepam	285.2→154.1	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.86
Gabapentin	172.2→137.1	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	0.88
Morphine	286.0→152.0	Atlantis C18 5×2.1, 5 μ ^a	A 95%, B 5%	A 10%, B 90 %	1.45
M3G	461.0→285.9	Atlantis C18 5×2.1, 5 μ ^a	C 100%	C 80%, B 20 %	1.25
M6G	462.3→286.1	Atlantis C18 5×2.1, 5 μ ^a	C 100%	C 80%, B 20 %	1.41
Norfloxacin	320.3→276.2	Polaris C18 5×2, 5 μ ^b	A 95%, B 5%	A 10%, B 90 %	1.07
Oxycodone	316.0→240.9	Atlantis C18 5×2.1, 5 μ ^a	C 100%	C 80%, B 20 %	2.01
Thiopental	241.0→100.9	Hypurity C18 5×2.1, 5 μ ^c	A 95%, B 5%	A 10%, B 90 %	1.64

A, 2 % acetonitrile in 0.2 % formic acid; B, 0.2 formic acid in acetonitrile; C, 0.2 % formic acid in de-ionized water.

a Waters corporation, Manchester, UK

b Varian Inc., Torrance, CA, USA

c Chromtech, Hägersten, Sweden

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Table 3.

$V_{u,brain}$ values for the model compounds determined by microdialysis, the slice uptake and homogenate binding methods, and by predictions from $\text{LogD}_{7.4}$. The values are given in $\text{mL} \cdot \text{g brain}^{-1}$ and are presented as means \pm SD. The microdialysis values are taken from the literature according to 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.

	In vivo microdialysis		<i>In vitro</i> brain slice uptake (n=5-7)	<i>In vitro</i> brain homogenate binding (n=3)	Predicted from $\text{LogD}_{7.4}$
Alovudine (A) ^{b e f}	(Linden et al., 2003)	0.52 ^m	1.07 \pm 0.091	1.05 \pm 0.30	0.66
R-Apomorphine (R-A) ^{c f}	(Sam et al., 1997)	15 ^m	18.1 \pm 1.9	15.5 \pm 0.38	16.8
S-Apomorphine (S-A) ^{c f}	(Sam et al., 1997)	26 ^m	14.0 \pm 1.4	14.4 \pm 0.76	15.5
R-Cetirizine (R-C) ^{a d e f}	(Gupta et al., 2006)	2.39 ⁿ	6.62 \pm 0.56	11.8 \pm 0.80	5.99
S-Cetirizine (S-C) ^{a d e f}	(Gupta et al., 2006)	2.86 ⁿ	6.56 \pm 0.61	11.8 \pm 0.80	4.90
Codeine (C) ^g	(Xie and Hammarlund-Udenaes, 1998)	3.6 \pm 1.2 ⁿ	2.77 \pm 0.22	1.46 \pm 0.18	1.41
CP-122721 (CP) ^f		207 \pm 14 ^o	210 \pm 17	152 \pm 19	21.3
Diazepam (D) ^{j k}	(Dubey et al., 1989)	22 ^m	17.7 \pm 0.80	24.6 \pm 1.2	28.1
Gabapentin (G) ^f	(Wang and Welty, 1996)	5.5 \pm 2.0 ⁿ	3.97 \pm 0.77 ^p	1.04 \pm 0.14	0.27
Morphine (M) ^f	(Tunblad et al., 2003)	1.7 ⁿ	2.69 \pm 0.43	2.00 \pm 0.20	0.67
Morphine-3-glucuronide (M3G) ⁱ	(Xie et al., 2000)	0.25 \pm 0.02 ⁿ	0.528 \pm 0.059	1.27 \pm 0.18	0.36
Morphine-6-glucuronide (M6G) ⁱ	(Bouw et al., 2001)	0.20 \pm 0.02 ⁿ	0.731 \pm 0.078	1.24 \pm 0.10	0.57
Norfloxacin (N) ^{i l}	(Ooie et al., 1997)	0.98 \pm 0.59 ⁿ	2.01 \pm 0.10	1.72 \pm 0.37	0.31
Oxycodone (O) ^g	(Bostrom et al., 2006)	2.20 \pm 0.53 ⁿ	3.60 \pm 0.35	1.95 \pm 0.33	4.60
Thiopental (T) ^h	(Mather et al., 2000)	10 ^m	5.07 \pm 0.29	6.51 \pm 0.89	34.8

a, Experiments performed in guinea pigs

b, Drug administration by subcutaneous bolus dose

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- c, Drug administration by continuous subcutaneous infusion
- d, Drug administration by short intravenous infusion
- e, Estimation of $V_{u,brain}$ during non-steady-state conditions
- f, Probe calibration by *in vivo* retrodialysis by drug
- g, Probe calibration by *in vivo* retrodialysis by calibrator
- h, Probe calibration by *in vivo* retrodialysis by drug and correction
- i, Probe calibration *in vivo* by reference compound
- j, Probe calibration *in vitro*
- k, Probe placement in cortex
- l, Probe placement in hippocampus
- m, $V_{u,brain}$ calculated from data in original literature report
- n, $V_{u,brain}$ reported in original literature
- o, $V_{u,brain}$ experimentally obtained from original data in this study
- p, $V_{u,brain}$ calculated from slices incubated for 1 hour.
- ns, Not stated

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Table 4.

Statistics of *in vitro* - *in vivo* agreement for $V_{u,brain}$ estimations. The statistical analysis was performed for all 15 compounds. Bias and confidence interval ratios (CIR) indicate the likely range of the difference between the *in vitro* or *in silico* estimations of $V_{u,brain}$ and *in vivo* values.

	Slice	Homogenate	LogD _{7.4}
Bias (ratio of <i>in vitro</i> to <i>in vivo</i> estimation)	1.34 (ns)	1.31 (ns)	0.807 (ns)
Bias excluding gabapentin	1.40 (ns)	1.50 (ns)	0.985 (ns)
90 % CIR	2.95	6.00	8.97
90 % CIR excluding gabapentin	2.96	4.91	6.02

ns: not significantly different from 1 (p>0.05)

Figure 1

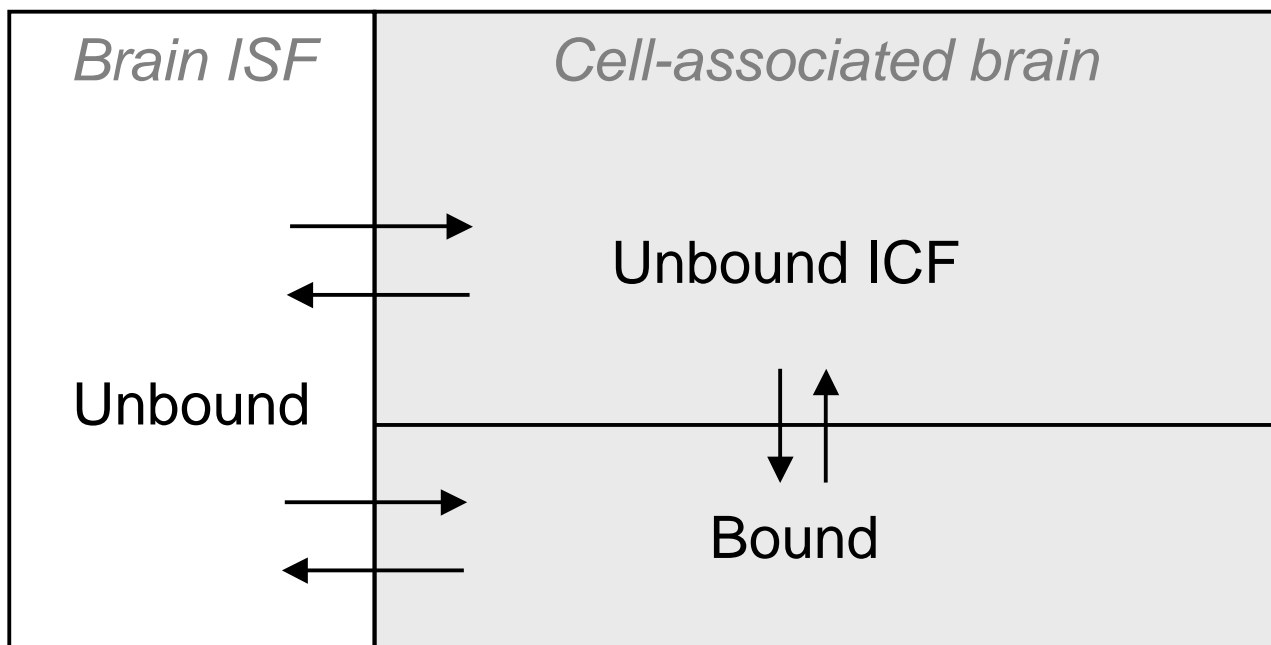


Figure 2

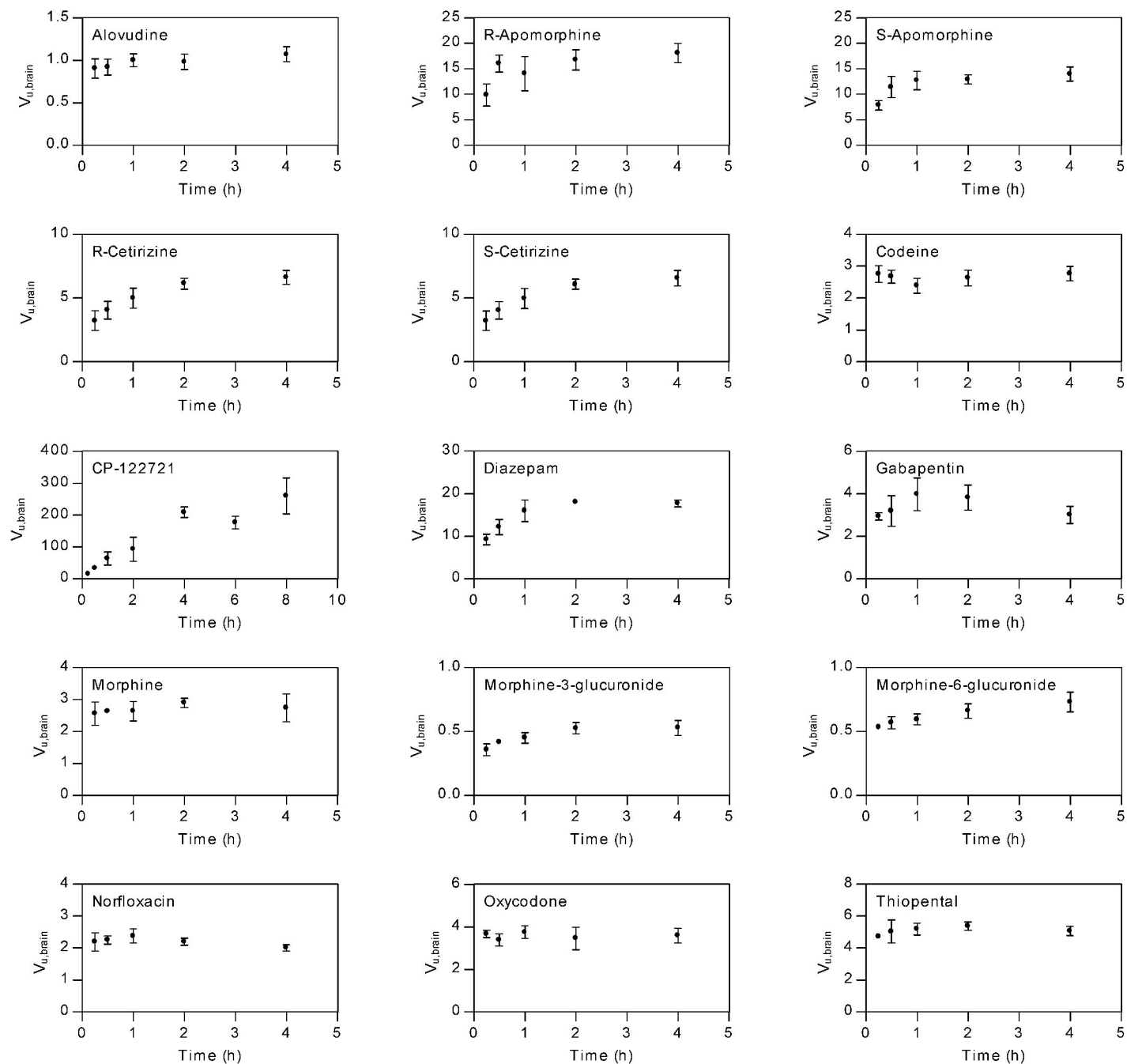


Figure 3

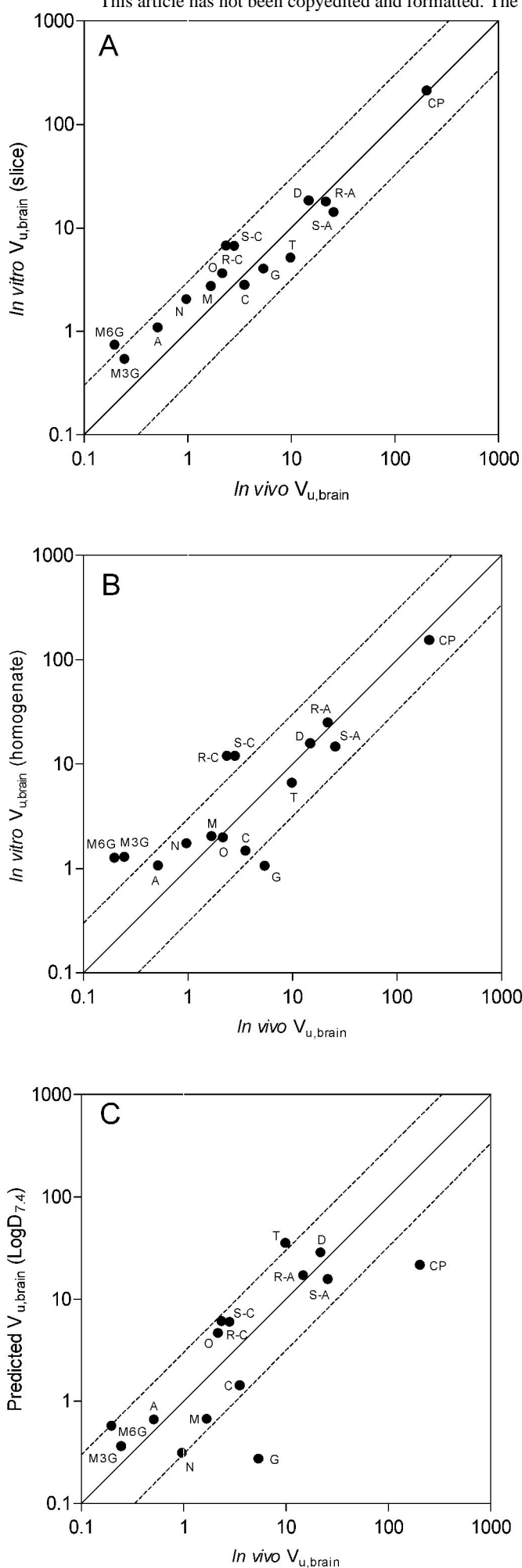


Figure 4

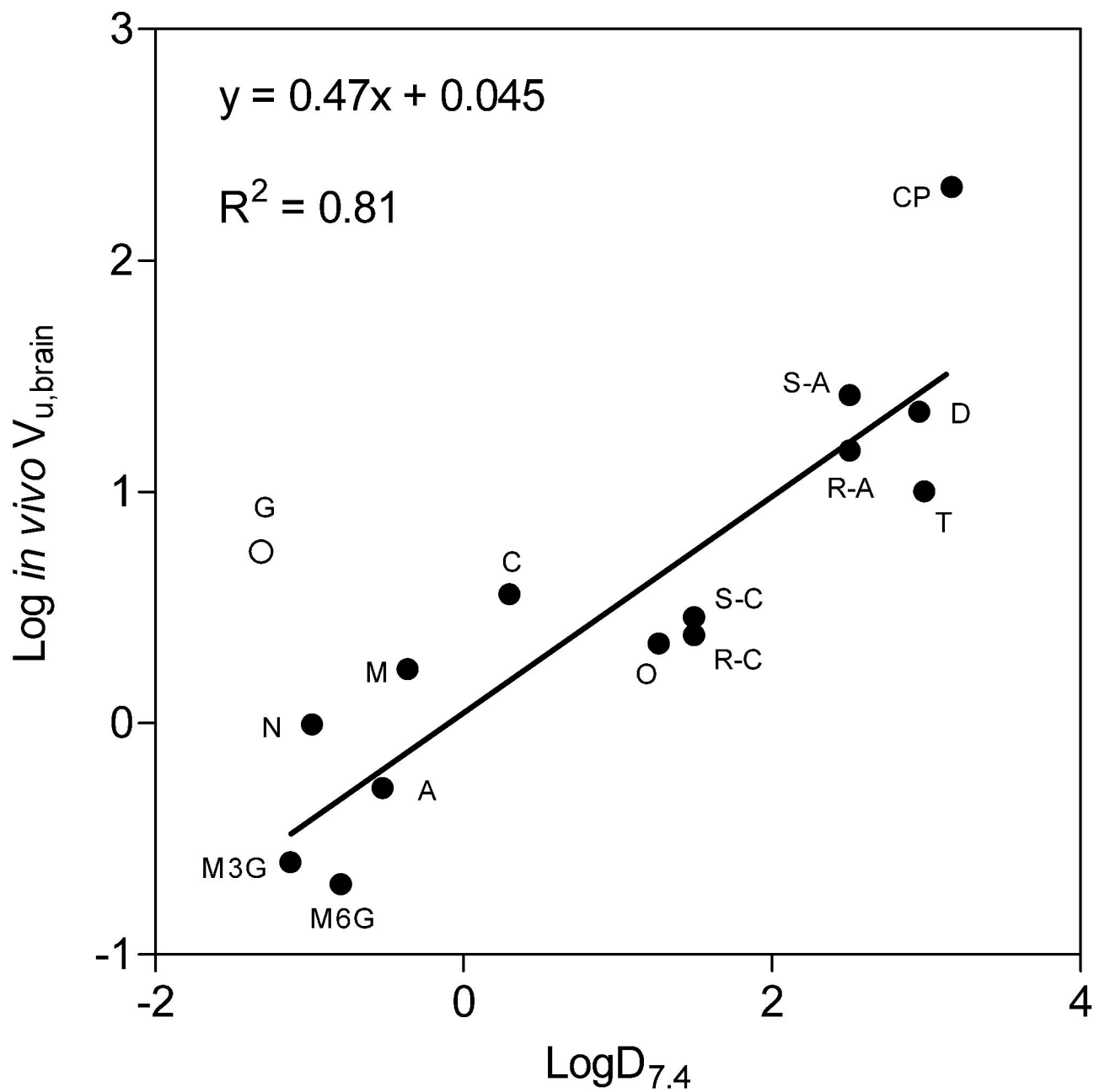


Figure 5

