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Comparison of in vitro BBMEC permeability and in vivo CNS uptake by microdialysis sampling

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Abstract

The studies presented in this report were designed to assess the correlation of the bovine brain microvessel endothelial cell (BBMEC) apparent permeability coefficient (P_{app}) and in vivo BBB penetration using microdialysis sampling. A mathematical model was developed to describe the relationship of brain extracellular fluid (ECF) concentration to free drug in plasma. The compounds studied have a broad range of physico-chemical characteristics and have widely varying in vitro and in vivo permeability across the blood–brain barrier (BBB). BBMEC permeability coefficients vary in magnitude from a low of 0.9×10^{-5} cm/s to a high value of 7.5×10^{-5} cm/s. Corresponding in vivo measurements of BBB permeability are represented by clearance (CL_{in}) into the brain ECF and range from a low of 0.023 μl/min/g to a high of 12.9 μl/min/g. While it is apparent that in vitro data from the BBMEC model can be predictive of the in vivo permeability of a compound across the BBB, there are numerous factors both prior to and following entry into the brain which impact the ultimate uptake of a compound. Even in the presence of high BBB permeability, factors such as high plasma protein binding, active efflux across the BBB, and metabolism within the CNS can greatly limit the ultimate concentrations achieved. In addition, concentrations in the intracellular space may not be the same as concentrations in the extracellular space. While these data show that the BBMEC permeability is predictive of the in vivo BBB permeability, the complexity of the living system makes prediction of brain concentrations difficult, based solely on the in vitro measurement.

Keywords: BBMEC permeability; CNS uptake; Microdialysis sampling

1. Introduction

The blood–brain barrier (BBB) functions to restrict the penetration of blood-borne substances into the CNS, regulates the concentration of compounds, such as amino acids, which are necessary for CNS function, and protects the brain from

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toxins. Due to its protective nature, the BBB often impedes treatment of CNS diseases by blocking the entry of potentially important drugs.

While lipophilicity and molecular weight have long been considered to be the most important factors in prediction of penetration into the CNS [1–3], recent studies have shown that the BBB is a complex active interface between the blood and the brain [4,5]. No longer is the BBB thought of as a passive barrier to the penetration of compounds. In addition to passive diffusion, numerous active processes have recently been discovered which regulate the passage of compounds into and out of the brain. Such active mechanisms as transcytosis [6], carrier-mediated influx by amino acid and peptide transporters [7] and carrier-mediated efflux by carriers like *p*-glycoprotein [8] are essential to CNS function.

Numerous methods are used to assess the degree to which a compound enters the brain. Of these methods, the bovine brain microvessel endothelial cell (BBMEC) model is widely used in initial screening efforts to identify compounds that have the potential for BBB penetration. Additionally, the BBMEC model is often used to elucidate BBB transport mechanisms.

The BBMEC culture system is an in vitro model of the BBB that utilizes endothelial cells which form the interface between the blood and the extracellular fluid of the brain. BBMEC primary cultures form confluent monolayers on solid supports; these monolayers retain many morphological and biochemical properties of the BBB. An in vitro BBB model permits an investigation where experimental parameters, such as temperature, pH and pressure, are easily controlled.

The BBMEC system has been used in studies of uptake [9], transport [10] and metabolism [11] by the BBB. Numerous studies have shown a correlation of BBMEC permeability and CNS uptake [12–15]. There have, however, been no studies that compare in vitro BBMEC permeability and in vivo permeability into the brain extracellular fluid (ECF). Since the brain ECF is the first compartment through which a compound must pass after penetrating the BBB, assessment of the temporal relationship between the ECF and free compound in the plasma can provide a direct measurement of barrier permeability. Microdialysis sampling is a method that provides access to the extracellular space of the brain thus allowing for this determination.

Microdialysis is a sampling method which employs a short length of hollow dialysis fiber, permeable to water and small solutes, but excludes large molecules such as proteins and enzymes due to the molecular weight cut-off of the fiber [16]. In an in vivo investigation, the microdialysis probe is implanted into the region of interest and a physiological buffer which closely matches the pH and ionic strength and composition of the surrounding tissue is pumped through the probe. Due to the concentration gradient across the membrane, the unbound fraction of analyte present in the ECF diffuses into the probe lumen and is collected for analysis. An in depth description of microdialysis sampling and its application can be found elsewhere [16–19].

Both in vivo microdialysis sampling [20–22] and the in vitro BBMEC culture system [5,9,23,24] have been shown to provide worthwhile information with regard to BBB permeability. The objective of this study was to assess the BBB penetration of several compounds by microdialysis sampling in the rat striatum during i.v. infusion. This data was then compared with information about the BBB permeation of these compounds found using the in vitro BBMEC model.

2. Materials and methods

2.1. Materials

Compounds A–D were obtained from Hoechst Marion Roussel (Cincinnati, OH). ^{14}C-mannitol was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Caffeine, tryptophan and kynurenic acid were obtained from Sigma (St. Louis, MO). Sodium phosphate was obtained from Mallinckrodt (Chesterfield, MO) and HPLC grade acetonitrile was obtained from Burdick and Jackson, Muskegon, MI. Water (18 MΩ) was purified prior to use using a NANOpure II system (Barnstead, Dubuque, IA). All reagents used in the preparation of LC mobile phase solu-

tions were of analytical reagent grade or better and were used as received. The microdialysis perfusion fluid used was artificial cerebral spinal fluid (aCSF) and consisted of 2.5 mM KCl (Sigma), 1.18 mM $MgCl_2$ (Sigma), 1.26 mM $CaCl_2$ (JT Baker, Phillipsburg, NJ) and 125 mM NaCl (Mallinckrodt).

2.2. In vitro BBMEC transport study design

Penetration across the BBMEC monolayer was monitored at 37 °C for 60 min for all compounds studied. A high and low concentration for each analyte was chosen based on the predicted in vivo steady-state plasma levels. Studies in both the apical-to-basolateral (A to B) and basolateral-to-apical (B to A) directions were conducted. An apparent permeability coefficient (P_{app}) was then determined for each compound using the equation

$$P_{app} = \text{slope}/(C_{DO}A) \quad (1)$$

were slope is the slope of the line (ng/s), C_{DO} is the initial concentration of the donor chamber and A is the membrane surface area (0.636 cm^2).

2.3. BBMEC cell isolation and culture procedures

BBMECs were isolated from the gray matter of the bovine cerebral cortex by enzymatic digestion followed by subsequent centrifugations and seeded into primary culture [23,24]. Polycarbonate membranes (13 mm; pore size 3.0 μm; diffusion area 0.636 cm^2) were placed in tissue culture dishes (100 mm; Corning, Corning, NY) and coated with rat-tail collagen and bovine fibronectin (Sigma). Isolated brain microvessel endothelial cells were seeded onto the prepared tissue culture dishes at a density of 50 000 cells/cm^2 in a culture medium consisting of 45% minimum essential medium, 45% Ham's F12 nutrient mix (Gibco, Life Technologies, Grand Island, NY, USA), 10 mM HEPES, pH 7.4, 13 mM sodium bicarbonate, 10% plasma-derived equine serum, 100 mg/ml heparin, 100 mg/ml streptomycin, 100 mg/ml penicillin G, 50 mg/ml polymyxin B and 2.5 mg/ml amphotericin B (Sigma Chemical Co.). The cells were cultured at 37 °C with 95% humidity and 5% CO_2 and were fed on the third day after seeding and then every 2 days until confluent monolayers were formed (10–14 days). Confluence was determined by inspecting the areas around the polycarbonate membranes with an inverted microscope. The basolateral side of the cells was defined as the side facing the collagen matrix.

2.4. In vitro BBMEC transport studies

Once confluence was reached (10–14 days), the membranes were placed in a horizontal side-by-side diffusion apparatus (Crown Glass Inc., Somerville, NJ, USA) for permeability studies. The receiver chamber was filled with 3.0 ml of culture medium and the temperature was maintained at 37 °C with an external circulating water bath. At $t = 0$, the donor chamber was filled with 3.0 ml of the drug or analyte dissolved in culture medium. The contents of each chamber were stirred with a Teflon coated magnetic stir bar. Aliquots of 100 μl were removed from the donor chamber at 0 and 60 min to be assayed for initial and final donor concentrations. Sample aliquots of 100 μl were removed from the receiver chamber at intervals between 0 and 60 min. After each receiver sample, the volume was replaced with fresh medium. Samples to be analyzed by LC were aliquoted into 300 μl autosampler vials (Chromacol, Trumbull, CT) and frozen at −20 °C until analysis. ^{14}C-mannitol samples were placed in scintillation vials with 5 ml of scintillation cocktail and assayed by liquid scintillation counting (LSC).

For each transport study the amount of analyte in the receiver reservoir was plotted versus time and a slope calculated by linear regression. The apparent permeability coefficient (P_{app}) was calculated using Eq. (1).

2.5. Microdialysis study design

For all studies, a microdialysis probe was implanted into the striatum, a Silastic cannula was implanted into the right external jugular vein and a PE-10 cannula was implanted into the right femoral vein. Following a 24 h recovery period each compound was administered to the rat ($n =$

3/compound) for 4 h. Brain dialysate and plasma were collected during the infusion and for 4 h after the end of the infusion. Plasma samples were collected at 0, 15, 30, 60, 120, 240, 245, 255, 270, 300, 360, 420 and 480 min after the start of the infusion and dialysate samples were collected every 10 min throughout the experiment. For Compound B plasma samples were collected at 0, 1, 5, 15, 30, 60, 120, 180 and 240 min after the start of infusion.

2.6. Microdialysis probe preparation

As a check of the integrity of the microdialysis probe before implantation, the in vitro recovery was calculated from the results of a delivery experiment. Following insertion into the striatum, the probe was perfused with aCSF at a flow rate of 1.0 μl/min until the start of the study. Prior to dosing, the probe was calibrated in vivo by perfusing it with a solution containing the analyte of interest and determining the concentration of the analyte in the perfusate relative to the initial concentration in the perfusion medium. The in vivo delivery of the probe was then calculated using the following equation:

$$\text{Delivery} = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}}} \quad (2)$$

In each case, recovery was assumed to equal delivery.

2.7. Animal preparation

Male Sprague-Dawley rats weighing between 250 and 350 g were anesthetized using a 0.2 ml/100 g body weight intramuscular dose of 38 mg/ml ketamine with 2.42 mg/ml xylazine. Using aseptic surgical procedures, a polyethylene dosing cannula (PE-10, 0.61 mm OD) was implanted into the right femoral vein and externalized at the back of the neck. A Silastic sampling cannula (0.037 in. O.D.) was implanted into the right external jugular vein and externalized at the back of the neck. After implantation of the dosing and sampling cannulas, the animal was placed in a stereotaxic frame (ASI Instruments) and a siliconized guide cannula (CMA/Microdialysis, Acton, MA) was inserted into the striatum at the coordinates A 1.2 mm, M 2.5 mm and V 2.5 mm relative to the bregma. The guide cannula was secured with dental acrylic (Sevriton, York, PA) and a microdialysis probe (CMA/12; 4 mm probe length) was inserted through the guide cannula; the tip of the probe was 6.5 mm ventral relative to the bregma.

After insertion of the microdialysis probe, the animal was placed in a Plexiglas containment system (CMA/120) and allowed to recover for approximately 24 h prior to dosing. The animal containment system was modified to accommodate the number of liquid lines required in this experimental set-up in a fashion similar to that reported by Malhotra et al. The liquid swivel was replaced with a length of rigid plastic tubing, connecting the balance arm and the rat. This tubing held the rat stationary relative to the bowl and prevented entangling the liquid lines. To facilitate movement, the Plexiglas containment bowl was placed on a laboratory turntable [23] which allowed the bowl to rotate as the rat moved. This modified system allowed free access to food and water and allowed for greater than four liquid lines to the animal. Prior to and during each study, the animals were housed in a temperature and humidity controlled room with a 12 h light (7 AM to 7 PM)/dark cycle and were allowed free access to food and water. The studies described in this report were conducted in accordance with the 'Principles of Laboratory Animal Care' (NIH publication # 85-23, revised 1985) and the institutional review committee.

2.8. In vitro plasma protein binding

The plasma protein binding of Compounds A, B and D was investigated in rat plasma by ultrafiltration (Amicon, Beverly, MA). Plasma samples, in triplicate, were prepared at concentrations from 0.1–20.0 μg/ml for Compounds A and B and 1.0–300 μg/ml for Compound D. Samples were allowed to equilibrate at 37 °C for 30 min prior to centrifugation. After equilibration, the samples were centrifuged at 3000 rpm for 10 min. The resulting ultrafiltrate samples were analyzed by the same methods used for dialysate samples. Nonspecific adsorption was investigated by treat-

ing aqueous standards of each compound in the same manner.

2.9. Dose preparation and administration

Compounds A and D dosing solutions were prepared by dissolving an appropriate amount of drug in 0.1 M NaOH and adjusting the pH to 7.2–7.4 with dilute (0.1 M) HCl. The resulting solution was brought to final volume (3 ml) with n-saline. The dosing solution was infused into the femoral vein using a Harvard Apparatus syringe pump (South Natick, MA) at a flow rate of approximately 0.6 ml/h, which was adjusted based on body weight to administer a dose of 10 mg/kg/h. Caffeine and tryptophan were dissolved in n-saline (3 ml) and infused into the femoral vein at a flow rate of 2.0 ml/h/kg to administer a dose of 10 mg/kg/h. A dosing solution of ^{14}C-mannitol was prepared by diluting a stock solution (200 μCi/ml) to approximately 30 μCi/ml with n-saline. An infusion rate of approximately 8.3 μl/min was used to administer a total dose of 50 μCi/kg of ^{14}C-mannitol. Compound B dosing solution was prepared by dissolving the appropriate amount of drug in a 3/7 (v/v) DMA/PEG 300 solution at a concentration of 100 mg/ml. Compound B dosing solution was administered at an infusion rate of 0.33 ml/kg/h. In all cases fresh dosing solution was prepared on the day of dosing.

2.10. Sample collection

The perfusion fluid was delivered via a CMA/100 syringe pump at a flow rate of 1.0 μl/min. Dialysate samples were collected at 10 min intervals into 200 μl autosampler vials (Chromacol) with a CMA/170 refrigerated (5 °C) sample collector. Following collection, dialysate samples were immediately analyzed by LC. Blood samples (approx. 100 μl/sample) were collected through the jugular vein cannula prior to dosing, and at 15, 30, 60, 120, 240, 245, 255, 270, 300, 360, 420 and 480 min following the start of infusion. Following each collection, the blood volume was replaced with an equal volume of normal saline through the indwelling jugular cannula. The jugular vein cannula was then rinsed and filled with a heparinized saline solution (50 U/ml) to maintain the patency of the cannula. Blood samples were collected into 64 × 10.25 mm heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ) and centrifuged at 3500 rpm for 10 min at 5 °C. The resulting plasma was transferred into 1 ml microcentrifuge tubes and were kept frozen at − 20 °C until analysis.

2.11. Sample analysis

Dialysate and plasma samples, following administration of Compound A, Compound D, caffeine, or tryptophan, were analyzed using a method based on LC/UV. Chromatographic separations were performed using a Hypersil 150 × 4.6 mm C18 column (Phenomenex, Torrence, CA) and an LDC pump (LDC Analytical, Riviera Beach, FL). Samples were injected (7.0 μl) using a Sample Sentinel autosampler (Bioanalytical Systems, Inc., West Lafayette, IN). Detection (Thermo Separation UV2000) was performed at 237 nm for the determination of Compound A, Compound D and caffeine, while a wavelength of 220 nm was used for tryptophan. The mobile phase consisted of 50 mM phosphate buffer, pH 7.0, with acetonitrile ranging from 10 to 22% (v/v). The column was maintained at ambient temperature with a flow rate of 1.0 ml/min. Plasma samples were prepared for analysis by adding 70% perchloric acid (50 μl/ml plasma) followed by centrifugation for 10 min. The resulting supernatant was added to autosampler vials and was injected onto the LC system. Dialysate samples were directly injected onto the LC system.

Compounds B and C plasma and dialysate samples were analyzed by LC/MS/MS. The method used for analysis of Compound C has been reported previously [25]. Compound B dialysate and plasma samples were injected using a Gilson Model 231 autosampler (Gilson, Middleton, WI). To the 10 μl dialysate sample was added 50 μl of internal standard solution. The resulting solution was mixed and 50 μl was injected on the LC system. The chromatographic system consisted of a Michrom UMA LC system (Michrom BioResources, Pleasanton, CA), a Phenomenex

Luna C18 (Phenomenex, Torrance, CA) (2.0 × 150 mm) at 50–60 °C and a Finnigan TSQ-B mass spectrometer (Finnigan MAT, San Jose, CA). The mobile phase consisted of 33.25% (v/v) water, 0.87% (v/v) isopropyl alcohol, 65.9% (v/v) acetonitrile, 0.035% HFBA and was pumped at a flow rate of 0.25 ml/min. A post-column make-up flow of 0.75 ml/min (water) was necessary to bring the flow-rate to 1.0 ml/min.

Dialysate samples containing ^{14}C-mannitol were transferred to a scintillation vial and 5 ml of Ultima Gold scintillation cocktail (Packard) was added. Following perchloric acid precipitation of the plasma samples, 10 μl of the supernatant was added to a scintillation vial with 5 ml of scintillation cocktail. All samples were analyzed by LSC.

2.12. Data analysis

Plasma and brain data were analyzed sequentially with plasma concentrations being used to estimate A, α, B and β (Eq. (3)). The resulting equation was then used as a forcing function to calculate CL_{in} and k_{in} based on the brain ECF concentrations determined by microdialysis sampling (Fig. 1 and Eqs. (4)–(10)). In this model, V_{ecf} was taken to be 0.15 ml/g tissue [26]. The free fraction (f) in plasma was determined for each compound either experimentally or through values reported in the literature. The ratio of the steady-state amount of drug in the ECF to the steady-state amount in the intracellular fluid (ICF) was generally assumed to be 0.15. This value assumes equal concentrations in the ECF and in the ICF at steady-state (i.e. passive diffusion and no binding to cellular constituents).

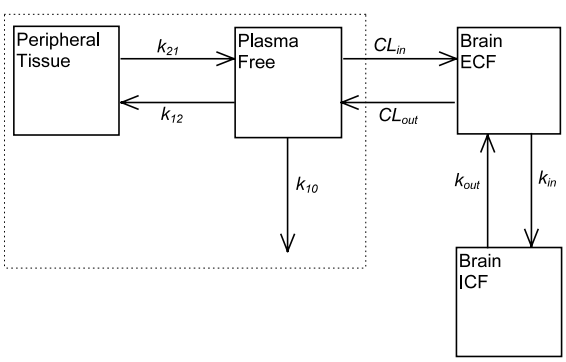

Fig. 1. Pharmacokinetic model of drug exchange between plasma, ECF and ICF of the brain.

$$C_p = A(e^{-t^*} - e^{-t}) + B(e^{-t^*} - e^{-t}) \quad (3)$$

If $t \leq$ infusion time then $t^* = 0$ otherwise $t^* = t -$ infusion time.

The concentration brain ecf and the amount in brain icf are given by the following equations:

$$\frac{dC_{ecf}}{dt} = \frac{CL_{in}}{V_{ecf}} C_p f - \frac{CL_{out}}{V_{ecf}} C_{ecf} - k_{in} C_{ecf} + k_{out} \frac{X_{icf}}{V_{ecf}} \quad (4)$$

$$\frac{dX_{icf}}{dt} = k_{in} C_{ecf} V_{ecf} - k_{out} X_{icf} \quad (5)$$

Replacing CL_{out} and k_{out} in Eqs. (4) and (5) with

$$CL_{out} = CL_{in}\left[\frac{C_{p,ss} f}{C_{ecf,ss}}\right] \quad (6)$$

and

$$k_{out} = k_{in}\left[\frac{X_{ecf,ss}}{X_{icf,ss}}\right] \quad (7)$$

gives

$$\frac{dC_{ecf}}{dt} = \frac{CL_{in}}{V_{ecf}}\left[C_p f - \left\{\frac{C_{p,ss} f}{C_{ecf,ss}}\right\} C_{ecf}\right] - k_{in} C_{ecf} + k_{in}\left[\frac{X_{ecf,ss}}{X_{icf,ss}}\right]\frac{X_{icf}}{V_{ecf}} \quad (8)$$

$$\frac{dX_{icf}}{dt} = k_{in} C_{ecf} V_{ecf} - k_{in}\left[\frac{X_{ecf,ss}}{X_{icf,ss}}\right] X_{icf} \quad (9)$$

Eqs. (8) and (9) were used for the purposes of modeling. In the cases of mannitol, Compound A, Compound D and tryptophan Eq. (10) was used instead of Eq. (8).

$$\frac{dC_{ecf}}{dt} = \frac{CL_{in}}{V_{ecf}}\left[C_p f - \left\{\frac{C_{p,ss} f}{C_{ecf,ss}}\right\} C_{ecf}\right] \quad (10)$$

Parameters given in the above equations are defined below:

C_p	concentration in plasma (ng/ml)
C_{ecf}	concentration in brain ECF (ng/ml)
$C_{p,ss}$	concentration in plasma at steady-state (ng/ml)

$C_{ecf,ss}$ concentration in brain ECF at steady-state (ng/ml)
CL_{in} clearance into the brain ECF (ml/min/g tissue)
CL_{out} clearance out of the brain ECF (ml/min/g tissue)
f free fraction of compound in plasma
k_{in} rate constant for cellular uptake (1/min)
k_{out} rate constant for cellular efflux (1/min)
V_{ecf} volume of the brain ECF (ml/g tissue)
$X_{ecf,ss}$ amount in ECF at steady-state (ng/g tissue)
X_{icf} intracellular amount of compound (ng/g tissue)
$X_{icf,ss}$ amount in ICF at steady-state (ng/g tissue)

3. Results

3.1. In vitro BBMEC system

The permeation of each compound across BBMEC monolayers was investigated at 37°C. In studying the marker compounds, it was found that the permeation (apical (A) to basolateral (B)) across the BBMEC monolayer is low for mannitol, kynurenic acid, Compound A, Compound D and tryptophan, and moderate/high for Compound B, Compound C and caffeine (Table 1). No significant differences were found in permeation in the A to B or B to A directions, which indicates the absence of an active efflux/influx mechanism for any of these compounds.

Table 1
Apparent permeability coefficients of various compounds in the BBMEC model

Compound	$P_{app} \times 10^5$ (cm/s)
Mannitol	1.66 (0.849)
Caffeine	7.54 (0.605)
Compound A	1.59 (0.286)
Tryptophan	3.00 (0.454)
Kynurenic acid	0.885 (0.171)
Compound B	5.00 (0.60)
Compound C	6.42 (0.80)
Compound D	1.57 (0.356)

Values are mean (std. dev.).

3.2. Plasma protein binding

In order to relate unbound plasma concentrations to the unbound brain ECF concentration obtained with microdialysis sampling, it was necessary to correct for the plasma protein binding of each compound. The average plasma protein binding for Compounds A, D, C and B was 97.5, 98.0, 57.9 and 98.4%, respectively. Plasma protein binding of 80% for tryptophan [27] and 11% for caffeine [28] have been reported, whereas mannitol does not significantly bind to plasma protein [29].

3.3. Microdialysis sampling

3.3.1. Microdialysis probe calibration

Prior to dosing, the relative recovery of the microdialysis probe was determined, both in vitro and in vivo, by perfusing the probe with an appropriate solution of the each compound, and comparing the concentration in the perfusate to the initial concentration in the perfusion fluid. Calculation of brain concentrations was based on in vivo recovery. The in vivo recovery was assumed to equal in vivo delivery. Results from the in vivo delivery/recovery experiments ($n = 3$/compound) were $24.6 \pm 4.7\%$ for Compound A, $38 \pm 17\%$ for Compound D, $82.5 \pm 4.7\%$ for mannitol, $31.8 \pm 2.6\%$ for caffeine, $18.5 \pm 5.1\%$ for Compound C, 60.7 ± 22 for Compound B and $84.1 \pm 9.7\%$ for tryptophan.

3.3.2. Brain ECF and plasma concentrations

Analyte concentration in brain ECF and plasma were monitored throughout the 4 h infusion and for 4 h following the end of the infusion. Concentration versus time profiles showing the free concentrations present in the striatum and the total plasma concentrations were then plotted for each compound. Figs. 2 and 3 show representative concentration versus time profiles for Compound A and caffeine, respectively.

3.3.3. Pharmacokinetic calculations

Pharmacokinetic parameters were calculated using Eqs. (3)–(10). Shown in Table 2 are the calculated parameters for each of the compounds in plasma. These values were used in Eq. (3) as a forcing function for the subsequent calculation of the brain uptake parameters. Table 3 shows calculated values of CL_{in}, CL_{out} and k_{in} along with

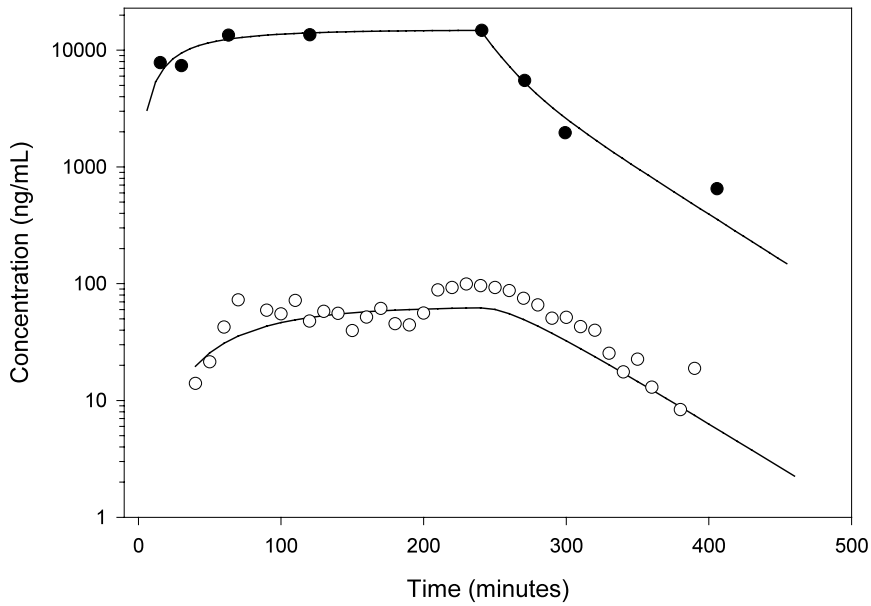

Fig. 2. Typical concentration–time profile of Compound A in brain ECF (○) and plasma (●) during an i.v. infusion for 4 h.

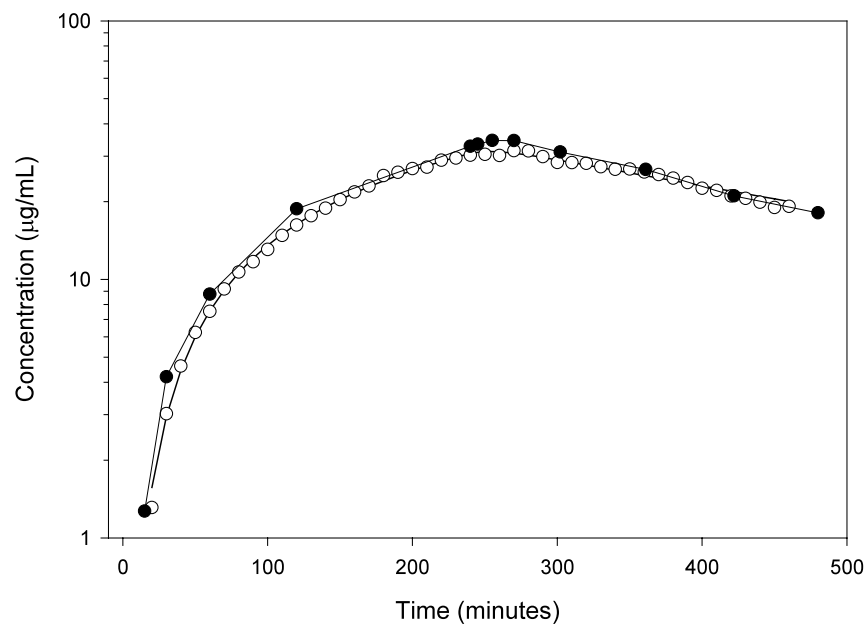

Fig. 3. Typical concentration–time profile of caffeine in brain ECF (○) and plasma (●) during an i.v. infusion for 4 h.

Table 2
Plasma parameters of each compound in rats

	Caffeine	Mannitol	Tryptophan	Compound A	Compound B	Compound C	Compound D
A (ng/ml)	96 600 (39 600)	149 (13.9)	15 516 (3453)	11 853 (9505)	3792 (3837)	1198 (303)	122 450 (38 765)
α (1/min)	0.00213 (0.000653)	0.039 (0.0200)	0.0268 (0.0154)	0.147 (0.188)	0.0107 (0.0107)	0.137 (0.0494)	0.0647 (0.0146)
B (ng/ml)	NA	83.3 (59.7)	NA	5290 (2010)	290 (153)	494 (57.6)	213 147 (123 719)
β (1/min)	NA	0.00653 (0.00755)	NA	0.0121 (0.00668)	NA	0.0157 (0.0033)	0.00837 (0.00177)
C (ng/ml)	NA	NA	NA	NA	4150 (3727)	NA	NA
Infusion time (min)	240	240	240	240	240	0	240
% of steady-state	36	84	91	81	100	NA	85
Dose	10 mg/kg/h iv infusion	50 μCi/kg/h iv infusion	10 mg/kg/h iv infusion	10 mg/kg/h iv infusion	100 mg/kg iv bolus + 33 mg/kg iv infusion	5 mg/kg iv bolus	10 mg/kg/h iv infusion

Values are mean (std. dev.).

Table 3
Brain uptake parameters

	Caffeine	Mannitol	Tryptophan	Compound A	Compound B	Compound C	Compound D
CL_{in} (μl/min/g)	12.9 (4.44)	0.023 (0.0161)	0.237 (0.126)	1.99 (2.09)	3.85 (2.35)	10.3 (2.10)	0.0310 (0.0236)
CL_{out} (μl/min/g)	13.2 (8.0)	1.39 (0.617)	3.90 (2.08)	8.36 (5.65)	13.1 (8.11)	5.83 (0.95)	2.61 (0.297)
k_{in} (1/min)	0.0121 (0.00671)	NA	NA	NA	0.00865 (0.00067)	0.000177 (0.000221)	NA
Protein binding (% bound)	11.0[a]	0.0[b]	80.0[c]	97.5	98.4	57.9	98.5
$C_{p,240}$ (total)[d] (ng/ml)	35400 (4280)	165000 (20500)	14050 (5660)	16000 (9230)	28900 (13700)	NA	284000 (86900)
$C_{p,240}$ (free)[d] (ng/ml)	31500 (3810)	165000 (20500)	2810 (1133)	400 (230)	435 (203)	NA	4265 (1304)
$C_{ecf,240}$ (free)[e] (ng/ml)	29900 (1500)	2280 (475)	172 (71)	101 (41)	152 (110)	NA	43.0 (22.7)
$\frac{C_{ecf,240}\ (\text{free})}{C_{p,240}\ (\text{total})}$	0.8501 (0.07087)	0.0141 (0.00487)	0.0122 (0.000107)	0.00941 (0.0100)	0.00572 (0.00511)	NA	0.000177 (0.000127)
$\frac{C_{ecf,240}\ (\text{free})}{C_{p,240}\ (\text{free})}$	0.9551 (0.0796)	0.0141 (0.00487)	0.0609 (0.000537)	0.376 (0.400)	0.325 (0.151)	NA	0.0118 (0.00849)

Values are mean (std. dev.); NA not applicable.
[a]Ref. [28].
[b]Ref. [29].
[c]Ref. [27].
[d]Plasma concentration at 240 min (i.e. end of infusion).
[e]Brain ecf concentration at 240 min (i.e. end of infusion).

plasma and brain ECF concentrations at the end of the infusion ($C_{p,240}$ and $C_{ecf,240}$). Values of CL_{in} ranged from a low of 0.023 μl/min/g for mannitol to a high of 12.9 μl/min/g for caffeine. Compound A, Compound D and tryptophan had CL_{in} values of 1.99, 0.0310 and 0.237 μl/min/g, respectively. Compounds C and B had moderate to high CL_{in} values of 10.3 and 3.85 μl/min/g, respectively. Calculated values of CL_{out} ranged from a low of 1.39 μl/min/g for mannitol to a high of 13.2 μl/min/g for caffeine. Cellular uptake appeared significant for only three of the seven compounds: caffeine, Compounds C and B.

4. Discussion

The studies presented in this report were designed to assess the correlation of the BBMEC apparent permeability coefficient (P_{app}) and in vivo BBB penetration by microdialysis. A mathematical model was developed to describe the relationship of brain ECF concentration to free drug in plasma. This model, while similar to those reported previously [22,30] is differentiated in that the volume of the ECF is set at the physiologic volume ($V_{ecf} = 0.15$ ml/g) and cellular uptake is an integral part of the model.

The compounds studied have a broad range of physico-chemical characteristics and have widely varying in vitro and in vivo permeability across the BBB (Tables 1 and 3). As shown in Table 1, the BBMEC permeability coefficients vary in magnitude from a low of 0.9×10^{-5} cm/s to a high value of 7.5×10^{-5} cm/s. Corresponding in vivo measurements of BBB permeability are represented by clearance into the brain ECF (CL_{in}) (Table 3). Values of CL_{in} range from a low of 0.023 μl/min/g to a high of 12.9 μl/min/g. The relationship of CL_{in} to steady-state brain ECF concentration ($C_{ecf,ss}$) is given by the equation

$$C_{ecf,ss} = \frac{CL_{in}}{CL_{out}} C_{p,ss} f \quad (11)$$

which rearranges to

$$\frac{C_{ecf,ss}}{C_{p,ss} f} = \frac{CL_{in}}{CL_{out}} \quad (12)$$

It is evident from inspection of the above equation (Eq. (12)) that the concentration of a compound in the brain cannot be predicted based solely on a measure of the ability of the compound to cross the BBB (i.e. CL_{in}). Since brain ECF concentrations are controlled by CL_{out} relative to CL_{in}, the ratio of CL_{in} to CL_{out} will establish the steady-state brain ECF concentration ($C_{ecf,ss}$) given a certain steady-state free plasma concentration ($C_{p,ss}$f).

Numerous factors including passive diffusion ($CL_{passive}$) from the brain ECF to plasma, cellular uptake and metabolism ($CL_{metabolism}$), active efflux (CL_{active}) across the BBB and bulk flow ($CL_{bulk\ flow}$) of the ECF contribute to the clearance out of the brain ECF (CL_{out}). In the model presented in this report, CL_{out} is a composite value that encompasses all clearances out of the extracellular space and does not represent transport only across the BBB.

$$CL_{out} = CL_{passive} + CL_{active} + CL_{metabolism} + CL_{bulk\ flow} \quad (13)$$

Due to the complexity of this parameter, no single in vitro model can be predictive of brain concentration given a certain plasma concentration. While transport of free drug across the BBB is, in most cases, necessary for uptake into the brain tissue, high in vitro and/or in vivo penetration across the BBB does not necessarily mean that the concentration of a specific compound will be high in the brain, as this is dependent on the magnitude of CL_{out}. On the other hand, in vitro models such as the BBMEC can give an indication of the magnitude to which a compound will cross the BBB (i.e. the permeability potential of a compound). Additionally, models such as the BBMEC can provide a simpler system with which to investigate mechanisms of transport across the BBB.

Rate constants for cellular uptake (k_{in}) are shown in Table 3. Mannitol, Compound A, Compound D and tryptophan all had k_{in} values which approached zero, indicating that cellular uptake is very slow. This is consistent with the literature which shows that mannitol does not readily penetrate cells [31]. Additionally, studies with Compound A have shown that total brain tissue and

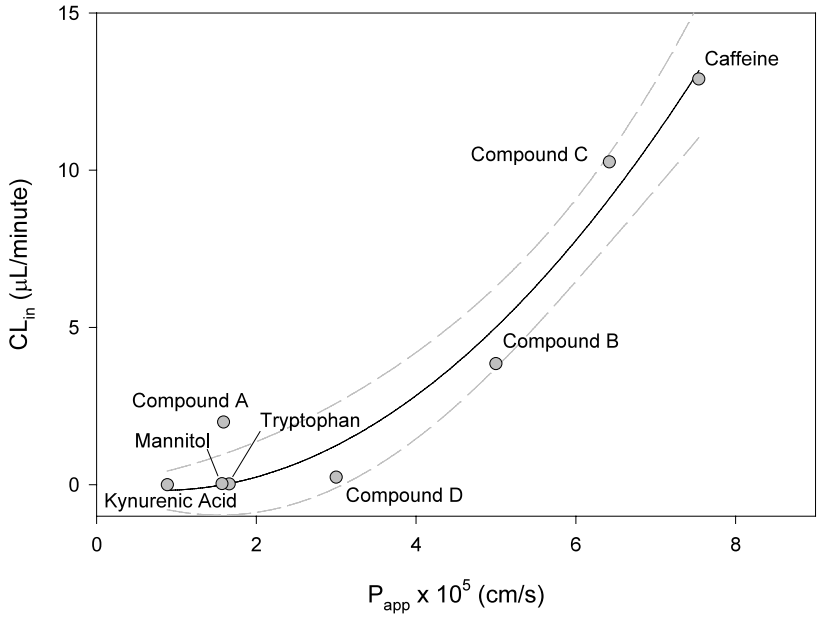

Fig. 4. Comparison of BBMEC apparent permeability coefficient (P_{app}) vs. clearance across the BBB (CL_{in}) as determined by in vivo microdialysis sampling.

CSF concentrations are less than or equal to brain ECF concentrations following an i.v. bolus plus 4.5 h infusion, indicating slow cellular uptake. Caffeine, Compounds C and B all had non-zero cellular uptake rate constants. It has previously been shown that caffeine [32] and Compound C distribute almost equally between the intracellular and the extracellular spaces. For Compound B the brain tissue concentration was measured at the end of the infusion and the measured value was used to calculate the extracellular/intracellular distribution ratio.

Assuming that transport across the BBB is equal in both directions and that there are no additional clearance mechanisms, steady-state brain ECF concentration will be equal to free concentration in plasma. Since the bulk flow of ECF (0.2–0.3 μl/min/g) [33,34] is a clearance mechanism that is always present under normal circumstances, as the permeability of a compound becomes lower $CL_{bulk\ flow}$ becomes more important and begins to limit the ratio of free concentration in the ECF relative to the free concentration in plasma. This situation is most likely the case for compounds like mannitol which have values of CL_{in} that are less than 1 μl/min/g.

In conclusion, both microdialysis and the BBMEC model are useful tools for the investigation of drug penetration into the CNS. While it is apparent from the data presented here that in vitro data from the BBMEC model can be predictive of the in vivo permeability of a compound across the BBB, there are numerous factors both prior to and following entry into the brain which impact the ultimate uptake of a compound. While it is clear that a compound will likely not enter the brain if the BBB permeability is low, good BBB permeability does not necessarily indicate that the concentration of a compound will be high within the CNS. Even in the presence of high BBB permeability, factors such as high plasma protein binding, active efflux across the BBB and metabolism within the CNS can greatly limit the ultimate concentrations achieved. In addition, concentrations in the intracellular space may not be the same as concentrations in the extracellular space. As an example, a compound could conceivably have extracellular concentrations which were equal to free plasma concentrations while simultaneously having low intracellular concentrations.

In the absence of information indicating the magnitude of cellular uptake, one could mistakenly determine that the compound had good BBB permeability. Conversely, a compound could have low extracellular concentrations while having high intracellular concentrations. Based exclusively on the ECF concentration it could be inferred that the permeability across the BBB was low when in fact it was high. While these data give an indication that the BBMEC permeability is predictive of the in vivo BBB permeability (Fig. 4), the complexity of the living system makes prediction of brain concentrations difficult if not impossible without additional data. Further study in this area is necessary to elucidate the similarities and differences of the in vitro and in vivo systems and to improve our understanding of the complexities of uptake of drugs into the CNS.

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