Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Cocktail-Dosing Microdialysis Study to Simultaneously Assess Delivery of Multiple Organic—Cationic Drugs to the Brain

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A R T I C L E   I N F O

Article history:
Received 20 August 2015
Revised 17 September 2015
Accepted 25 September 2015
Available online 10 November 2015

Keywords:
active transport
blood--brain barrier
cocktail dosing
drug transport
membrane transporter
microdialysis
organic cation transporters

A B S T R A C T

Brain microdialysis is a powerful tool to estimate brain-to-plasma unbound concentration ratio at the steady state \(K_{p,uu}\) of compounds by direct measurement of the unbound concentration in brain interstitial fluid. Here, we evaluated a method to estimate \(K_{p,uu}\) values of multiple organic—cationic drugs simultaneously, by means of brain microdialysis combined with cocktail dosing. Five cationic drugs (diphenhydramine, memantine, oxycodone, pyrilamine, and tramadol), substrates of the proton-coupled organic cation antiport system, were selected as model drugs, and compared under single-dosing and cocktail-dosing conditions. We selected doses of the drugs at which no significant drug—drug interaction occurs at the proton-coupled organic cation antiport system in the blood—brain barrier (BBB). This was confirmed by uptake studies in hCMEC/D3 cells, an in vitro BBB model. The \(K_{p,uu}\) values after cocktail administration were in the range of 1.8–5.2, and were in good agreement with those after single administration. These results suggest that the microdialysis method with cocktail dosing is suitable to estimate \(K_{p,uu}\) values of several cationic drugs simultaneously, if there is no drug—drug interaction during BBB transport. The method could be useful for evaluating drug candidates with high \(K_{p,uu}\) values at an early stage in the development of central nervous system-acting drugs.

Introduction

The blood—brain barrier (BBB) is composed of brain capillary endothelial cells linked by tight junctions, and dynamically regulates the transfer of drugs, as well as nutrients and waste metabolites, between blood and brain interstitial fluid (ISF).1 Owing to the function of the BBB, most compounds (>98%) screened as candidate drugs for the central nervous system (CNS) are rejected during preclinical development.2 Therefore, quantitative prediction of drug delivery to the brain is indispensable for efficient CNS drug development.3

The extent of drug delivery to the brain has usually been evaluated in terms of the brain-to-plasma concentration ratio of total drug in the steady state \(K_p\). However, the \(K_p\) value is greatly influenced by many factors, including plasma protein binding, brain tissue binding, and net transport activity across the BBB.4,5 On the contrary, the brain-to-plasma ratio of unbound drug in the steady state \(K_{p,uu}\), which is the ratio of the ISF and plasma-unbound concentrations, is an indicator of net BBB permeability clearance, as well as unbound drug distribution to the brain. Thus, this parameter is independent of plasma and brain tissue bindings.2,4 A drug of interest with similar influx and efflux BBB clearances would give \(K_{p,uu}\) value of unity \((K_{p,uu} = 1)\). Thus, a \(K_{p,uu}\) Value greater than unity \((K_{p,uu} > 1)\) indicates that influx clearance predominates over efflux clearance, whereas a \(K_{p,uu}\) value smaller than unity \((K_{p,uu} < 1)\) indicates that efflux clearance predominates over influx clearance. Thus, the \(K_{p,uu}\) Value is a useful indicator for evaluation of transport across the BBB.

\(K_{p,uu}\) can be evaluated by means of in vivo brain microdialysis study and/or in vitro brain homogenate or brain slice methods.6 Of these experimental techniques, only brain microdialysis can directly measure \(K_{p,uu}\) in living animals.7 Further, the microdialysis method avoids the issue of intermethodological variation when \(K_{p,uu}\) values are obtained by extrapolation from various in vitro experimental methods. Although the skill-intensive and time-consuming experimental procedures that are required by the use of brain microdialysis limit throughput in obtaining \(K_{p,uu}\) values, recent progress in analytical procedures, such as highly sensitive liquid chromatography–mass spectrometry (LC–MS/MS),8,9 has

Abbreviations used: BBB, blood—brain barrier; ISF, interstitial fluid; CNS, central nervous system.

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made it possible to overcome the through-put issue, at least in part, by means of the cocktail dosing approach. Thus, this approach offers the advantages of reducing the time required to develop CNS drugs and also reducing the number of animals used.

Deshmukh et al.\textsuperscript{11} recently reported that the use of cocktail dosing enhances throughput in rat brain microdialysis studies, but they mainly investigated drugs with $K_{p,u}^{uu}$ values less than 1. On the contrary, several clinically used CNS-acting drugs, including diphenhydramine,\textsuperscript{12} oxycodone,\textsuperscript{13} tramadol,\textsuperscript{14} apomorphine,\textsuperscript{15} and memantine,\textsuperscript{16} have been confirmed to have $K_{p,u}^{uu}$ greater than 1. These cationic drugs are actively transported into the brain by a proton-coupled organic cation antiport system, which is dependent on an oppositely directed proton gradient and metabolic energy, but not on sodium ion or membrane potential.\textsuperscript{12,14,17-20} Though the molecular entity of this antiport system has not yet been identified, it can generate high unbound drug concentration gradients in the brain across the BBB. This is important, because CNS drugs with $K_{p,u}^{uu}$ greater than 1 are not only desirable in terms of good efficacy in the brain, but also exhibit reduced peripheral side effects (i.e., the unbound drug concentration in the plasma is lower than that in the brain). For these reasons, it is anticipated that the brain microdialysis method coupled with cocktail dosing would be useful for efficient selection of CNS drug candidates with $K_{p,u}^{uu}$ greater than 1 in the early stages of drug development.

The purpose of the present study was to establish methodology to evaluate CNS-acting drug candidates with large $K_{p,u}^{uu}$ values by means of rat brain microdialysis with cocktail dosing. For this purpose, we focused on the proton-coupled organic cation antiport system, and selected oxycodone, diphenhydramine, tramadol, memantine, and pyrilamine as model drugs.\textsuperscript{12,14,17-20} Further, in cocktail dosing studies, it is important to avoid drug–drug interactions among candidates sharing the same influx transport system at the BBB, and therefore, we checked drug–drug interactions in hCMEC/D3 cells, an in vitro BBB model,\textsuperscript{11} because these cells are known to express the proton-coupled organic cation antiport system.\textsuperscript{18} The $K_{p,u}^{uu}$ values for these drugs were evaluated and compared under single-dosing and cocktail-dosing conditions.

**Materials and Methods**

**Reagents**

Antipyrine, diphenhydramine hydrochloride, memantine hydrochloride, pyrilamine maleate, and tramadol hydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan), or Sigma-Aldrich Company (St. Louis, MO). Oxycodone was kindly provided by Takeda Pharmaceutical Company Ltd. (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

**Cell Culture**

hCMEC/D3 cells immortalized by lentiviral transduction of the catalytic subunit of human telomerase and SV40-T antigen\textsuperscript{21} were cultivated at 37°C in EBDM-2 medium (Lonza, Basel, Switzerland) supplemented with 2.5% fetal bovine serum, 0.025% VEGF, 0.025% R3-JGF, 0.025% hEGF, 0.01% hydrocortisone, 5 μg/mL bFGF, 1% penicillin streptomycin, and 10 mM HEPES on rat collagen type I-coated dishes in an atmosphere of 95% air and 5% CO₂.

**Animals**

Adult male Wistar rats purchased from Japan SLC (Shizuoka, Japan) were housed, 2 or 3 per cage, with free access to food and water. The room was maintained on a 12-h dark/12-h light cycle with controlled temperature (24 ± 2°C) and humidity (55 ± 5%). This study was conducted according to guidelines approved by the Experimental Animal Ethical Committee of Teikyo University.

**In Vitro Cell Uptake Studies**

hCMEC/D3 cells between passages 25 and 35 were used for experiments. The cells were seeded on rat collagen I-coated 24-well plates (BD Biosciences, Franklin Lakes, NJ) at a density of 0.2 × 10⁶ cells/cm². At 3 or 4 days after seeding, the cells reached confluence. For uptake experiments, they were washed with 1 mL of transport buffer (122 mM NaCl, 3 mM KCl, 25 mM NaHCO₃, 1.2 mM MgSO₄, 1.4 mM CaCl₂, 10 mM D-glucose, 10 mM HEPES, pH 7.4) and preincubated with 0.25 mL of transport buffer for 20 min at 37°C. After preincubation, 0.25 mL of the transport buffer containing a single compound (1 μM each) or a cocktail of compounds (1 μM tramadol, 1 μM memantine, 1 μM pyrilamine, 1 μM oxycodone, and 3 μM diphenhydramine) was added to initiate uptake. The cells were incubated at 37°C for 30 s, and then washed three times with 1 mL of ice-cold incubation buffer to terminate the uptake. The cells were collected with a scraper in 200 μL of H₂O containing 100 nM propranolol as an internal standard and stored in a freezer set at −30°C until analysis. The cells were solubilized with an equal volume of 1 M NaOH at 37°C for 60 min and three volumes of H₂O were added. The cellular protein content was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA). Uptake was expressed as the cell-to-medium ratio (μL/(mg protein·30 s)), obtained by dividing the uptake amount by the concentration of substrate in the transport medium.

**Plasma Protein Binding Assay**

Compounds were added to 1 mL of blank rat plasma to give a drug concentration of 0.1, 1, or 10 μM. Aliquots of spiked plasma were equilibrated for 20 min at 37°C, then ultrafiltered (MPS-1; Millipore Corporation, Billerica, MA) and centrifuged at room temperature for 5 min (1000g). The total concentration in spiked plasma ($C_{p, tot}$) and the unbound concentration ($C_{p,u}$) in ultrafiltrate samples were measured by LC–MS/MS. The unbound fraction in plasma ($f_p$) was determined by dividing $C_{p,u}$ into $C_{p, tot}$.

**In Vitro Microdialysis**

_In vitro_ brain microdialysis was carried out according to our previous report.\textsuperscript{14,22} A CMA12 microdialysis probe (3 mm; CMA, Stockholm, Sweden) was inserted into a tube containing oxycodone, diphenhydramine, tramadol, memantine, pyrilamine, and antipyrine (each 1 μg/mL, 2.5–5.3 μM) in Krebs–Ringer phosphate (KRP) buffer (120 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 0.9 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, pH 7.4). KRP buffer was perfused for 240 min through the probe at a constant flow rate of 5 μL/min by means of a syringe infusion pump (Model 22; Harvard Apparatus, South Natick, MA). The dialysate was collected every 30 min and the concentrations of test drugs and antipyrine in the dialysate and the buffer were measured by LC–MS/MS.

**Brain Microdialysis with Cocktail Dosing**

_In vivo_ brain microdialysis studies were carried out according to our previous report.\textsuperscript{14,22} The rats were anesthetized with pento-barbital and a hole was drilled 2.7 mm lateral and 0.8 mm anterior to the bregma, and 3.8 mm ventral to the surface of the brain. A CMA12 guide cannula (CMA) was implanted into the striatum and
fixed to the skull with a screw and dental cement (GC Fuji I; GC Corporation, Tokyo, Japan). A CMA12 probe was inserted through the guide cannula 24 h after the surgery.

Brain microdialysis was performed 48 h after surgery. The rats were anesthetized with pentobarbital and SP31 polyethylene tubes (inner diameter: 0.5 mm, outer diameter: 0.8 mm; Natsume Seisakusho Company, Ltd., Tokyo, Japan) were inserted into the femoral vein to administer drugs and into the femoral artery for blood sampling. The cannulas were filled with 100 IU/mL heparin—saline solution to prevent clotting. The cocktail injection solution of oxycodone, diphenhydramine, tramadol, memantine, and pyrilamine was administered as an intravenous bolus dose of 0.28, 1.2, 2.0, 2.0, and 0.52 mg/kg, respectively, followed by a 4-h constant infusion of 8.9, 150, 10, 4.5, and 3.6 μg/(kg·min), respectively, into the femoral vein by using a Harvard 22 pump. The bolus and infusion doses are included in Table 1. Antipyrine was coadministered as a reference compound, and its bolus and constant infusion doses were 0.3 mg/kg and 0.6 μg/(kg·min), respectively. KRP buffer was perfused for 240 min through the probe at a flow rate of 5 μL/min. The dialysate solution and blood were collected every 30 min. Blood samples were centrifuged at 3000 g for 10 min at 4°C to obtain plasma.

The concentrations of test drugs and antipyrine in dialysate, plasma, and CSF were measured by LC–MS/MS, as described below.

Extrapolation of Brain ISF Concentration

The steady-state concentration of each drug in the ISF (C_{isf}) was estimated according to the reported method, as follows.

\[ R_{vitro} = \frac{C_{d,vitro}}{C_f} \times \left(1 - \exp(-\frac{PA_{vitro}}{F})\right) \]  

(1)

\[ C_{isf} = \frac{C_{d,vivo}}{\left(1 - \exp(-R_d \times \frac{PA_{vitro}}{F})\right)} \]  

(2)

\[ PA_{vivo} = R_d \times PA_{vitro} \]  

(3)

where PA_{vivo} and PA_{vitro} are the in vitro and in vivo permeability area products, respectively, of each drug through the microdialysis probe. F is the dialysis flow rate, and C_{d,vitro} and C_{d,vivo} are the dialysate drug concentrations obtained in vitro and in vivo microdialysis studies, respectively. C_f is the reservoir concentration in the in vitro microdialysis study. R_d is the effective dialysis coefficient, which is the ratio of the in vivo and in vitro permeability rate constants.

\[ R_d = \frac{PA_{vivo}}{PA_{vitro}} \]  

(4)

In the reference method, the R_d value is assumed to be the same as that of a reference compound, antipyrine, because it is well established that the binding of antipyrine to plasma and tissue proteins is negligible, and there is rapid equilibration between ISF and plasma.

Accordingly, the K_{pu,u} value in the steady state can be determined according to the following formula:

\[ K_{pu,u} = \frac{C_{isf}}{f_p \times C_{p,tot}} \]  

(5)

LC–MS/MS Analysis

Cells and plasma were deproteinized with four volumes of acetonitrile, kept in a freezer (−30°C) for at least 30 min and filtered (pore size: 0.2 μm). The filtered samples, ultrafiltrate, and dialysate were analyzed on an LC–MS/MS system composed of an Acela HPLC system and a TSQ Quantum Ultra (Thermo Fisher Scientific Inc.) mass spectrometer with an electrospray ionization interface in positive ion mode. Chromatographic separation was achieved on a Synergi Hydro-RP column (2.0 × 50 mm², 2.5 μm; Phenomenex, Torrance, California) at a flow rate of 0.3 mL/min. The gradient program was composed of solvent A [ammonium acetate buffer (10 mM, pH 4.0)] and solvent B (methanol) as follows: 0% B for 0-0.5 min, 0%–80% B for 0.5-2 min, 80% B for 2-3.5 min, and 0% B for 3.5-5 min. The column temperature was set at 40°C. Xcalibur version 2.10 software was used to control the instrument and to collect data. All values are presented as average ± standard error.

Results

Uptake Study Using hCMEC/D3 Cells

Initial uptake by hCMEC/D3 cells from medium containing each single drug or the cocktail of all five drugs was measured to confirm the absence of drug–drug interaction in the uptake process from blood to brain. Uptake data for each of the five drugs tested showed

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Values of Steady-State Total Plasma Concentration (C_{p,tot}) and Unbound Plasma Concentration (C_{p,u}), Brain Interstitial Fluid Concentration (C_{isf}), Plasma Unbound Fraction (f_p), and K_{pu,u} for the Test Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Oxycodone</td>
</tr>
<tr>
<td>Dose</td>
<td>(μM)</td>
</tr>
<tr>
<td>C_{p,tot}</td>
<td>(μM)</td>
</tr>
<tr>
<td>C_{p,u}</td>
<td>(μM)</td>
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<tr>
<td>C_{isf}</td>
<td>(μM)</td>
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<td>C_{i,v}</td>
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<td>C_{i,s}</td>
<td>(μM)</td>
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<tr>
<td>C_{i,t}</td>
<td>(μM)</td>
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<td>f_p</td>
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<tr>
<td>K_{pu,u}</td>
<td>Single</td>
</tr>
<tr>
<td>K_{pu,u}</td>
<td>Cocktail</td>
</tr>
</tbody>
</table>

The values of f_p were determined by the ultrafiltration method. C_{p,u} was calculated as C_{p,tot} × f_p. C_{isf} was estimated by the reference method (Eq. (2)). K_{pu,u} was calculated according to Eq. (5). Each result is the mean ± SE of 4 or 6 experiments. The single-dosing value for oxycodone was calculated from the results in Ref. 13.
no significant difference between a single-drug-containing medium and a cocktail-containing medium at the concentration of 1 μM for each drug except diphenhydramine (3 μM) (Fig. 1). This supports the idea that drug–drug interaction is not likely to occur in the uptake process at brain capillary endothelial cells at these concentrations. In the subsequent studies, the doses were adjusted to obtain plasma unbound concentrations of about 1 μM for oxycodone, tramadol, memantine, and pyrilamine, and 3 μM for diphenhydramine.

In Vitro and In Vivo Permeability Rate Constants

To extrapolate the ISF concentration by the reference method using Eq. (2), the in vitro and in vivo permeability area product of microdialysis probes (PA_vitro and PA_vivo) of test drugs were determined. The results are summarized in Table 2. PA_vitro was estimated by multiplying PA_vitro by R_f of antipyrine, assuming that the R_f values of the test drugs are the same as that of antipyrine. The PA_vitro values determined for a drug cocktail solution ranged from 0.43 μL/min for tramadol to 0.53 μL/min for pyrilamine. To obtain PA_vivo of drugs according to Eq. (3), R_d value was measured in the same individual rats using antipyrine as a reference compound. The average R_d value of antipyrine was 0.240 ± 0.028, suggesting that PA_vitro value of drugs is decreased to about 25% of the PA_vitro Value. There were no major differences in PA_vitro and PA_vivo values between cocktail dosing and single dosing (Table 2).

Comparison of In Vivo Microdialysis Data Between Single Dosing and Cocktail Dosing

Figure 2 shows the time courses of unbound plasma concentration (C_p,u) and brain ISF concentration (C_isf) after single dosing or cocktail dosing of test drugs. Total plasma concentration (C_p,tot, data not shown), C_p,u, and C_isf of all drugs tested reached a steady state by 120 min after administration. Therefore, the average values of C_p,tot, C_p,u, and C_isf during 120-240 min after administration were used in the comparison of single dosing and cocktail dosing. The values of unbound fraction in plasma (f_p) were measured by in vitro ultrafiltration, and the values of the 5 test drugs did not show any significant difference between measurement of each drug alone and in the presence of the other four drugs, suggesting no apparent interaction of the five drugs in plasma protein binding (Table 1).

The total concentration in spiked plasma, C_p,u, and C_isf values at the steady state after cocktail dosing of test drugs were almost identical to those after a single dosing of each drug, except for memantine. C_p,tot, C_p,u, and C_isf of memantine after cocktail dosing were approximately 4.5-fold higher than those after single dosing (Table 1). K_p,u values of all drugs were greater than unity, confirming that these drugs are actively taken up by the brain. Further, there is no marked difference of K_p,u values between single dosing and cocktail dosing for all the drugs (Table 1).

Discussion

The establishment of effective methodology for evaluating K_p,u would be helpful in the development of CNS-acting drugs with high K_p,u values. Here, we simultaneously determined the K_p,u values for five CNS-acting cationic drugs, substrates of proton-coupled organic cation antiport system, by using the rat brain microdialysis method combined with cocktail dosing. The obtained K_p,u values for these drugs were all greater than unity, and comparable with those obtained by after dosing of each drug alone. This is the first evidence that the cocktail-dosing microdialysis method is applicable for evaluation of K_p,u values for drugs whose influx clearances predominate over efflux clearances under conditions where drug–drug interactions do not occur in the BBB transport process.

Thus, it is important to test preliminarily whether or not drug–drug interactions are likely to occur in the BBB transport process. Proton-coupled organic cation antiport system accepts substrates with K_m values around 15–100 μM and is inhibited by many cationic drugs at relatively high concentrations. Therefore, to examine the possibility of drug–drug interaction during the BBB transport, we carried out an in vitro uptake study using hCMEC/D3 cells. It has been shown that functional expression of the proton-coupled organic cation antiport system in hCMEC/D3 cells is similar to that in rat BBB model cells, in terms of oxycodone and diphenhydramine uptakes. As shown in Figure 1, no appreciable drug–drug interaction was seen in cellular uptake under conditions where each drug concentration was 1 μM (except for diphenhydramine: 3 μM) and the sum of the concentrations was 7 μM. Taking into account that the K_m values are around 15–100 μM, it was expected that drug–drug interactions at the transporter would not occur under these conditions. Indeed, the K_p,u values determined by cocktail-dosing brain microdialysis study were consistent with those of each single-dosing study under conditions where the sum of the C_p,u was less than 7 μM (Table 1). These results suggest that drug interactions at the proton-coupled organic cation antiport system can be minimized by selection of appropriate experimental conditions for cocktail dosing. Further, the K_p,u values for these five drugs were all greater than unity, indicating that influx clearance predominates over efflux clearance for these drugs, in line with previous reports on oxycodone, diphenhydramine, memantine, and tramadol. The K_p,u value of pyrilamine, a typical substrate of the proton-coupled organic cation antiport system at the BBB, has not been reported previously, but was shown to be higher than unity in this study. Thus, cocktail-dosing microdialysis study at appropriate concentrations can be used to estimate K_p,u values and to identify drug candidates with high K_p,u values. In addition, to avoid the possibility of misjudgment because of potent inhibitory activity toward the proton-coupled organic cation antiport system, it would be desirable to check the inhibitory effects by means of in vitro uptake assay.
or by addition of a known substrate of the antiport system to the cocktail of tested compounds as a positive control.

Microdialysis using the reference method can be employed to estimate ISF concentration from the in vitro permeability area product of the microdialysis probe (PA_{vitro}) and the R_d value estimated using antipyrine as a reference compound (Eqs. (1)-(4)).^{23-26} The reference method is useful to correct for differences in diffusion through the fluid environment around the microdialysis probe between in vitro and in vivo situations.^{24,25} Although it is arguable whether or not the reference method is applicable to a wide variety of compounds, it should at least be suitable for compounds with similar molecular weights, because the diffusivity of a compound around the microdialysis probe is predominantly determined by molecular weight.^{23} Indeed, the BBB transport mechanisms of several drugs have been characterized by means of the reference method.^{14,22,26}

In the present study, we confirmed that there were no appreciable differences of PA_{vitro} and plasma protein binding (f_p) of each drug between single drug and cocktail determinations (Tables 1 and 2). The differences in unbound concentrations in brain ISF and plasma between single-dosing and cocktail-dosing assay were within twofold, except for memantine (Table 1). These results suggest that there is no apparent interaction in plasma pharmacokinetics or brain delivery of oxycodone, diphenhydramine, tramadol, and pyrilamine after cocktail dosing. Memantine concentrations in plasma after cocktail dosing were higher than after single dosing. Though the mechanism underlying the increase in memantine concentration in the plasma is unclear, memantine ISF concentration was elevated with increasing plasma concentration after cocktail dosing. Accordingly, the K_{p,uu} value of memantine (1.82) after cocktail dosing was very close to that after single dosing (1.80), and this suggests that there was no appreciable interaction between memantine and the other four drugs in the BBB transport process.

This study shows that the cocktail-dosing brain microdialysis method is applicable to evaluate a wide range of K_{p,uu} values for drugs that are actively transported into the brain, in addition to drugs with smaller K_{p,uu} values because of active efflux transport from the brain across the BBB. Substrates of MDR1 and BCRP, which are major efflux transporters at the BBB, are usually discontinued early in drug development on the basis of in vitro cell assay. However, if such compounds were also substrates of influx transporter(s), such as the proton-coupled organic cation antiport system, a pharmacologically relevant concentration might be obtained in the brain ISF. In other words, the present results indicate that the proton-coupled organic cation transport system can play an important role as an efficient blood-to-brain influx transporter, and therefore, cocktail-dosing microdialysis could be a useful method for precise estimation of K_{p,uu} values of selected candidates in drug discovery.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Oxycodone</th>
<th>Diphenhydramine</th>
<th>Tramadol</th>
<th>Memantine</th>
<th>Pyrilamine</th>
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<tbody>
<tr>
<td>R_{vitro}</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Single</td>
<td>0.0838 ± 0.0040</td>
<td>0.0866 ± 0.0016</td>
<td>0.0849 ± 0.0082</td>
<td>0.136 ± 0.003</td>
<td>0.129 ± 0.010</td>
</tr>
<tr>
<td>Cocktail</td>
<td>0.0915 ± 0.0019</td>
<td>0.0983 ± 0.0119</td>
<td>0.0815 ± 0.0069</td>
<td>0.0888 ± 0.0120</td>
<td>0.100 ± 0.009</td>
</tr>
<tr>
<td>PA_{vitro} (μL/min)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>0.438 ± 0.022</td>
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<tr>
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<td>0.426 ± 0.037</td>
<td>0.467 ± 0.066</td>
<td>0.531 ± 0.050</td>
</tr>
</tbody>
</table>

R_d was estimated using antipyrine, a reference compound, in each rat. Each result is the mean ± SE of four or six experiments.
Conclusions

Our results indicate that the cocktail-dosing brain microdialysis method can be used for evaluating drug candidates with $K_{puu}$ greater than 1, provided that the cocktailed drugs do not interact with each other in the BBB transport process. In vitro uptake studies using hCMEC/D3 cells, which functionally express proton-coupled organic cation antiport system, are helpful to determine the concentrations of drugs to be used in the cocktail. Further developments of analytical LC–MS/MS technology should increase the number of drugs that can be included in a cocktail. We believe this approach will be a powerful tool for identifying promising CNS-acting drug candidates early in the development process.

Acknowledgments

We would like to thank Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France) for supplying hCMEC/D3 cells under license from INSERM. We also thank Ms. Kana Sobue, Mr. Toshiki Kurosawa, and Mr. Kosho Higashi. This work was supported in part by a Grant-in-Aid for Scientific Research and by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities provided by The Ministry of Education, Culture, Sports, Science and Technology.

The authors declare no conflict of interest.

References