Diphenhydramine Active Uptake at the Blood–Brain Barrier and Its Interaction with Oxycodone *in Vitro* and *in Vivo*

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ABSTRACT: Diphenhydramine (DPHM) and oxycodone are weak bases that are able to form cations. Both drugs show active uptake at the blood–brain barrier (BBB). There is thus a possibility for a pharmacokinetic interaction between them by competition for the same uptake transport system. The experiments of the present study were designed to study the transport of DPHM across the BBB and its interaction with oxycodone *in vitro* and *in vivo*. *In vitro*, the interaction between the drugs was studied using conditionally immortalized rat brain capillary endothelial cells (TR-BBB13 cells). The *in vivo* relevance of the *in vitro* findings was studied in rats using brain and blood microdialysis. **DPHM was actively transported across** the **BBB *in vitro* (TR-BBB13 cells).** Oxycodone competitively inhibited DPHM uptake with a K_i value of 106 μM. DPHM also competitively inhibited oxycodone uptake with a K_i value of 34.7 μM. **In rats, DPHM showed** fivefold higher unbound concentration in brain interstitial fluid (ISF) than in blood, confirming a net active uptake. There was no significant interaction between DPHM and oxycodone *in vivo*. This accords with the results of the *in vitro* experiments because the unbound plasma concentrations that could be attained *in vivo*, without causing adverse effects, were far below the K_i values. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3912–3923, 2011

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INTRODUCTION

Diphenhydramine (DPHM) is a histamine H1-receptor antagonist that is widely used against hypersensitivity reactions, and for its sedative/hypnotic and antiemetic effects. Like other first-generation antihistamines, DPHM causes sedative effects and hypnosis by occupying H_1 receptors in central nervous system (CNS), indicating its ability to cross the blood–brain barrier (BBB). Oxycodone is an opioid analgesic, often used for the treatment of moderate-to-severe pain such as malignant and postsurgical pain.

Diphenhydramine had a higher total concentration in the brain than in plasma in rats and guinea pigs when administered by different routes.[1,2] It was also reported to enter the brain tissue from blood with a rapid and saturable influx.[3] Mahar Doan et al.[4] studied the steady-state brain concentration of DPHM and other antihistamines in rats. They found a brain-to-plasma ratio, K_p, of 18.4, and a brain-to-unbound plasma ratio, $K_{p,u}$, of 115, confirming a high distribution of DPHM to and within the brain. Au-Yeung et al.[5] found that the free concentration exposure of DPHM for sheep was at least two times higher in the brain than in blood. Higher free concentration in the brain than in blood strongly suggests the presence of

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an active uptake transport mechanism at the BBB for DPHM. Boström et al.[6,7] reported that the unbound steady-state concentration of oxycodone in rats was threefold higher in the brain than in plasma.

Diphenhydramine is a weak base having a tertiary amine moiety, and is thus able to form a cation. In a few studies, cationic compounds have been found to be transported with a proton-coupled transport system. Methylenedioxymethamphetamine is one of them and its transport was inhibited by DPHM in a concentration-dependent manner.[8] Oxycodone, a tertiary amine, and clonidine were also found to be transported by this H^+ antiporter, which has different functional properties than previously known organic cation transporters (OCTs).[9,10] Andre et al.[11] suggested that clonidine transport across the luminal BBB involves a distinct amine/H^+ antiporter, and that this transporter interacts with organic compounds that have secondary or tertiary amine moieties. The molecular nature of this amine/H^+ transporter still remains to be revealed. At the BBB, this system is responsible for the transport of pyrilamine, an H_1 antagonist. The uptake of pyrilamine was inhibited by DPHM.[12–14] Okura et al.[10] reported that this pyrilamine transporter is also involved in oxycodone transport across the BBB.

Diphenhydramine and oxycodone may thus be transported across the BBB by the same transporter. If it is so, then there is a possibility for a pharmacokinetic interaction between them via competition for the same uptake transport system. It is important to understand this interplay as it may influence the pharmacodynamics of the two drugs and thereby the dose requirements if coadministered.

The present experiments were designed to study the transport of DPHM across the BBB and its interaction with oxycodone *in vitro* and *in vivo*. Interaction between these drugs was studied *in vitro* in conditionally immortalized rat brain capillary endothelial cells (TR-BBB13 cells). The *in vivo* relevance of the interaction was studied in rats using brain and blood microdialysis.

MATERIALS AND METHODS

All *in vitro* experiments were performed at Teikyo University (Sagamihara, Japan), and all *in vivo* experiments were performed concomitantly at Uppsala University (Uppsala, Sweden).

Chemicals

Diphenhydramine hydrochloride was purchased from Sigma–Aldrich (Stockholm, Sweden). Oxycodone was kindly provided by Takeda Pharmaceutical Company Ltd. (Osaka, Japan) or purchased from Apoteket AB, Production & Laboratory (Stockholm, Sweden. The molecular mass of DPHM is 255.36 g/mol and of oxycodone is 315.36 g/mol. D3-DPHM, D3-oxycodone, and D6-oxycodone were purchased from Cerilliant Corporation, (Round Rock, Texas). The saline solution was from Frezenius Kabi (Bad Homburg, Germany), isoflurane was from Baxter medical AB (Kista, Sweden), and heparin of 100 IU/mL was from Leo Pharma AB (Malmö, Sweden). Acetonitrile (ACN), formic acid, and ammonium acetate were purchased from Merck (Darmstadt, Germany). The water was purified using Milli-Q system (Millipore, Bedford, Massachusetts). The Ringer perfusion solution consisted of 145 mM NaCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.6 mM KCl, and 0.1 mM ascorbic acid in 2.0 mM phosphate buffer (pH 7.4). Ammonium formate buffer was prepared by mixing 100 μL formic acid and 750 μL of 25% NH_3 solution (Merck) with 2 L Milli-Q water. All other chemicals and reagents were commercial products of reagent grade.

In Vitro Experiments

Uptake Studies in TR-BBB13 Cells

TR-BBB13 cells were seeded on collagen-coated multiwell dishes at a density of 0.1×10^5 cells/cm^2. Three days after seeding, the cells were washed twice with 1 mL of phosphate buffer saline and preincubated with incubation buffer (122 mM NaCl, 3 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM D-glucose, and 10 mM *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES), pH 7.4) for 20 min at 37°C. After preincubation, the buffer (2 mL) containing DPHM (30 μM) was added to initiate uptake. The cells were incubated at 37°C for a designated time to identify time-dependent linearity of uptake, and then washed three times with 2 mL of ice-cold incubation buffer to terminate the uptake. To measure the effect of extracellular pH, the uptake was measured in acidic and alkaline medium (pH 6.0–8.4). The uptake was also measured in the presence or absence of 10 μM *p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore. When the influence of intracellular pH (pHi) was examined, the uptake was measured in the presence of 30 mM NH_4Cl to elevate pHi.[15,16] To measure the uptake at acidic pHi, extracellular NH_4Cl was removed after the preincubation with 30 mM NH_4Cl because intracellular NH_3 rapidly diffuses out of the cells, resulting in the accumulation of protons released from NH_4^+ during NH_3 generation in the cells. After treatment with 25 μM rotenone or 0.1% NaN_3 for 20 min to reduce metabolic energy, the uptake of DPHM was measured as described above. In the Lineweaver–Burk plot analysis, DPHM uptake was measured in the presence or absence of oxycodone (500 μM). The uptake of oxycodone (50–500 μM, for 15 s) into TR-BBB13 cells was also measured as described previously,[10] in the presence or absence of DPHM (50 μM). The cells were collected in 400 μL

of 0.5% KH_2PO_4 solution or 300 μL of water for determination of DPHM or oxycodone, respectively. The collected samples were stored at −20°C until DPHM or oxycodone was determined as described below.

Cellular protein content was determined with a BCA protein assay kit (Pierce Chemical Comapny, Rochford, Illinois). Uptake was expressed as the cell-to-medium ratio (μL/mg protein) obtained by dividing the uptake amount by the concentration of substrate in the incubation buffer. In order to estimate the kinetic parameters for the uptake by TR-BBB13 cells, the initial uptake rate for DPHM (30–1000 μM, for 15 s) was determined by subtracting the uptake at a concentration of 5 mM DPHM. The following equation was fitted to the initial uptake rate data by means of nonlinear least-squares regression analysis with Prism software (Graphpad, San Diego, California):

$$v = \frac{v_{max} \times s}{K_m + s} \quad (1)$$

where v is the initial uptake rate of substrate [nmol/(min mg protein)], s is the substrate concentration in the medium (μM), K_m is the Michaelis–Menten constant (μM), and v_{max} is the maximum uptake rate [nmol/(min mg protein)].

Chemical Analysis

Diphenhydramine. The collected cells in 0.5% KH_2PO_4 solution were homogenized by sonication. To the cell homogenate (300 μL), orphenadrine hydrochloride solution (50 μM, 6.3 μL) as internal standard was added, as well as 60 μL of saturated K_2CO_3 solution. After mixing, samples were extracted with 1.5 mL of hexane–isopropanol (98:2, v/v) by shaking in a horizontal shaker for 15 min and then centrifuging for 10 min at 800 × g. The upper organic layer was transferred into a tube containing 300 μL of 0.5% KH_2PO_4 solution. The tube was shaken for 15 min and centrifuged for 10 min at 800 × g. The upper organic layer was aspirated off and the lower aqueous phase was added to 60 μL of saturated K_2CO_3 solution, and the extraction and back-extraction step was repeated. The final K_2CO_3 solution phase was extracted again with 1.5 mL of hexane–isopropanol, and the extract was dried under a nitrogen stream. The residue was reconstituted in 100 μL of mobile phase.

Diphenhydramine was determined by ultra-performance liquid chromatography (UPLC®) with ultraviolet (UV) detector, by modification of the previous methods.[17,18] A 7.5 μL aliquot was injected into the UPLC®. The UPLC® system (Waters ACQUITY, Milford, Massachusetts) consisted of a binary solvent manager, sample manager, and UV detector. The analytical column used was an ACQUITY UPLC BEH C18 (2.1 × 50 mm^2, 1.7-μm particle size; Waters). The UPLC separation was carried out at a flow rate of 0.15 mL/min with a mobile phase containing 25% ACN and 0.22 mM phosphate buffer. UV detection was performed at 205 nm. The retention times of DPHM and orphenadrine were 6.4 and 9.1 min, respectively. The detection limit for quantification of DPHM was 75 pmol.

Oxycodone. Oxycodone in collected cells was determined by the same high-performance/pressure liquid chromatography (HPLC) method reported previously.[10] Briefly, the collected cells in water were homogenized by sonication. To the cell homogenate (200 μL), 20 μL of codeine solution (250 ng/mL) was added as an internal standard, and 100 μL of 4 M NaOH and 800 μL of butyl chloride were also added. The samples were mixed and centrifuged for 10 min at 800 × g at 4°C, and then the butyl chloride layer was transferred. The butyl chloride extract was evaporated to dryness. The residue was reconstituted in 200 μL of mobile phase, and a 40-μL aliquot was injected into the HPLC. The HPLC system for measuring oxycodone consisted of a pump (301E; Eicom, Kyoto, Japan) and an electrochemical detector (ECD-300; Eicom). The HPLC analytical column used was an XTerra® RP18 (4.6 × 50 mm^2, 5-μm particle size; Waters). The HPLC separation for oxycodone and codeine was carried out at a flow rate of 0.5 mL/min with a mobile phase containing 10% ACN, 20% methanol, and 5 mM phosphate buffer (pH 8.0) at 40°C.

Statistical Analysis of In Vitro Data

Statistical analysis of the data was performed by employing Student's t-test and by one-way analysis of variance followed by Dunnett's test for single and multiple comparisons, respectively. Differences were considered statistically significant at a P value of less than 0.05.

In Vivo Experiments

Animals

The studies were approved by the Ethics Committee for Animal Research in Tierp, Sweden (Ref. numbers C 3/08 and C 2/08). The rats were divided into three groups. Male Sprague–Dawley rats (Scanbur B&K, Sollentuna, Sweden) were used in the DPHM uptake study in Group 1. For Groups 2 and 3 male Sprague–Dawley rats were purchased from Taconic (Lille Skensved, Denmark). The rats were acclimatized for 7 days prior to surgery in 12 h light–dark cycle with free access to food and water at 22°C. The weights were between 0.256 and 0.304 kg on the day of surgery.

Surgery

The rats were anesthetized with 2.5% isoflurane balanced with oxygen (2 L/min) and nitrous oxide (1.5 L/min), administered with a mask. To maintain body temperature at 38°C during the surgery, the rats were placed on a heating pad (CMA/150 temperature controller; CMA, Stockholm, Sweden). A PE-50 cannula (CMA, Solna, Sweden) fused with a short piece of PE-10 connected to a piece of silicon tubing was inserted into the left femoral vein and also into the left jugular vein for rats in Groups 2 and 3 for drug administration. A PE-50 cannula fused with PE-10 tubing was inserted into the femoral artery for blood sampling. The cannulas were filled with heparin to avoid clotting (0.4 mL of 100 IU/mL heparin to 20 mL saline).

A flexible CMA 20 Elite PAES (polyarylethersulphone) probe with a membrane length of 10 mm and 20,000 Da cutoff (CMA, Stockholm, Sweden) was inserted into the right jugular vein and fixed with two stitches to the pectoral muscle. The rat was placed in a stereotactic instrument (David Kopf instruments, Tujunga, California), and the skull was exposed by making a midline incision. 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 CMA/12 guide cannula was implanted into the striatum and fixed to the skull by a screw and dental cement (Dentalon® Plus; Heraeus, Hanau, Germany). A CMA/12 (3 mm) probe was inserted after fixing the guide cannula. Both probes were perfused with Ringer solution before the insertion. The inlet and outlet were then sealed to prevent air from entering the probes. In order to allow the perfusion solution to adjust to body temperature before entering the brain probe, a 15-cm PE-50 tube was looped subcutaneously on the back of the rat to the surface of the neck. The catheters were passed subcutaneously to the posterior surface of the neck and placed in a plastic cup sutured to the skin out of reach of the rat. The rats were placed in a CMA/120 system for freely moving animals after surgery, with free access to water and food, and were given 24 h to recover.

Pharmacokinetic Study of DPHM

To determine basic pharmacokinetic parameters for DPHM in rats, a plasma pharmacokinetic study after a DPHM bolus infusion was carried out in four rats. DPHM was administered to the rats as a 150 μg/(min kg) constant rate intravenous bolus infusion over 5 min [0.59 μmol/(min kg)]. Blood samples of 150 μL were collected before (0 min) and 2, 4, 10, 15, 30, 60, 90, 120, 180, 240, and 300 min after the infusion started. The samples were collected in heparinized tubes to avoid clotting and centrifuged for 5 min at 10,000 rpm ($7200 \times g$) immediately after sampling. The plasma was then transferred to Eppendorf tubes and stored at −20°C until analysis. The information obtained was used in the calculation of bolus and constant rate infusion doses.

DPHM Active Uptake Study (Group 1)

The microdialysis probes were initially perfused with Ringer solution and spiked with 10 ng/mL DPHM at a flow rate of 1 μL/min. A 1 h stabilization period was followed by 1 h of retrodialysis to calibrate the probes. The perfusion solution was then switched to blank Ringer solution for 1 h before the drug was administered. A 5 min bolus infusion of 234 μg/(min kg) [0.92 μmol/(min kg)] DPHM in saline solution was followed by a constant rate infusion of 30 μg/(min kg) [0.12 μmol/(min kg)] for 2 h. Dialysate fractions from blood and brain ISF were collected in polypropylene vials every 10 min (10 μL) after starting the stabilization period, lasting until 1 h after the end of infusion and then every 20 min for two more hours. Blood samples were collected in heparinized Eppendorf tubes before the study starts (0 min) and at 25, 55, 115, 175, 230, and 300 min after the infusion starts. Eight animals were studied. Four of them were decapitated directly before the end of the infusion to obtain information on the total drug amount in the brain at a steady state.

DPHM and Oxycodone Interaction Study (Groups 2 and 3)

Two groups, each consisting of six rats, were studied. The day after the surgery, microdialysis experiment started with a stabilization and retrodialysis period for Group 1. Deuterated calibrators of DPHM and oxycodone were used to estimate the recovery in all animals except for DPHM in Group 3 (Table 1). The flow rate was 1 μL/min in the microdialysis probes. The dialysate fractions were collected from blood and brain microdialysis probes every 10 min into polypropylene vials. In Group 3, a washout period of 60 min followed the retrodialysis with DPHM. Both Groups 2 and 3 received infusions in three periods according to Figure 1 and Table 1. For Group 2, drug A was DPHM and drug B was oxycodone. In Group 3, the sequence of drugs was opposite, that is, drug A was oxycodone and drug B was DPHM (Fig. 1). All infusions started with a 10 min bolus infusion followed by a 80 min constant rate infusion. During the first infusion period, a low infusion rate of drug A was administered. In the second period, a high infusion rate of drug B was started along with drug A. In the final period, the low infusion rate of drug A was replaced by a high infusion rate with continued rate of drug B infusion (Fig. 1). The experiments in Group 2 were designed to study the effect of oxycodone on DPHM transport across the BBB, and in Group 3 to study the effect of DPHM on oxycodone transport

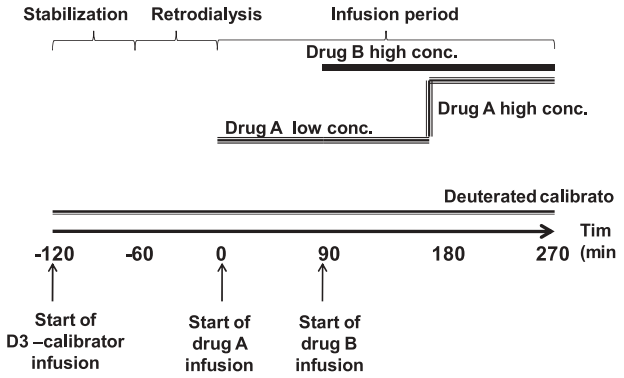

Figure 1. Study design for *in vivo* interaction. For Group 2, drug A was DPHM and drug B was oxycodone, and for Group 3, it was the opposite, that is, drug A was oxycodone and drug B was DPHM.

across the BBB. All rates of administration for both drugs are shown in Table 1.

Blood samples were collected in heparinized Eppendorf vials at 0, 25, 55, 85, 115, 145, 175, 205, 235, and 265 min. Plasma was separated by centrifugation at 10,000 rpm ($7200 \times g$) for 5 min and stored at -20°C until analysis. The rats were decapitated at the end of the experiment (270 min), and the brain was checked for possible brain tissue damage after the probe insertion, divided in half and immediately frozen at -20°C.

Chemical Assay

Diphenhydramine

Standards and Quality Control. The analytical methods for the Ringer solution and plasma samples were validated using quality controls (QCs). For the analysis of the microdialysis samples, a series of 11 standards of DPHM in Ringer solution was prepared in the range of 0.104–125 ng/mL. QCs with the concentrations 0.7, 7.4, 36.9, and 73.8 ng/mL were also prepared in Ringer solution, using a different stock solution of DPHM. A standard series and QCs for the analysis of DPHM in plasma were prepared from blank plasma spiked with DPHM stock solutions. The 11 standards were in the range of 0.2–416 ng/mL, and the QCs had the concentrations 3.5, 17.3, 86.5, and 346 ng/mL.

In the interaction study (Groups 2 and 3), concentrations of DPHM administered to animals were much higher, so the range of standards increased to 0.5–832 ng/mL for microdialysis samples with four QCs from 2.9 to 576.5 ng/mL, whereas for plasma samples, standards ranged from 10 to 5200 ng/mL with three QCs from 57.7 to 1153 ng/mL.

For the brain tissue analysis, standards and QCs were prepared by adding DPHM stock solution in saline to blank brain tissue before homogenization. Seven standards in the brain homogenate were made

Table 1. Doses of DPHM and Oxycodone Administered with Achieved Plasma Concentrations. The Table also Describes the Different Calibrators Used for Recovery Estimation and Resulting Recoveries

	Drug Administered (10 min Bolus + 80 min Constant Rate Infusion)	Dose [μg/(kg min)]		Plasma Conc. (C_{ss}) Achieved Average ±SD (μM)	Calibrator Used for Recovery Estimation	Recovery Average ±SD%	
		Bolus	Constant Rate inf.			Brain	Blood
Group 2 ($n = 6$)	DPHM low	58.5	15	0.80 ± 0.39	D3-DPHM	32 ± 7	85 ± 7
	DPHM high	585	150	10.15 ± 4.61			
	Oxycodone high	70	22.2	0.75 ± 0.14	D3-Oxycodone	17 ± 5	65 ± 10
Group 3 ($n = 6$)	Oxycodone low	7	2.22	0.05 ± 0.01	D3-Oxycodone	16 ± 3	68 ± 14
	Oxycodone high	70	22.2	0.60 ± 0.01			
	DPHM high	585	150	8.05 ± 4.2	DPHM	23 ± 8	78 ± 7

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Table 2. Estimation of the Pharmacokinetic Parameters of DPHM in Rats (Group 1) After Bolus Infusion + Constant Rate Infusion

Parameter	Mean ± SD	Units	Number of Animals (n)
Rec_{brain}	32.5 ±4.0	%	($n = 6$)
Rec_{blood}	85.2 ± 3.8	%	($n = 5$)
$C_{tot,pl,ss}$	298 ± 72	ng/mL	($n = 8$)
$C_{u,pl,\ ss}$	50.2 ± 4.0	ng/mL	($n = 8$)
$C_{u,brain,ss}$	284 ± 37	ng/mL	($n = 8$)
$A_{tot,brain,ss}$	3486 ± 197	ng/g brain	($n = 4$)
$K_{p,brain}$	11.3 ± 3.7		($n = 4$)
$K_{p,u,brain}$	68.2 ± 4.7		($n = 3$)
$K_{p\ uu,brain}$	5.54 ± 0.67		($n = 7$)
CL	28.8 ± 7.0	mL/min	($n = 4$)
V_{ss}[a]	4.56 ± 1.06	L/kg	($n = 8$)
f_u	0.18 ± 0.05		($n = 8$)
$V_{u,brain}$	12.5 ± 1.8	mL/g brain	($n = 4$)
$t_{\frac{1}{2}}$ blood	47.1 ± 4.4	min	($n = 4$)
$t_{\frac{1}{2}}$ plasma	46.6 ± 4.6	min	($n = 4$)

Data are presented as mean ± SD.
[a]Calculated with mean residence time (MRT) obtained from the plasma pharmacokinetic study with a single bolus infusion.

in the range of 51.2–8360 ng/g brain with three QCs in the range of 201–6700 ng/g brain.

The microdialysis samples were diluted with 100 µL mobile phase and 53 µL was transferred to new vials to obtain duplicates. Fifty microliters of plasma was precipitated with 150 µL ACN. After vortex mixing and centrifugation at 10,000 rpm (7200 × *g*) for 5 min, 50 µL of the supernatant was mixed with 500 µL ammonium formate buffer and 55 µL of the diluted samples was then transferred to new vials for analysis. The brain tissue was analyzed by mixing half a brain with 4 mL saline solution per gram brain to make a homogenate. One hundred microliters of the homogenate was then precipitated with 1 mL of ACN. After centrifugation, the supernatant was diluted with 1 mL ammonium formate buffer and transferred to new vials.

Liquid Chromatography–Tandem Mass Spectrometry Method. The quantitative analysis was carried out on a liquid chromatography–tandem mass spectrometry (LC –MS/MS) system. The LC system consisted of an LC-10AD pump (Shimadzu, Kyoto, Japan) and a SIL-HTc autosampler (Shimadzu). Separations were carried out on a 50 × 4.6 mm^2 reversed-phase C18 column with a particle diameter of 3 µm (Thermo Hypersil-Keystone, Bellefonte, Pennsylvania). A guard column with the same material was placed before the analytical column. The detector was a triple quadruple mass spectrometer Quattro Ultima (Micromass, Manchester, UK). The spectra processing was carried out using MassLynx software, version 4.0 (Micromass).

The analytical method was developed by optimizing the parameters to obtain high resolution in a reasonable analysis time. The mobile phase contained 40% ACN in 5 mM ammonium formate buffer (pH 3.4). The mobile phase was degassed with helium. A constant flow rate of 0.8 mL/min was used and the flow was split, allowing 0.3 mL/min to enter the detector. The injection volume was 40 µL for the microdialysis samples and 30 µL for the plasma and brain tissue samples. The method used for injection was partial loop fill. The desolvation temperature was set at 400°C and the source temperature at 130°C. The collision gas was argon, with a pressure of 3×10^{-3} mbar and energy of 16.0 eV. The cone gas was nitrogen with a flow rate of 200 L/h. The desolvation gas flow rate was 1000 L/h. The capillary and the cone voltage were set to 0.90 kV and 20 V, respectively. The transition mode for DPHM was set to *m/z* 256 → 166.9 and for D3-DPHM *m/z* 259 → 166.9. The analysis was carried out in positive ion mode.

Oxycodone

Oxycodone concentrations in plasma and brain ISF were determined with the LC –MS/MS method developed by Boström et al.[19] Briefly, microdialysis sample was mixed with 10 µL of Milli-Q water containing 40 ng/mL of D6-oxycodone as an internal standard and then 8 µL of the sample was injected into the LC–MS–MS system. The standard curves consisted of 12 standards ranging from 0.2 to 300 ng/mL. Four QCs ranged from 1.6 to 106.9 ng/mL. Fifty microliters of plasma was mixed with 100 µL of ACN containing 50 ng/mL D6-oxycodone as an internal standard, on a vortex for 5 s. After centrifugation at 10,000 rpm (7200 × *g*) for 5 min, 20 µL of the supernatant was injected into the column. The standard curve for plasma samples was in the range from 1.1 to 1061 ng/mL and three QCs ranged from 7.2 to 716 ng/mL.

Data Analysis of In Vivo Samples

The recovery of DPHM in Groups 1 and 3 was estimated by retrodialysis with drug for each probe,[20] and in Group 2, it was estimated by using the calibrator D3-DPHM in the perfusate.[21] The calibrator D3-oxycodone was used to estimate the oxycodone recovery for each probe in both Groups 2 and 3. Recovery was calculated as

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

where C_{in} is the concentration of D3-calibrator or drug in the perfusate and C_{out} is the concentration of D3-calibrator or drug in the dialysate. For each probe, the average recovery for the whole experiment was used, as the recoveries of both drugs did not change with time.

The concentration of unbound drug in the blood and brain ISF were calculated as

$$C_{\text{u,tissue}} = \frac{C_{\text{dialysate}}}{\text{Recovery}} \quad (3)$$

The different partition coefficients for DPHM K_{p}, $K_{\text{p,u}}$, and $K_{\text{p,uu}}$ were calculated as

$$K_{\text{p}} = \frac{C_{\text{brain,ss}}}{C_{\text{blood,ss}}} \quad (4)$$

$$K_{\text{p,u}} = \frac{C_{\text{brain,ss}}}{C_{\text{u,blood,ss}}} \quad (5)$$

$$K_{\text{p,uu}} = \frac{C_{\text{u,brain,ss}}}{C_{\text{u,blood,ss}}} \quad (6)$$

$K_{\text{p,uu}}$ was determined for each rat at each concentration using the last three samples from the blood and brain probes for both DPHM and oxycodone.

The volume of distribution of unbound drug in the brain, $V_{\text{u,brain}}$, was calculated as

$$V_{\text{u,brain}} = \frac{A_{\text{brain}} - v_{\text{plasma brain}} \times C_{\text{plasma}}}{C_{\text{u,brainISF}}} \quad (7)$$

where A_{brain} is the total amount of DPHM per gram of brain at the steady state, obtained from rats decapitated at the end of infusion, and $C_{\text{u,brain}}$ is the unbound drug concentration at the steady state. To compensate for the amount of drug in the brain vascular space, the plasma concentration times the volume of plasma in the brain was subtracted from the total amount in brain. A literature value for $v_{\text{plasma,brain}}$ of 14 μL/g brain in the rat was used.[22]

Statistical Methods for *In Vivo* Studies

All values are represented as average ± standard deviation. One way analysis of variance was used to compare the $K_{\text{p,uu}}$ values between experimental periods of DPHM in Group 2 and of oxycodone in Group 3. Student's *t*-test was used to compare the $K_{\text{p,uu}}$ of DPHM in Group 3 and oxycodone in Group 2.

RESULTS

In Vitro Experiments

Uptake of DPHM by TR-BBB13 Cells

The uptake of DPHM in TR-BBB13 cells increased with time until 30 s and the cell-to-medium ratio reached 84.5–87.0 at 30–60 s (Fig. 2), suggesting that DPHM is concentrated into TR-BBB13 cells and/or bound to the intracellular constituents of the cells. The initial uptake rate of DPHM was concentration dependent (Fig. 3). The Eadie–Hofstee plot gave a single straight line, indicating a single saturable process. Kinetic analysis provides a K_{m} value of 49.7 ± 16 μM and a v_{max} of 5.6 ± 0.4 nmol/mg protein/15 s for DPHM in TR-BBB13 cells.

Driving Force and Metabolic Energy Dependence of Uptake of DPHM by TR-BBB13 Cells

The cell-to-medium ratio of DPHM was decreased in acidic transport medium (pH 6.0) and increased in alkaline medium (pH 8.4), compared with that at pH 7.4 (Fig. 4a). Intracellular acidification of TR-BBB13 cells by pretreatment with NH_4Cl resulted in significant stimulation of DPHM uptake by TR-BBB13 cells, whereas intracellular alkalization by acute treatment with NH_4Cl markedly reduced the DPHM uptake to 30% of control value (Fig. 4b). The uptake was

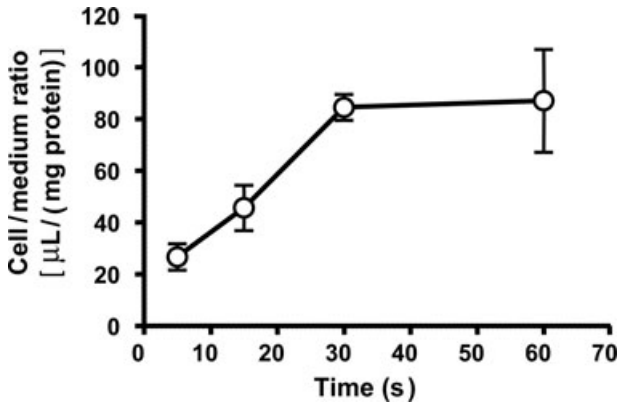

Figure 2. Time course of DPHM uptake into TR-BBB13 cells. Uptake of DPHM (30 μM) was measured at 37°C. Each point represents the mean ± SE of three to four determinations.

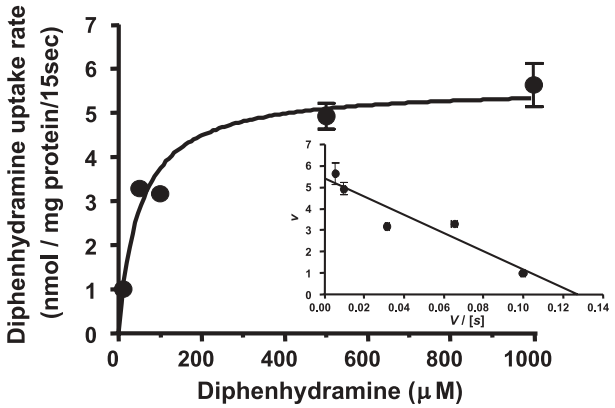

Figure 3. Concentration dependence of DPHM uptake into TR-BBB13 cells. The initial uptake rate for DPHM (10–1000 μM, for 15 s) was determined by subtracting the uptake at the concentration of 5 mM DPHM from the observed uptake rate to eliminate nonspecific uptake. Kinetic analysis provided a K_m value of 49.7 μM and a v_{max} of 5.59 nmol/mg protein/15 s. (Inset) Eadie–Hofstee plots of the initial uptake rate for DPHM. v, Uptake rate (nmol/mg protein/15 s), s, DPHM concentration (μM). Each point represents the mean ± SE from three determinations.

also decreased by 59% by treatment with FCCP, a protonophore. Furthermore, the DPHM uptake was significantly inhibited by pretreatment with rotenone and sodium azide in TR-BBB13 cells (Fig. 4c).

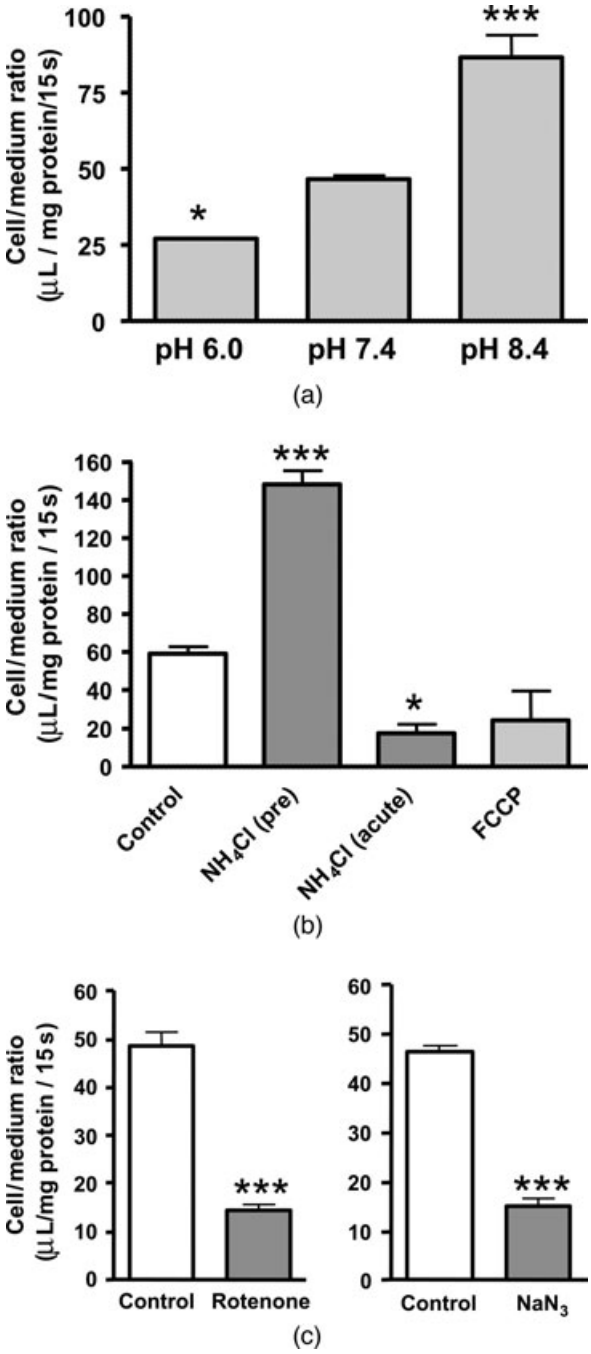

Figure 4.

Figure 4. (a) Effects of extracellular pH on uptake of DPHM into TR-BBB13 cells. Uptake of DPHM (30 μM) was measured at 37°C for 15 s. The cells were preincubated with an incubation medium (pH 7.4) for 20 min. Then, the preincubation medium was removed and the cells were incubated with 30 μM DPHM (pH 6.0, 7.4, or 8.4) for 15 s at 37°C. Each column represents the mean ± SE of three determinations. Asterisks show a significant difference, $^{*}P < 0.05$, $^{***}P < 0.001$ versus pH 7.4. (b) Effects of changes in intracellular pH and protonophore treatment on DPHM uptake into TR-BBB13 cells. The cells were preincubated with an incubation medium (pH 7.4) in the absence (control and acute) or presence (pre) of 30 mM ammonium chloride (NH_4Cl) for 20 min. Then, the preincubation medium was removed and the cells were incubated with 30 μM DPHM (pH 7.4) in the absence (control and pre) or presence (acute) of 30 mM NH_4Cl for 15 s at 37°C. Uptake of DPHM was also measured in the absence and presence of 10 μM *p*-trifluoromethoxyphenylhydrazone (FCCP). Each column represents the mean ± SE of three determinations. Asterisks show a significant difference, $^{*}P < 0.05$, $^{***}P < 0.001$ versus control. (c) Effect of ATP depletion on uptake of DPHM into TR-BBB13 cells. Uptake of DPHM (30 μM) was measured at 37°C for 15 s after treatment with 25 μM rotenone or 0.1% sodium azide for 20 min to reduce metabolic energy. Each column represents the mean ± SE of three determinations. Asterisks show a significant difference, $^{***}P < 0.001$ versus control.

Interaction Between DPHM and Oxycodone

To examine commonality in the transport between DPHM and oxycodone, mutual inhibitory kinetic studies were performed. In the Lineweaver–Burk plot analyses of mutual inhibitory effect on uptake of DPHM and oxycodone (Fig. 5), the plots of DPHM uptake in the presence and absence of oxycodone intersected at the ordinate axis. This result indicated that oxycodone competitively inhibited DPHM uptake with a K_i value of 106 μM (Fig. 5a). DPHM also

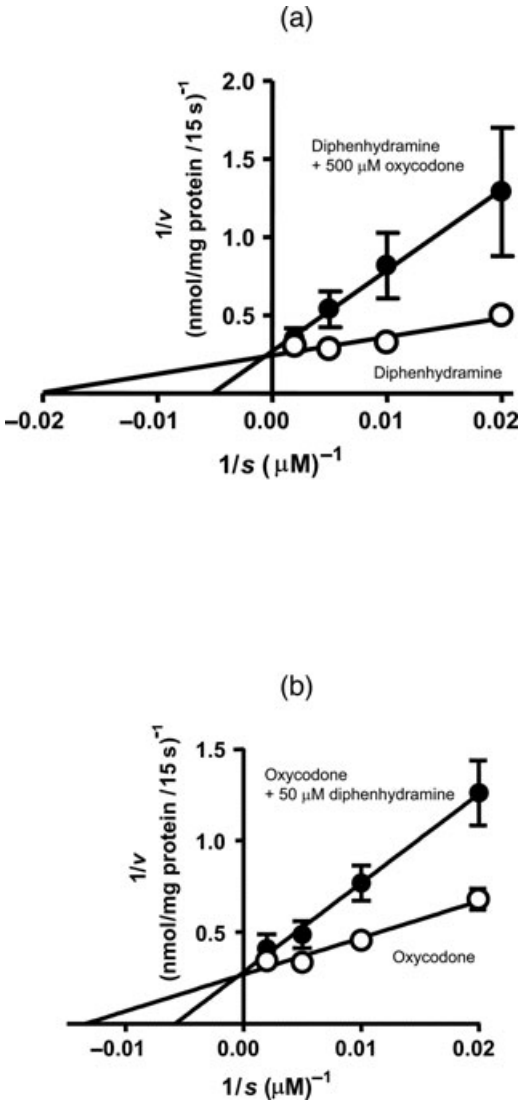

Figure 5. Lineweaver–Burk plot of mutual inhibitory effects on uptake of DPHM and oxycodone into TR-BBB13 cells. (a) Uptake of DPHM was measured at 37°C for 15 s in the presence (•) or absence (○) of 500 μM oxycodone. (b) Uptake of oxycodone was also measured at 37°C for 15 s in the presence (•) or absence (○) of 50 μM DPHM. Each point represents the mean ± SE of four determinations.

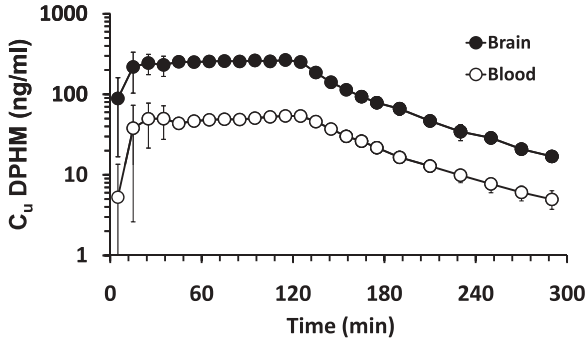

Figure 6. The concentration–time profile for unbound drug in brain ISF (•) and blood (○) after 5 min bolus infusion followed by a 120 min constant rate infusion DPHM (0–120 min, $n = 8$; 0–240 min, $n = 4$; 0–300 min, $n = 3$).

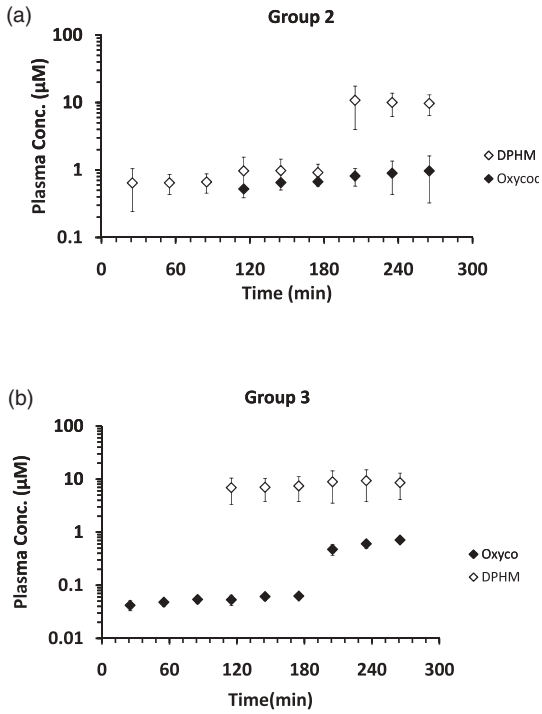

Figure 7. Steady-state plasma concentrations, μM (mean ± SD), for (a) Group 2 and (b) Group 3. Filled markers represent oxycodone and empty markers represent DPHM.

competitively inhibited oxycodone uptake with a K_i value of 34.7 μM (Fig. 5b).

In Vivo Experiments

DPHM Active Uptake at the BBB

The pharmacokinetic parameters determined during the microdialysis experiments are shown in Table 2. The data from the pilot bolus study were similar; DPHM followed two compartmental pharmacokinetics. The volume of distribution and clearance were 5.5 ± 0.6 L/kg and 39.2 ± 3.7 mL/min, respectively, resulting in a terminal half-life, $t_{\frac{1}{2}}$, of 55.8 ± 7 min. DPHM was rapidly distributed to the brain and reached steady state very fast (Fig. 6). The unbound drug concentrations were higher in the brain than in the blood, with a $K_{p,uu}$ of 5.5 (Table 2). The drug was also extensively distributed within the brain with a $V_{u,brain}$ of 12.5 mL/g brain, indicating that 12.5-fold more drug is bound than unbound in the brain tissue. After the end of infusion, the unbound brain and plasma concentrations decreased in parallel, giving the same $t_{\frac{1}{2}}$ in blood and the brain.

In Vivo Interaction Between DPHM and Oxycodone

Total drug concentration in plasma for both DPHM and oxycodone achieved steady state rapidly,

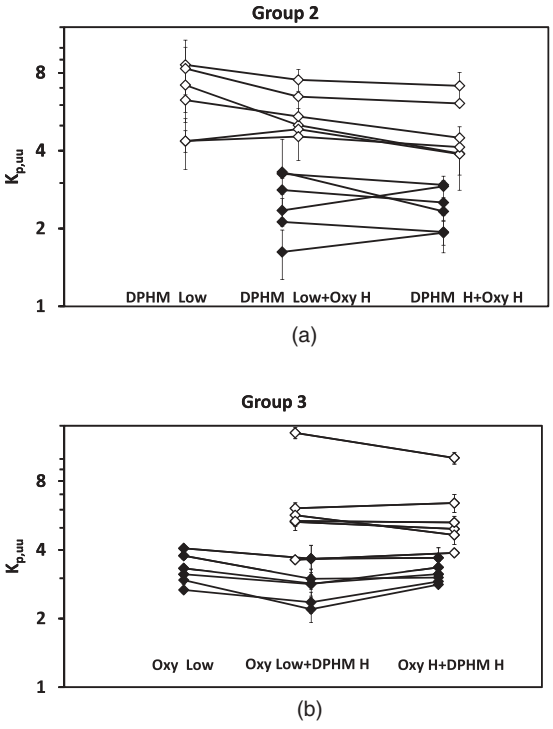

Figure 8. (a) Individual $K_{p,uu}$ of DPHM (empty markers) for three infusion periods, that is, the low infusion rate of DPHM, with coadministration of oxycodone and high infusion rate of DPHM with oxycodone. $K_{p,uu}$ of oxycodone (filled markers) are also showing no effect of DPHM on oxycodone transport. (b) Individual $K_{p,uu}$ of oxycodone (filled markers) for three infusion periods, that is, the low infusion rate oxycodone, with coadministration of DPHM and high infusion rate of oxycodone with DPHM. $K_{p,uu}$ of DPHM (empty markers) showing no effect of oxycodone on DPHM transport.

corroborating the choice of infusion scheme (Figs. 7a and 7b). The highest plasma concentrations achieved *in vivo* of DPHM and oxycodone were 21.5 and 2.3 μM, respectively. $K_{p,uu}$ for the last three samples of the control infusion period was 6.5 ±2 for DPHM and 3.3 ±0.5 for oxycodone, confirming active uptake of both drugs.

Oxycodone had no significant influence on DPHM transport across the BBB (Group 2, Fig. 8a). No statistically significant effect of coadministration of oxycodone was found on the $K_{p,uu}$ for either the low or high concentration of DPHM (p =0.2). Also, there was no significant change in the BBB transport of oxycodone when DPHM concentration was changed from low to high (p =0.5, Fig. 8a).

The effect of DPHM on oxycodone BBB transport (Group 3) showed similar results (Fig. 8b). There was however a trend to a lower $K_{p,uu}$ of oxycodone, decreasing from 3.3 ±0.5 to 2.8 ±0.5, when DPHM was added. Increasing oxycodone infusion rate while keeping DPHM at the same infusion rate reversed the trend. The difference between different infusion periods was not statistically significant (p =0.2). $K_{p,uu}$ for DPHM was not significantly affected either, by the increase of the oxycodone infusion rate (p =0.3).

DISCUSSION

Drug–drug interaction is a critical issue in clinical practice that can change the outcome of a therapy. In the present study, the active uptake of DPHM at the BBB was investigated both *in vitro* in TR-BBB13 cells and *in vivo* in rats. It was clearly shown *in vitro* that DPHM and oxycodone competitively inhibit each other's transport, proving that they are substrates for the same active transport system. The *in vivo* results, on the contrary, did not show any significant change in BBB transport of DPHM by concomitant administration of oxycodone or vice versa.

The *in vitro* results suggest that the active uptake transporter for DPHM is dependent on energy and that it is an oppositely directed proton gradient. The K_m and v_{max} values were in a fairly good agreement with those of oxycodone (K_m = 89 μM, v_{max} = 3.5 nmol/mg protein/15 s) and [^{3}H] pyrilamine (K_m = 28 μM, v_{max} = 1.05 nmol/mg protein/15 s).[10] In addition, the calculated uptake clearance (v_{max}/K_m) for DPHM was 450 μL/min/mg protein. The value corresponded well with that for oxycodone (157 μL/min/mg protein) and [^{3}H] pyrilamine (150 μL/min/mg protein), suggesting that a transporter with similar affinity and transport clearance is probably involved in uptake by TR-BBB13 cells. Thus, the transport characteristics of DPHM obtained here were very similar to the pyrilamine/oxycodone transporter, a putative OCT, in the brain endothelial cells.[10,23] A similar energy-dependent and proton-coupled antiporter was recently found *in vivo* in mouse brain[11] and Caco-2 cells[8] for clonidine and 3,4-methylenedioxy-methamphetamine, respectively. The mutual and competitive inhibition between DPHM and oxycodone is a further indication that a common transporter, likely the pyrilamine/oxycodone transporter, is involved in the transport of DPHM into TR-BBB13 cells. It has previously been shown that P-glycoprotein inhibition has no effect on oxycodone brain pharmacokinetics[24] and that DPHM is not a substrate for P-glycoprotein.[4]

The *in vivo* results of DPHM BBB transport with a $K_{p,uu}$ of 5.5 confirm the presence of an active transport mechanism for DPHM at the BBB in rats (Fig. 6). Not many drugs have been reported to have net active uptake into the brain. The K_p and $K_{p,u,brain}$ values of 11.7 and 68.2 from the present study, respectively, were similar to those from the study by Mahar Doan et al.[4] (K_p = 18.4 and $K_{p,u,brain}$ = 115). The K_p of a drug is dependent on properties such as protein binding in plasma, BBB transport, and affinity to the brain tissue. By compensating for protein binding, $K_{p,u,brain}$

is only dependent on BBB transport and affinity to the brain tissue. High K_p and $K_{p,u,brain}$ values are mainly indicators of high drug affinity to the brain tissue and cannot be directly related to the pharmacologically active unbound drug concentration in the brain. At the steady state, $K_{p,uu}$ gives a direct quantitative description of how the BBB handles the drug with regard to passive transport and active influx/efflux.[22]

Oxycodone is the only opiate analgesic yet reported to have an unbound drug exposure that is higher in the brain than in blood.[6] This was confirmed in the present study with a $K_{p,uu}$ of 3.1. It was evident from the *in vitro* studies that DPHM and oxycodone interacted for active transport at the BBB in a concentration-dependent manner. They show that oxycodone competitively inhibits the DPHM transport with a K_i value of 106 μM. Also, DPHM has a K_i value of 34.7 μM for inhibition of oxycodone transport. It is crucial to see the relevance of an interaction *in vivo* to weigh its clinical importance. The K_i value of oxycodone required to inhibit DPHM transport was 147 times higher than the average unbound plasma concentration obtained ($C_{u,oxycodone} = 0.72 \pm 0.26$ μM). For DPHM to competitively inhibit oxycodone transport, the K_i value estimated in cell culture was 27 times higher the average unbound plasma concentration achieved ($C_{u,DPHM} = 1.27 \pm 0.23$ μM) in rats. Thus, oxycodone had no significant effect on the BBB transport of DPHM and vice versa *in vivo*. However, there was a trend in all animals in the oxycodone group (Group 3), which suggested a slight inhibition of oxycodone transport at the BBB by DPHM. Further increases of the doses were not possible as it resulted in convulsions due to DPHM or respiratory depression due to oxycodone. Nakazawa et al.[23] had a similar conclusion based on results from studying the interaction between oxycodone and adjuvant analgesics, where the relatively higher concentrations required for interaction make them pharmacologically irrelevant.

In human volunteers, the total plasma concentration of DPHM obtained with an oral therapeutic dose of the drug reached a peak of approximately 200 ng/mL or 0.8 μM.[25] Assuming $f_u = 0.22$ in human plasma,[26] this corresponds to an unbound concentration of 0.2 μM, that is, about one-sixth of that achieved in our rats. The therapeutic plasma concentration of oxycodone with normal dosing may range between approximately 10 and 50 ng/mL, or between 0.03 and 0.16 μM.[27,28] Assuming $f_u = 0.55$ in human plasma,[29] this corresponds to unbound concentrations of 0.02–0.09 μM, that is, less than one-eighth of that achieved in the rats. In humans, the probability of interactions is thus even lower than in the rats.

CONCLUSION

Diphenhydramine had fivefold higher unbound concentration in the brain ISF than in blood, in rats. Results from *in vitro* experiments indicated an interaction between the transports of DPHM and oxycodone. *In vivo*, however, no such interaction was observed due to much lower unbound concentrations in blood compared with the K_i values found *in vitro*. It is thus very unlikely that a combination of DPHM and oxycodone would result in any clinically relevant pharmacokinetic interaction at the BBB.

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