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Application of a sample pooling method for the accelerated assessment of the rate of uptake of drugs by the brain in rats

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Abstract

The purpose of this study was to examine the feasibility of applying a sample pooling method to the accelerated estimation of the uptake clearance of drugs to the brain in rats. Brain uptake clearances (CL_{uptake}) were estimated for five model compounds using the sample pooling method and an integration plot analysis. CL_{uptake} was also evaluated for caffeine and theophylline by brain microdialysis. The parameters and throughput of the pooling method were compared with those of typically used standard methods. The correlation for CL_{uptake} was statistically significant ($P < 0.005$) between the integration plot and the current method; the throughput of evaluation was 15-fold higher for the sample pooling method. A comparison of CL_{uptake} values indicated that the three methods showed comparable results for caffeine while the CL_{uptake} of theophylline using the proposed method was significantly different from those of the other methods. A kinetic analysis indicated that a compound with a slower CL_{uptake} and longer half-life (e.g., theophylline) is more prone to error and that the lower limit of the CL_{uptake} of 0.17 mL min^{-1} (g brain)$^{-1}$ may be set so as to have an error less than 20% of the estimation. These results suggest that the sample pooling method is applicable for use in the accelerated estimation of the uptake clearance of compounds in the brain for which the value is greater than 0.17 mL min^{-1} (g brain)$^{-1}$.

Introduction

The transport of certain xenobiotics via the blood–brain barrier (BBB), a monolayer of tightly connected endothelial cells of the brain microvessels, is significantly limited by the presence of a tight junction (Reese & Karnovsky 1967; Pardridge 2003) and the functional expression of efficient efflux transport systems (Sugiyama et al 1999) in the cells. In addition, metabolic enzymes (e.g., alkaline phosphatase, cytochrome P450 enzymes and glutathione-S-transferase), which are known to be present in endothelial cells (Lawrenson et al 1999), further prevent the access of drugs to the central nervous system. Collectively, the BBB represents one of the least accessible barriers for the transport of xenobiotics in the body.

In the field of drug discovery, increasing the rate of pharmacokinetic screening has become an important and urgent issue. A sample pooling technique, involving cassette-dosing and the simultaneous quantification of substrates via a liquid chromatography-mass spectrometry (LC-MS) assay, has been used to accomplish this objective (Hsieh et al 2002; Smith et al 2004). In this method, the area under the plasma concentration–time curve (AUC) can be readily estimated by multiplying the drug concentration in the pool sample of equally spaced serial samples by the time interval (Atherton et al 1999; Zhao et al 2003). The systemic clearance can then be calculated using the standard pharmacokinetic equation (i.e., the dose divided by the AUC). Such a sample pooling method was recently extended in calculating the steady-state volume of distribution (Cheung et al 2005), suggesting an additional application of the method. Unfortunately, a rapid estimation method has not been proposed to date for brain permeability.

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Traditionally, the penetration of xenobiotics into the brain is evaluated by brain uptake index, an in-situ brain perfusion method (Takasato et al 1984) and a brain microdialysis technique. However, some of these methods require the use of radiolabelled substrates (brain uptake index, in-situ brain perfusion method), which may not be readily available in drug discovery settings. In addition, other experimental difficulties (e.g., the necessity for the depletion of brain capillaries in the in-situ method, the necessity for extensive calibration in the brain microdialysis technique) further limit the application of these methods to the accelerated evaluation of brain permeability (Bungay et al 1990; Skarlatos et al 1995). Among the in-vivo techniques reported in the literature, integration plot analysis has been used to estimate brain uptake kinetics (Kusuhara et al 1997; Takanaga et al 1998). In this method, the amount of drug in the brain at a given time, t, is plotted against the AUC from time zero to the time of brain collection; the slope of the plot represents the brain uptake clearance. The primary determining factor for throughput using this technique is the calculation of the AUC, because this parameter is typically calculated by measuring the concentration in each of the serially collected plasma samples. Since it has been shown that the sample pooling technique is sufficiently accurate (Atherton et al 1999; Zhao et al 2003) for estimating the AUC for a fixed time interval, the pooling method, in principle, could also be used in the determination of brain uptake clearance. Unfortunately, an experimental verification of the application has not been reported for estimating brain uptake kinetics.

The objective of this investigation, therefore, was to examine the feasibility of the sample pooling method in estimating brain uptake clearance by comparing the kinetic parameters from a standard integration plot analysis and the sample pooling method for model drugs with varying brain permeabilities. In addition, brain uptake kinetics were evaluated by brain microdialysis and the results of this method were compared with those for the pooling method. Our results indicate that the sample pooling method is reasonably accurate for the determination of brain uptake kinetics and that the method is applicable in conjunction with a cassette-dosing situation for the accelerated assessment of brain uptake kinetics in-vivo.

Materials and Methods

Materials

Caffeine, theophylline, metoclopramide, quinine and dexamethasone were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ketamine (Ketalar; Yuhan Co., Seoul, Korea, K.P.VII) and acepromazine (Sedaject; Samu Chemical Co., Seoul, Korea, K.P.VII) were also used in this study. All other chemicals were of reagent grade or better, and were used without further purification.

Animals

Male Sprague-Dawley rats (Dae-Han Biolink, Eumsung, Korea), 300 ± 20 g, were used in this study. Experimental protocols were reviewed by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University, according to National Institutes of Health guidelines (NIH publication number 85-23, revised 1985) Principles of Laboratory Animal Care.

Estimation of brain uptake clearance by traditional integration plot analysis

Sprague-Dawley rats were anaesthetized with an intramuscular injection of ketamine ($50\,mg\,kg^{-1}$) and acepromazine ($10\,mg\,kg^{-1}$). After confirming anaesthesia, the rats were subjected to surgery that involved the implantation of catheters to the femoral artery and vein of the rat with polyethylene tubing (PE-50; Clay Adams, Parsippany, NJ, USA) filled with heparinized saline ($25\,U\,mL^{-1}$) to prevent blood clotting.

After recovery from anaesthesia, the model drug (caffeine, theophylline, metoclopramide, quinine or dexamethasone) was administered via the femoral vein by a bolus injection (the total injection volume was 0.28–0.32 mL) to the rat. Each dosing solution contained 5 mg of the drug in 1 mL of saline. Blood samples were collected five times at intervals of 15 s (viz, 15, 30, 45, 60 and 75 s), 30 s (viz, 30, 60, 90, 120 and 150 s), 1 min (viz, 1, 2, 3, 4 and 5 min) or 2 min (viz, 2, 4, 6, 8 and 10 min). The blood samples were centrifuged immediately, and a 100-μL volume of the plasma was stored in a freezer ($-20^{\circ}C$). Immediately after the last blood collection (viz, 75 s, 150 s, 5 min or 10 min), the rat was sacrificed by decapitation and the brain tissue collected and weighed. The brain tissue was also stored in a freezer ($-20^{\circ}C$). The brain uptake clearance was estimated by a standard integration plot analysis (Kusuhara et al 1997), in which the uptake clearance was obtained from the slope of a plot of drug in the brain at time t and the area under the plasma concentration–time curve to the time for the model drugs.

Estimation of brain uptake clearance by microdialysis

Implantation of microdialysis probes

In the literature, the spatial dependence on different regions of the brain has not been reported for drug distribution. Since the striatum is typically selected (Terasaki et al 1991; Ahn et al 2004) in the study of neuropharmacokinetics via microdialysis sampling for its ease of access, we chose this location to study the kinetics of drugs. Seven days before the experiment, male Sprague-Dawley rats were anaesthetized with an intramuscular injection of ketamine ($50\,mg\,kg^{-1}$) and acepromazine ($10\,mg\,kg^{-1}$). After confirmation of the absence of a reflex reaction from a toe-pinch test, the rat was mounted on a stereotaxic instrument (Stoelting Co, Wood dale, IL, USA) for the implantation of a guide cannula (MD-2201; Bioanalytical System Inc., West Lafayette, IN, USA) to the striatal region of the brain.

The surgical procedure was essentially identical to that described in a previous study (Sung et al 2005). Briefly, a midsagital incision was made to expose the skull, and the guide cannula, fitted with a dummy probe, was implanted into the striatum with the coordinates of 2.6 mm lateral, 0.2 mm anterior, 6.5 mm ventral to the bregma. After the insertion, the cannulae were anchored to two screws with dental cement and the rat was allowed to return to the individual cage for recovery. One week after the implantation, the rat was anaesthetized again and the femoral artery and vein were catheterized with PE50 tubes. The polyethylene tubes were then externalized to the back of the rat and a syringe was filled with a heparin solution and connected to the cannulae to prevent blood clotting. The dummy probe was removed from the guide cannulae and the microdialysis probe, presoaked in a solution containing appropriate retrocalibrator (see below), was carefully introduced through the cannula. The perfusion of the probe with dialysate medium was then initiated.

Measurement of in-vitro recovery

For the determination of the probe recovery in-vitro, a microdialysis probe (MD-2200, 2 mm; Bioanalytical System Inc.) was presoaked in saline containing caffeine or theophylline (final concentration 5 μg mL^{-1}). The probe was perfused with saline containing a calibrator (i.e., caffeine for the theophylline study, theophylline for the caffeine study, depending on the drug, respectively) at a flow rate of 2 μL min^{-1} using a Harvard-22 infusion pump (Harvard Apparatus, Holliston, MA, USA). After equilibration, the dialysate samples were collected for 3 h at 10- to 15-min intervals. The concentrations of drug and calibrator were determined by HPLC (see below). The recovery of drug (R_d) and the loss of calibrator (L_c) in-vitro were determined using the following equations:

$$R_d = C_{dialysate}/C_{medium} \quad (1)$$

$$L_c = (C_{perfusate} - C_{dialysate})/C_{perfusate} \quad (2)$$

The relative recovery of the drug and the calibrator was calculated using equation 3.

$$F_{in\text{-}vitro} = L_c/R_d \quad (3)$$

Measurement of in-vivo recovery

Using a separate group of rats, the loss of caffeine and theophylline from the dialysate to the striatal extracellular fluid (ECF) was determined. In this method, in-vivo recovery was estimated by assuming that the movement of the drug across the dialysis probe is essentially identical regardless of the direction (Yang et al 1997). Stereotaxic surgery, cannulae implantation and animal recovery were carried out in a manner similar to that described in the previous section. Upon insertion, the microdialysis probe was perfused at a flow rate of 2 μL min^{-1} using saline containing 5 μg mL^{-1} caffeine and theophylline. The perfusate leaving the probe was collected at 10- to 20-min intervals for a period of up to 8 h and the concentration of caffeine and theophylline in the perfusate was determined by HPLC. The in-vivo relative recovery by loss was calculated using equation 4.

$$F_{in\text{-}vivo} = L_c/L_d \quad (4)$$

where L_c represents the loss of calibrator and L_d represents the loss of drug across the probe.

Brain pharmacokinetic study of caffeine and theophylline

Caffeine (5 mg kg^{-1}) or theophylline (2 mg kg^{-1}) was intravenously administered to the rats via the left femoral vein. The dialysis medium that equilibrated with the striatal ECF was continuously collected at 10- to 15-min intervals up to 8 h. Blood samples (250 μL) were collected from the catheter connected to the artery at predetermined time points (blank, 10, 20, 30, 60, 120, 240, 360 and 480 min) and centrifuged to obtain plasma. The plasma (100 μL) and dialysate samples (20–30 μL) were stored in the freezer (−20°C) until used for analysis. The concentration of caffeine and theophylline in the striatal ECF was calculated as follows:

$$C_{ECF} = (C_d/L_c) \times F_{in\text{-}vivo} \quad (5)$$

where C_{ECF}, C_d and L_c represent the concentration of the drug in the striatal ECF, the concentration of drug in dialysate and the loss of calibrator from microdialysis probe, respectively. The concentration of the striatal ECF and the plasma was simultaneously fitted to obtain the brain uptake clearance by a nonlinear regression analysis (see Data analysis section).

Estimation of brain uptake clearance by the sample pooling method

To examine the applicability of the sample pooling method for estimating brain uptake clearance, the apparent brain uptake clearance ($CL_{apparent,\ uptake}$) was determined by dividing the amount of drug in brain at time t by the area under the curve up to the time of brain collection (see Data analysis section). In this study, we set a sampling time of 75 s. The procedures for animal surgery were similar to those used for a traditional integration plot analysis.

When it was necessary to determine $CL_{apparent,\ uptake}$ after the administration of the individual model drugs, the compound (caffeine, theophylline, metoclopramide, quinine or dexamethasone) was administered via the femoral vein cannula by a bolus injection (i.e. each dosing solution contained 5 mg of the compound per 1 mL of saline; the total injection volume was 0.28–0.32 mL).

When it was necessary to determine the brain uptake clearances after the simultaneous administration of the model drugs, a cassette of five model drugs was administered and the uptake clearance estimated. After recovery from anaesthesia a bolus of the cassette-dosing solution (mixture of 1 mg of each of the five model drugs in 1 mL saline) was administered via the femoral vein (the total injection volume was 0.28–0.32 mL) to rats.

For both the individual administration and the cassette-dosing situation, blood samples were collected at appropriate times (15, 30, 45, 60 and 75 s). The blood

samples were centrifuged immediately, and a 100-μL volume of plasma was collected. For all samples, except for the first and the last sample, 20-μL volumes were then pooled. For the first and the last sample, one half (i.e., 10 μL) of the volume was added. Immediately after the last blood collection, the rats were sacrificed by decapitation and brain tissue was collected and weighed. The pool plasma sample and the brain tissue were stored in the freezer at -20°C.

The $CL_{apparent,\ uptake}$ was obtained by dividing the amount of drug in brain at 75 s after the administration by the estimated area under the plasma concentration time curve from the pool sample (see Data analysis section).

Analytical methods

Analytical procedure for samples from standard integration plot analysis and sample pooling method

To determine the concentration of the model compounds (caffeine, theophylline, metoclopramide, quinine or dexamethasone) in plasma, an LC-MS assay was used. Two-hundred microlitres of acetonitrile was added to the plasma (i.e., 100 μL) and the mixture was then vortexed for 5 min. The mixture was then centrifuged at 3000 rev min^{-1} for 10 min and 250 μL of the supernatant was evaporated to dryness under a stream of nitrogen (Eyela Dry thermo bath MG-2100; Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The residue was reconstituted with 100 μL of 70% acetonitrile in water, and 10 μL was injected directly into the LC-MS (see below) system. The brain tissue was homogenized in two volumes (assuming the density of the tissue to be unity) of tissue with acetonitrile using a tissue homogenizer (Ultra-Turrax T25; IKA-Labortechnik, Staufen, Germany). The homogenate was then centrifuged at 3000 rev min^{-1} for 10 min and the supernatant collected. A 250-μL volume of the supernatant was evaporated to dryness in a stream of nitrogen. The residue was reconstituted with 100 μL of mobile phase (see below) and a 10-μL volume was injected directly into the LC-MS system.

LC-MS was carried out on a Waters ZQ (Milford, MA, USA) single quadrupole mass spectrometer and interfaced via an electrospray interface probe to a liquid chromatograph, consisting of a Waters 2695 separation module (quaternary pump and autoinjector) and a vacuum degasser. A Luna C-18 reversed phase column (2.0 mm i.d. $\times$ 150 mm 5 μm) supplied by Phenomenex (Torrance, CA, USA) was used. The mobile phase consisted of solvent A (50% acetonitrile in methanol) and solvent B (distilled de-ionized water containing 0.1% formic acid), and the gradient used. The gradient was as follows: 5-min linear increase from 30 to 70% solvent A; 5-min linear decrease from 70 to 30% solvent A. Total analysis time was 10 min. In this study, a flow rate of 0.2 mL min^{-1} was used. Electrospray interface probe conditions were as follows: 3.00 kV capillary voltage, 45 V cone voltage, 2 V extractor voltage, 0.2 V RF lens voltage, 100°C source temperature, 150°C desolvation temperature, 250 L h^{-1} desolvation gas flow and 50 L h^{-1} cone gas flow. The LC-MS run allowed adequate separation of quinine, metoclopramide, theophylline, caffeine and dexamethasone, with retention times of 2.2, 2.5, 2.9, 3.5 and 13.6 min, respectively. Each model compound was detected using five selected ion-monitoring (SIM) modes (m/z 325.4, m/z 300.2, m/z 181.2, m/z 195.2 and m/z 393.5, respectively). For all model compounds, calibration studies indicated that the detector response was linear over 0.05–10 μg mL^{-1} in both the plasma and the brain homogenate. The inter- and intra-day precision and accuracy of the assay was below 15% in this range and was approximately 12.7% at the quantification limit (i.e. 0.05 μg mL^{-1}), indicating that the assay was valid in the range of concentrations studied.

Analytical procedure for samples from microdialysis study

An HPLC assay was used to determine the concentration of caffeine and theophylline in the microdialysates. The HPLC system consisted of a pump (Model PU610; GL Sciences, Tyoko, Japan), a UV detector (UV620; GL Sciences) and a Luna C18 reversed-phase column (5-μm particle, 1.0 mm i.d. $\times$ 150 mm; Phenomenex, Torrance, CA, USA). A sample of the dialysate was directly injected into the HPLC system. The mobile phase consisted of acetonitrile–0.05 M ammonium phosphate buffer (10:90, v/v), delivered at a rate of 0.05 mL min^{-1}. The column eluent was monitored by means of a UV detector at 270 nm.

To determine the concentration of caffeine and theophylline, plasma samples were deproteinated by the addition of 200 μL acetonitrile containing 50 μL of internal standard (2 mg mL^{-1} β-hydroxyethyl-theophylline), then vortexed for 5 min and centrifuged for 10 min at 13 000 rev min^{-1}. A 250-μL volume of the supernatant was collected and evaporated to dryness in a stream of nitrogen. The residue was reconstituted with 100 μL of mobile phase and injected into the HPLC system. The mobile phase for the caffeine assay consisted of methanol–0.05 M ammonium phosphate buffer (30:70, v/v); the mobile phase for the theophylline assay consisted of acetonitrile–0.01 M ammonium acetate buffer (4:96, v/v). The flow rate was 1 mL min^{-1} and the eluent was monitored at a wavelength of 254 nm (for the assay of caffeine in the plasma) or 280 nm (for the assay of theophylline in the plasma).

For both xanthine analogues, the calibration curve was constructed in the plasma and the dialysate; concentration range was 0.05–50 μg mL^{-1}. The inter/intra-day precision and the accuracy of the assay was below15% in the range and was approximately 13.4% for the quantification limit (i.e. 0.05 μg mL^{-1}), indicating that the assay was valid in the range of concentrations studied.

Data analysis

Determination of brain uptake clearance by sample pooling method

When it was necessary to determine the brain uptake clearance by the sample pooling method, the rate of

uptake can be expressed by equation 6 (Kusuhara et al 1997).

$$dX_{brain}/dt = CL_{uptake} \times C_{plasma} - CL_{efflux} \times C_{brain} \quad (6)$$

where X_{brain} represents the amount of drug in the brain, CL_{uptake} the uptake clearance to the brain and CL_{efflux} the efflux clearance from the brain. At early times, C_{brain} is likely to be negligible and, thus:

$$dX_{brain}/dt \cong CL_{uptake} \times AUC_0^t \quad (7)$$

By integration of both sides:

$$X_{brain}(t) = CL_{uptake} \times AUC_0^t \quad (8)$$

However, when the brain is sampled from the rat, a small volume of blood is likely to remain in the collected tissue and, thus:

$$X_{collected\ brain}(t) = X_{brain}(t) + X_{remaining\ blood}(t) \quad (9)$$

where $X_{collected\ brain}(t)$ represents the amount of drug in the brain sample at time t and $X_{remaining\ blood}(t)$ the amount of drug in the blood trapped in the brain sample at time t. Therefore, the apparent uptake clearance can be defined in equation 10:

$$\begin{aligned} CL_{apparent,\ uptake} &= X_{collected\ brain}(t)/C_{pooled}.\Delta t \\ &= X_{collected\ brain}(t)/AUC_0^t \\ &= (X_{brain}(t)/AUC_0^t) \\ &\quad + (X_{remaining\ blood}(t)/AUC_0^t) \\ &= CL_{uptake} + (X_{remaining\ blood}(t))/AUC_0^t) \end{aligned} \quad (10)$$

where C_{pooled} and t represent the concentration of drug in the pooled plasma sample and the interval of the blood collection (0.25 min in this study). Since the plasma concentration may be readily described by a monoexponential function for a short period of time (e.g. from time zero to 1.25 min in this study), thus:

$$\begin{aligned} X_{remaining\ brain}(t)/AUC_0^t &= (V_{blood,\ trapped} \times C(t))/AUC_0^t \\ &= V_{blood,\ trapped}\{C_0 \times e^{-\lambda t}/[C_0 \times 1/\lambda(1 - e^{-\lambda t})]\} \\ &= V_{blood,\ trapped} \times (\lambda/e^{\lambda t} - 1)) \end{aligned} \quad (11)$$

Therefore, the % error in the estimation of CL_{uptake} by the sample pooling method may be calculated as:

$$\%\ \text{Error in } CL_{uptake} = ([V_{blood,\ trapped} \times \lambda/(e^{\lambda t} - 1)]/CL_{apparent,\ uptake}) \times 100 \quad (12)$$

We previously demonstrated that $V_{blood,\ trapped}$ was approximately 42 μL (g brain)$^{-1}$ (Ahn et al 2004) in rats. In this study, the sampling time of the study (i.e. t) was 1.25 min.

Determination of brain uptake clearance by brain microdialysis

To determine the clearances into the brain for caffeine and theophylline, first-order kinetics were assumed for the uptake and efflux processes between the systemic circulation and the brain. Since the polyexponential function may be used for the concentration in the plasma–time curve for the xanthine derivatives, equations 13 and 14 may be used for the pharmacokinetic analysis.

$$V_{brain} \times dC_{ECF}/dt = CL_{uptake} \times C_{plasma} - CL_{efflux} \times C_{ECF} \quad (13)$$

$$C_{plasma} = \sum_{i=1}^{n} C_i \times e^{-\lambda_i t} \quad (14)$$

where C_{plasma}, C_i and λ_i represent the drug concentration in the plasma, the intercept terms for the λ_i phase and the slope term, respectively. Preliminary studies indicated that monoexponential and biexponential functions may be used for the analysis of the pharmacokinetics of caffeine and theophylline in the plasma, respectively. The equation was fitted to the data to obtain the clearance terms, the slope and intercept terms in the equations using a PC running WinNonlin software (version 3.1; Pharsight, Mountain View, CA). In the kinetic analysis, V_{brain} was assumed to be 1.8 mL (Davies & Morris 1993).

Statistical analysis

Data are expressed as the mean ± the standard deviation (s.d.), except for the data generated from the regression analysis in which the standard error was used. When it was necessary to compare mean values, one-way analysis of variance was used. For comparison of the uptake clearances from the individual and cassette dosing, Student's *t*-test was used. $P < 0.05$ denoted statistical significance.

Results

Assessment of brain uptake clearance by integration plot method

The rates of brain uptake for the model drugs were estimated using a standard integration plot analysis (Figure 1). For the times up to 10 min after intravenous administration, the amount of drug in the brain at a given time was plotted against the area under the concentration–time curve from time zero to the sampling time. In general, the amount of drug in the brain increased with the area under the curve (Figure 1) but did not appear to reach a plateau, indicating that the uptake clearance for the model drugs can be estimated from the corresponding slope of the plots. Based on a linear regression analysis, the true brain uptake clearances (CL_{uptake}) and standard error of estimation for caffeine, theophylline, metoclopramide, quinine and dexamethasone were 0.558 ± 0.082 ($n = 15$), 0.045 ± 0.009

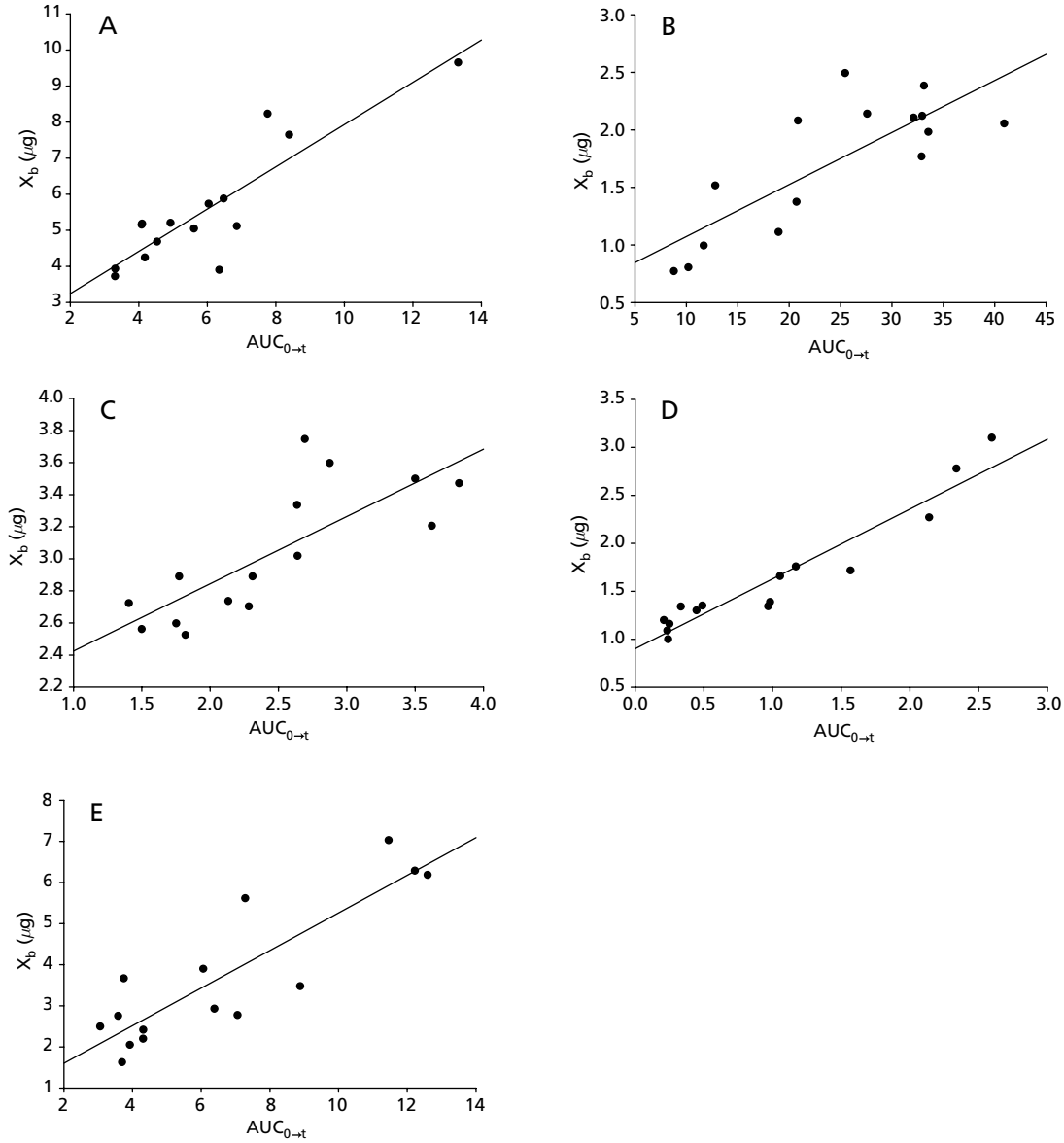

Figure 1 Plots of the amount of drug in the brain (X_b) M at various times and the area under the plasma concentration–time curve up to the time of brain collection. Caffeine (A), theophylline (B), metoclopramide (C), quinine (D) and dexamethasone (E) were intravenously injected to rats at a dose of 5 mg kg^{-1}.

(n = 15), 0.416 ± 0.105 (n = 15), 0.727 ± 0.063 (n = 15) and 0.457 ± 0.072 (n = 15) mL min^{-1} (g brain)$^{-1}$, respectively. The intercept (i.e. the amount of drug remaining per g of brain sample) ranged from 0.621 ± 0.246 (for theophylline) to 2.290 ± 0.300 (for metoclopramide) μg drug/g brain.

Assessment of apparent brain uptake clearance by sample pooling method

In this study, the concentration of model compounds in the pooled sample and brain homogenate was measured for determination of the $CL_{apparent,\ uptake}$ by the sample pooling method (equation 10). $CL_{apparent,\ uptake}$ for caffeine, theophylline, metoclopramide, quinine and dexamethasone were 0.746 ± 0.110, 0.084 ± 0.005, 0.459 ± 0.074, 0.879 ± 0.050 and 0.474 ± 0.124 mL min^{-1} (g brain)$^{-1}$ (n = 3 for each model drug), respectively. When CL_{uptake} values from the standard integration plot method were plotted against $CL_{apparent,\ uptake}$, obtained by the sampling pooling method (Figure 2), the r square value for the regression analysis was 0.975, indicating that the two parameters have a statistically significant correlation ($P < 0.005$). Furthermore, the intercept was not statistically different from zero. These observations, therefore, indicate that the $CL_{apparent,\ uptake}$ is comparable with

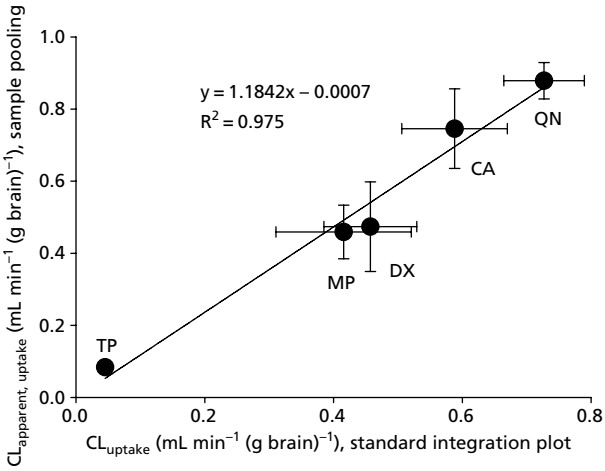

Figure 2 Relationship between the uptake clearances by the brain obtained by the standard integration plot analysis (x-axis) and the sample pooling method (y-axis) after the individual administration of the model drugs to rats. The error bar in the direction of x-axis represents the standard error (mean ± s.e.) estimated by the linear regression analysis for the data from the standard integration plot; the error in the direction of y-axis represents the s.d. of the triplicate determinations of the apparent brain uptake clearance ($CL_{apparent,\ uptake}$) based on the sample pooling method (mean ± s.d.). TP, theophylline; MP, metoclopramide; DX, dexamethasone; CA, caffeine; QN, quinine.

CL_{uptake}, obtained by the standard integration plot analysis. In this study, a total of 75 rats were used to evaluate CL_{uptake} for the 5 model drugs via the standard integration plot analysis (viz, 450 LC-MS determinations), while only 15 rats were required to estimate the clearance values for the model drugs when the sample pooling method was used (viz, 30 LC-MS determinations). Therefore, these observations indicate that the extension of the sample pooling method requires fewer experimental animals and, thus, fewer biological samples to obtain a comparable estimation of CL_{uptake}.

Assessment of brain uptake clearance by brain microdialysis

For estimating brain uptake clearance, the standard integration plot analysis method and the current sample pooling method share almost identical kinetic principles, suggesting that these methods may also share similar limitations. In this study, we attempted to evaluate CL_{uptake} using a method that does not share common kinetic principles (viz, brain microdialysis followed by model-dependent pharmacokinetic analysis) and to compare the kinetic parameters. We selected theophylline and caffeine as models for brain microdialysis since the CL_{uptake} values for these two drugs were at the extreme ends of the uptake clearance values (Figure 1).

To determine whether the brain microdialysis was adequately carried out for the two model drugs, in-vivo and in-vitro recovery/loss was first evaluated. Since the two drugs, xanthine analogues, share a common chemical structure, one analogue may be used as the retrocalibrator for the microdialysis of the other. The equilibration across the dialysis membrane was rapid, as demonstrated by the fact that the recovery of the model drug reached 10.2% for caffeine and 10.6% for theophylline within 15 min (i.e. the first collection time) of the dialysis. The recovery was found to be constant throughout the study (i.e. up to 180 min, not significantly different with the collection by one-way analysis of variance, data not shown). The ratios of caffeine recovery to theophylline loss and theophylline recovery to caffeine loss in-vitro ($F_{in\text{-}vitro}$) were determined to be 1.070 ± 0.118 ($n = 3$) and 0.970 ± 0.124 ($n = 3$), respectively. Furthermore, the in-vivo recovery/loss ratio ($F_{in\text{-}vivo}$), was 1.080 ± 0.078 ($n = 5$) for caffeine (viz, theophylline as the retrocalibrator) and 0.959 ± 0.058 ($n = 5$) for theophylline (viz, caffeine as the retrocalibrator). Therefore, these observations suggest that these xanthine derivatives may be used as retrocalibrators in the microdialysis of the other analogues.

Using the $F_{in\text{-}vivo}$ value obtained in the control study, the concentration of the model compound in the striatal ECF (see equation 5) was estimated. The concentrations of caffeine and theophylline in the plasma and the brain ECF are shown in Figure 3. The concentration of caffeine in the ECF was comparable with that in the plasma while the ECF concentration for theophylline was significantly lower than that in the plasma (Figure 3). In addition, the plasma concentration decreased with time in a monoexponential manner in the case of caffeine while a slight biexponential decline was observed in the case of theophylline in the plasma (Figure 3). The data were fitted to equations 13 and 14 and the resulting pharmacokinetic parameters are summarized in Table 1. The kinetic analysis indicated that the CL_{uptake} was 0.639 ± 0.018 and 0.047 ± 0.006 mL min^{-1} (g brain)$^{-1}$ for caffeine and theophylline, respectively, assuming the weight of the brain in a 250-g rat to be 1.8 g (Davies & Morris 1993). The CL_{uptake} for caffeine was comparable with that obtained from the integration plot analysis (0.588 mL min^{-1} (g brain)$^{-1}$, −7.98% from the dialysis data) or the sample pooling method (0.746 mL min^{-1} (g brain)$^{-1}$, 16.7% from the dialysis data). For the case of theophylline, the CL_{uptake} value from the microdialysis study (0.047 mL min^{-1} (g brain)$^{-1}$) was found to be comparable with that obtained from the integration plot analysis (0.045 mL min^{-1} (g brain)$^{-1}$) while the estimated clearance was higher when the sampling pooling method (0.084 mL min^{-1} (g brain)$^{-1}$) was used. Since the origin of the error in the assessment is due to blood trapped in the collected tissue and is a function of the slope of the concentration–time profile (equation 11), the error may be readily calculated for theophylline. Assuming the half-life of theophylline and the volume of blood contained in the brain tissue to be approximately 9 h (Vestal et al 1986) and 0.042 mL g^{-1} (Ahn et al 2004), respectively, the error associated with the estimation is calculated to be approximately 0.034 mL min^{-1} (g brain)$^{-1}$. Therefore, the CL_{uptake} (i.e. $CL_{apparent,\ uptake}$ (0.084 mL min^{-1} (g brain)$^{-1}$) – error (0.034 mL min^{-1} (g brain)$^{-1}$)) for theophylline may be close to 0.050 mL min^{-1} (g brain)$^{-1}$, a

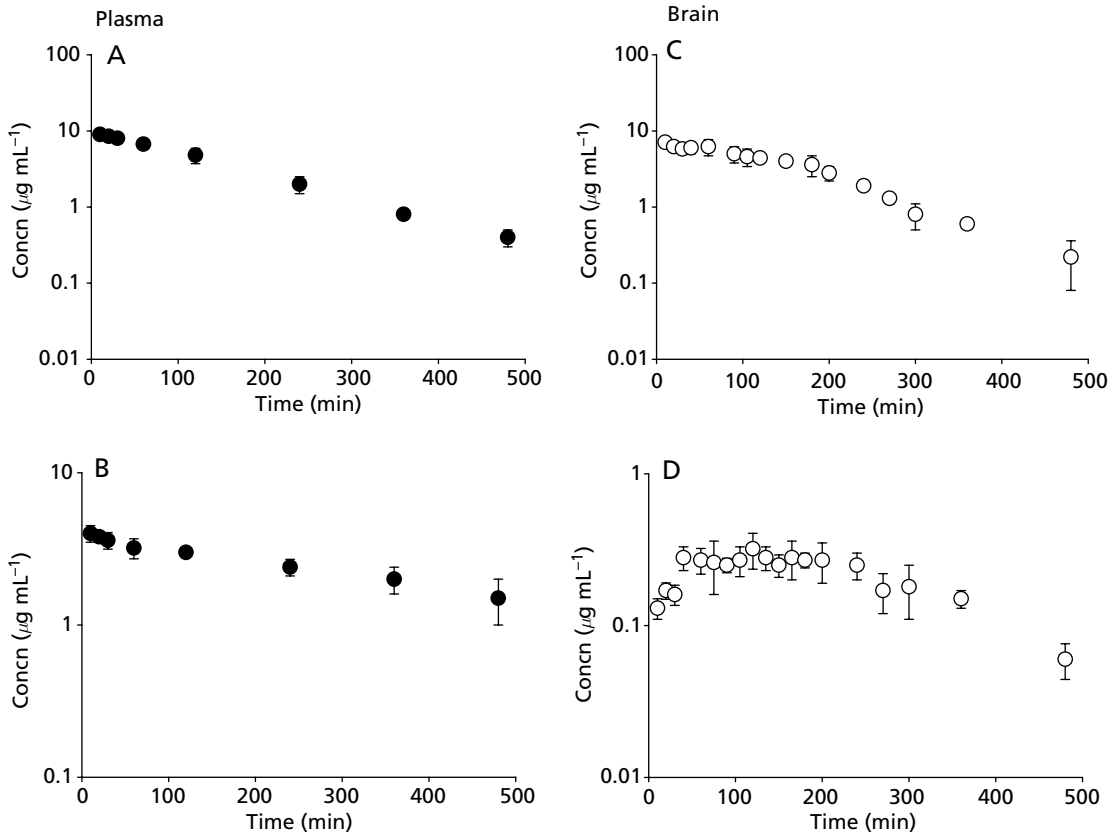

Figure 3 Temporal profiles for plasma (closed circles; A, B) and the striatal ECF (open circles; C, D) concentration of caffeine (A, C) after a 5 mg kg^{-1} intravenous administration or theophylline (B, D) after a 2 mg kg^{-1} intravenous administration in rats. The data represent the mean ± s.d. from triplicate runs.

Table 1 Summary of kinetic parameters for caffeine and theophylline after the intravenous injection of 5 and 2 mg kg^{-1}, respectively, to rats

Parameter	Caffeine	Theophylline
C_1 (μg mL^{-1})	9.370 ± 0.260	3.787 ± 0.060
C_2 (μg mL^{-1})	NA	0.976 ± 1.059
λ_1 (10^{-3} min^{-1})	5.760 ± 0.303	1.830 ± 0.730
λ_2 (min^{-1})	NA	0.120 ± 0.106
CL_{uptake} (mL min^{-1} (g brain)$^{-1}$)	0.639 ± 0.018	0.047 ± 0.006
CL_{efflux} (mL min^{-1} (g brain)$^{-1}$)	0.778 ± 0.018	0.548 ± 0.058

The data represent the mean ± s.e. of triplicate runs of the brain microdialysis study. NA, not applicable.

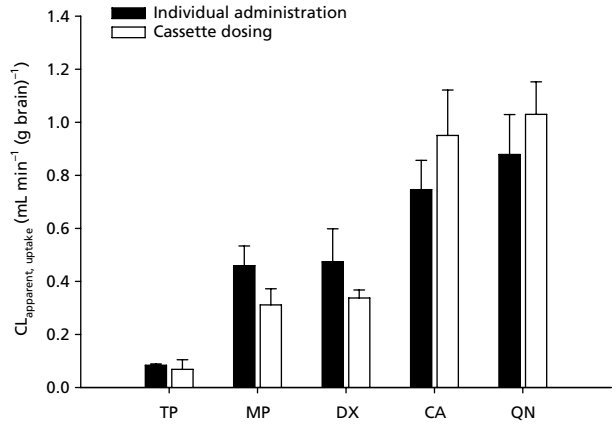

Figure 4 Comparison of the apparent brain uptake clearances ($CL_{apparent,\ uptake}$) for theophylline (TP), metoclopramide (MP), dexamethasone (DX), caffeine (CA) and quinine (QN) from cassette-dosing and individual administration to rats. The data represent the mean ± s.d. from triplicate runs.

value more comparable with the CL_{uptake} obtained from the brain microdialysis study (viz, 0.047 mL min^{-1} (g brain)$^{-1}$).

Cassette-dosing method

To determine whether the current method was applicable for the cassette-dosing situation, a combination of model drugs (viz, caffeine, theophylline, metoclopramide, quinine and dexamethasone) were simultaneously administered to rats and the $CL_{apparent,\ uptake}$ evaluated (Figure 4). The $CL_{apparent,\ uptake}$ values were not statistically

different between individual and cassette dosing for any of the five model drugs. Therefore, these observations suggest that the estimation of $CL_{apparent,\ uptake}$ from cassette dosing is comparable with that for individual administration (Figure 4). In this estimation, because the five model drugs were simultaneously administered and analytically determined, three rats and six analytical determinations were required to simultaneously assess the brain uptake clearance for the five model drugs. Therefore, by using the cassette dosing, the number of experimental animals and the number of analyses can be significantly reduced.

Discussion

For the discovery of a drug that is targeted to the brain, the evaluation of brain permeability for a list of compounds is likely to be essential. Considering the rapid growth in the numbers of available chemical libraries, the rate of assessment of brain permeability may be the determining step in the development of such new drugs. Unfortunately, however, existing methods for evaluating brain permeability are not consistent with such an accelerated screening (Takakura et al 1991; Terasaki et al 2003). In this study, we examined the feasibility of an extension of the sample pooling method in the accelerated evaluation of brain uptake kinetics. The findings showed that the $CL_{apparent,\ uptake}$ obtained using the current method was quite comparable with the CL_{uptake} obtained from the traditional integration plot analysis (Figure 2) and the rate of estimation by the current method was significantly faster than that by the traditional method. Furthermore, the sample pooling method is readily applicable to the simultaneous determination of $CL_{apparent,\ uptake}$ via cassette dosing. Collectively, these observations indicate that the current method has practical merits in cases where the accelerated estimation of brain uptake kinetics is required.

We also attempted to evaluate the brain uptake kinetics of model drugs by a brain microdialysis technique. The F values (i.e. the ratio of recoveries of the drug and the retrocalibrator) obtained in this study for caffeine and theophylline were close to unity (i.e. for caffeine, 1.090 ± 0.074; for theophylline, 0.959 ± 0.058). This indicates that the selection of retrocalibrators is adequate for these model compounds. Under these experimental conditions, the CL_{uptake} was similar amongst the three experimental methods for caffeine, with xanthine showing a higher clearance value. In contrast, however, a significant difference in CL_{uptake} values by the sample pooling method was noted for theophylline, the less permeable xanthine analogue. An examination of equation 11 indicated that a drug with a longer half-life and smaller $CL_{apparent,\ uptake}$, such as theophylline, is more prone to error in estimating brain uptake clearance. Since the error (i.e. equation 11) is given by $V_{blood,\ trapped}/t$ when λ approaches zero (i.e. where the error term reaches the maximum value), the maximum error of this method may be close to $0.034\,mL\,min^{-1}$ (g brain)$^{-1}$ (viz, $V_{blood,\ trapped}$ to be $0.042\,mL$ (g brain)$^{-1}$) for a brain collection time of 1.25 min. Assuming a 20% error in the estimation to be reasonable for the purpose of accelerated screening, the minimum brain uptake clearance that can be tolerated without a significant error may be set to $0.17\,mL\,min^{-1}$ (g brain)$^{-1}$. Among the five model drugs used in this study, only theophylline was found to have a CL_{uptake} below the lower limit.

Theoretically, the maximum error given in equation 11 is inversely proportional to the collection time. Therefore, extended collection times (e.g. 5 min) may result in a further reduction in the error associated with the estimation. Since the amount of the drug in the brain increased linearly with the area, up to a collection of 10 min in this study (Figure 1), the extension of the brain collection time would be reasonable in terms of reducing the error associated with the estimation.

In these studies the sample pooling technique was performed to increase throughput in estimating brain permeability. The use of the concept of sample pooling not only markedly reduced the sample analysis time, but also was found to accurately estimate the area under the concentration–time curve and brain uptake clearance. The primary objective of this investigation was to study the feasibility of the accelerated estimation of brain uptake kinetics via the sample pooling method. Similarly, this principle may be applied to estimating uptake kinetics to other organs (e.g., the liver). Indeed, the integration plot analysis was originally developed for other organs, such as the liver and kidney (Yamazaki et al 1993; Kino et al 1999), thus suggesting that the application of the principle may be feasible for the estimation of uptake kinetics to other organs. This aspect of sample pooling may warrant further investigation.

Conclusions

By utilizing a sample pooling method, brain uptake clearance in rats was estimated with a reasonable degree of accuracy with an accelerated rate of assessment and less experimental load. This method was also applicable to the simultaneous determination of brain uptake kinetics via the cassette dosing of multiple drugs. Therefore, the current method has considerable practical merit in the accelerated estimation of the uptake clearance to the brain in rats.

References

Ahn, S. H., Jeon, S. H., Tsuruo, T., Shim, C. K., Chung, S. J. (2004) Pharmacokinetic characterization of dehydroevodiamine in the rat brain. *J. Pharm. Sci.* **93**: 283–292

Atherton, J. P., Van Noord, T. J., Kuo, B. S. (1999) Sample pooling to enhance throughput of brain penetration study. *J. Pharm. Biomed. Anal.* **20**: 39–47

Bungay, P. M., Morrison, P. F., Dedrick, R. L. (1990) Steady-state theory for quantitative microdialysis of solutes and water in vivo and in vitro. *Life Sci.* **46**: 105–119

Cheung, B. W., Cartier, L. L., Russlie, H. Q., Sawchuk, R. J. (2005) The application of sample pooling methods for determining AUC, AUMC and mean residence times in pharmacokinetic studies. *Fundam. Clin. Pharmacol.* **19**: 347–354

Davies, B., Morris, T. (1993) Physiological parameters in laboratory animals and humans. *Pharm. Res.* **10**: 1093–1095

Hsieh, Y., Bryant, M. S., Brisson, J. M., Ng, K., Korfmacher, W. A. (2002) Direct cocktail analysis of drug discovery compounds in pooled plasma samples using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.* **767**: 353–362

Kino, I., Kato, Y., Lin, J. H., Sugiyama, Y. (1999) Renal handling of biphosphonate alendronate in rats. *Biopharm. Drug. Dispos.* **20**: 193–198

Kusuhara, H., Suzuki, H., Terasaki, T., Kakee, A., Lemaire, M., Sugiyama, Y. (1997) P-Glycoprotein mediates the efflux of quinidine across the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **283**: 574–580

Lawrenson, J. G., Reid, A. R., Finn, T. M., Orte, C., Allt, G. (1999) Cerebral and pial microvessels: differential expression of gamma-glutamyl transpeptidase and alkaline phosphatase. *Anat. Embryol. (Berl.)* **199**: 29–34

Li, H., Chung, S. J., Kim, D. C., Kim, H. S., Lee, J. W., Shim, C. K. (2001) The transport of a reversible proton pump antagonist, 5, 6-dimethyl-2-(4-fluorophenylamino)-4-(1-methyl-1,2,3, 4-tetrahydroisoquinoline-2-yl) pyrimidine hydrochloride (YH1885), across caco-2 cell monolayers. *Drug Metab. Dispos.* **29**: 54–59

Pardridge, W. M. (2003) Molecular biology of the blood-brain barrier. *Methods Mol. Med.* **89**: 385–399

Park, H. W., Chung, S. J., Kuh, H. J., Lee, M. G., Shim, C. K. (2001) The transport of a hepatoprotective agent, isopropryl 2-(1-3-dithiethane-2-ylidene)-2[*N*-(4-methyl-thiazole-2-yl) carbamoyl] acetate (YH439), across Caco-2 cell monolayers. *Arch. Pharm. Res.* **24**: 584–589

Reese, T. S., Karnovsky, M. J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* **34**: 207–217

Skarlatos, S., Yoshikawa, T., Pardridge, W. M. (1995) Transport of [125I]transferrin through the rat blood-brain barrier. *Brain Res.* **683**: 164–171

Smith, N. F., Hayes, A., Nutley, B. P., Raynaud, F. I., Workman, P. (2004) Evaluation of the cassette dosing approach for assessing the pharmacokinetics of geldanamycin analogues in mice. *Cancer Chemother. Pharmacol.* **54**: 475–486

Sugiyama, Y., Kusuhara, H., Suzuki, H. (1999) Kinetic and biochemical analysis of carrier-mediated efflux of drugs through the blood-brain and blood-cerebrospinal fluid barriers: importance in the drug delivery to the brain. *J. Control. Release* **62**: 179–186

Sun, H., Dai, H., Shaik, N., Elmquist, W. F. (2003) Drug efflux transporters in the CNS. *Adv. Drug Deliv. Rev.* **55**: 83–105

Sung, J. H., Yu, K. H., Park, J. S., Tsuruo, T., Kim, D. D., Shim, C. K., Chung, S. J. (2005) Saturable distribution of tacrine into the striatal extracellular fluid of the rat: evidence of involvement of multiple organic cation transporters in the transport. *Drug Metab. Dispos.* **33**: 440–448

Takakura, Y., Audus, K. L., Borchardt, R. T. (1991) Blood-brain barrier: transport studies in isolated brain capillaries and in cultured brain endothelial cells. *Adv. Pharmacol.* **22**: 137–165

Takanaga, H., Murakami, H., Koyabu, N., Matsuo, H., Naito, M., Tsuruo, T., Sawada, Y. (1998) Efflux transport of tolbutamide across the blood-brain barrier. *J. Pharm. Pharmacol.* **50**: 1027–1033

Takasato, Y., Rapoport, S. I., Smith, Q. R. (1984) An in situ brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* **247**: H484–H493

Terasaki, T., Deguchi, Y., Sato, H., Hirai, K., Tsuji, A. (1991) In vivo transport of a dynorphin-like analgesic peptide, E-2078, through the blood-brain barrier: an application of brain microdialysis. *Pharm Res.* **8**: 815–820

Terasaki, T., Ohtsuki, S., Hori, S., Takanaga, H., Nakashima, E., Hosoya, K. (2003) New approaches to in vitro models of blood-brain barrier drug transport. *Drug Discov. Today* **8**: 944–954

Vestal, R. E., Thummel, K. E., Mercer, G. D., Koup, J. R. (1986) Comparison of single and multiple dose pharmacokinetics of theophylline using stable isotopes. *Eur. J. Clin. Pharmacol.* **30**: 113–120

Yamazaki, M., Suzuki, H., Hanano, M., Tokui, T., Komai, T., Sugiyama, Y. (1993) Na(+)-independent multispecific anion transporter mediates active transport of pravastatin into rat liver. *Am. J. Physiol.* **264**: G36–G44

Yang, Z., Brundage, R. C., Barbhaiya, R. H., Sawchuk, R. J. (1997) Microdialysis studies of the distribution of stavudine into the central nervous system in the freely-moving rat. *Pharm. Res.* **14**: 865–872

Zhao, Y., Woo, G., Thomas, S., Semin, D., Sandra, P. (2003) Rapid method development for chiral separation in drug discovery using sample pooling and supercritical fluid chromatography-mass spectrometry. *J. Chromatogr. A* **1003**: 157–166