Characterization of the Transport Properties of a Quinolone Antibiotic, Fleroxacin, in Rat Choroid Plexus

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Purpose. It is reported that the cerebrospinal fluid (CSF) to plasma unbound concentration ratio of fleroxacin at steady-state is approximately 0.5 in experimental animals. These results can be accounted for by assuming the presence of an active transport system for the efflux of this compound across the choroid plexus. In the present study, the transport system for fleroxacin was characterized in isolated rat choroid plexus.

Methods. Choroid plexus was isolated from the lateral ventricles of rats. The accumulation of [^{14}C] fleroxacin or [^{3}H] benzylpenicillin by the choroid plexus was examined by the centrifugal filtration method.

Results. The accumulation of [^{14}C] fleroxacin by the rat isolated choroid plexus was significantly inhibited by metabolic inhibitors (rotenone, 30 μM and carbonyl cyanide *p*-trifluorometh oxyphenylhydrazone (FCCP), 100 μM) and sulfhydryl reagent (*p*-chloromercuribenzenesulfonic acid (PCMBS), 100 μM). This accumulation was composed of a saturable component (V_{max} = 240 pmol·min^{-1}·μl tissue^{-1}, K_m = 664 μM) and non-saturable one (P = 0.424 min^{-1}·μl tissue^{-1}). Accumulation of fleroxacin was competitively inhibited by benzylpenicillin and probenecid with K_i values of 29 μM and 51 μM, respectively. These values are comparable with the K_m of benzylpenicillin transport and the K_i of probenecid for the benzylpenicillin transport at the choroid plexus, respectively. Furthermore, fleroxacin inhibited competitively the accumulation of [^{3}H] benzylpenicillin with a K_i of 384 μM, a value comparable with the K_m of [^{14}C] fleroxacin transport.

Conclusions. Fleroxacin and benzylpenicillin showed mutual competitive inhibition, suggesting that both are transported *via* a common transport system in the choroid plexus and are pumped out from CSF into the circulation.

KEY WORDS: quinolone antibiotics; cerebrospinal fluid; blood-CSF barrier; choroid plexus; anion transport system.

INTRODUCTION

The fluorinated quinolones are used frequently in the treatment of various bacterial infections (1). However, these drugs have been reported to induce headache and dizziness as side-effects, presumably resulting from their action on the central nervous system (CNS). Furthermore, concomitant administration of these quinolones with fenbufen, a nonsteroidal anti-inflammatory drug, causes serious convulsions. It has been suggested that the interaction of quinolones and γ-aminobutyric acid at its receptor may be responsible for the CNS excitation (2). It is important, therefore, to determine the factors which affect the distribution of quinolones in the CNS.

Fleroxacin (FLRX), one of the fluorinated quinolone antibiotics, is absorbed almost completely and the substantial plasma concentrations are maintained after oral administration (3, 4). It has also been shown that the FLRX concentration in most tissues and biological fluids, except in the CNS, exceeds the plasma level in several animal species (3, 4). The CNS to plasma concentration ratio of FLRX, however, is much lower than unity; in rats with normal meninges, the CSF to serum unbound concentration ratio of FLRX at steady-state was reported to be 0.42 (5). This low CSF penetration of FLRX might be accounted for by assuming the presence of an active efflux system by which this compound is transported from the CSF into blood.

The choroid plexus, an epithelial tissue that forms the blood-CSF barrier, has been postulated to play an important role in transporting organic anions from CSF to blood (6). Several organic anions, including the β-lactam antibiotics, can be substrates for this system. We have reported that the affinity of β-lactam antibiotics for the transport system is an important factor in determining their disposition in the CSF (7, 8). In a previous study from this laboratory, we (6) reported that the choroid plexus plays the predominant role in eliminating benzylpenicillin from CSF (6). We thus demonstrated that isolated choroid plexus can be a useful tool to predict the *in vivo* elimination clearance of organic anions from CSF.

The purpose of the present study is to characterize the transport system for a quinolone, fleroxacin, located on the choroid plexus.

MATERIALS AND METHODS

Chemicals

[2,3-^{14}C-piperazinyl] FLRX (254 MBq/mmol) was synthesized at the Central Research Laboratories of Kyorin Pharmaceutical Co., Ltd. (Tochigi, Japan). Throughout the present study, [^{14}C] FLRX solutions were prepared immediately before use from the dry powder. To avoid any possible degradation of the isotope in the aqueous solutions, [^{14}C] FLRX was stored in a freezer at −50°C as dry powder. The purity (> 99%) of [^{14}C] FLRX was confirmed by inverse isotope dilution (solvent; chloroform/methanol 1:1 vol./vol.).

[*phenyl*-4(n)-^{3}H] Benzylpenicillin (740 GBq/mmol) was purchased from Amersham International Ltd. (Buckinghamshire, UK). [1-^{14}C] *n*-Butanol (37 MBq/mmol) and tritiated water (925 MBq/g) were purchased from Du Pont-New England Nuclear (Boston, MA). Benzylpenicillin sodium salt, probenecid, rotenone, *p*-chloromercuribenzenesulfonic acid (PCMBS), *p*-chloromercuribenzoic acid (PCMB), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulfonic acid disodium salt (SITS) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI) and Nutritional Biochemicals (Cleveland, OH), respectively. All other chemicals were commercial products and of analytical grade.

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Male Wistar rats weighing 230–260 g were used throughout the experiments, which were carried out according to the guidelines provided by the Institutional Animal Care Committee (Faculty of Pharmaceutical Sciences, University of Tokyo).

Accumulation Study

The accumulation of [^{14}C] FLRX and [^{3}H] benzylpenicillin by the isolated rat choroid plexus was examined by the centrifugal filtration method described in detail previously (7). The rats were decapitated with a guillotine and the choroid plexus was isolated from the lateral ventricles. The isolated choroid plexus was incubated in 150 μl artificial CSF containing: NaCl, 122 mM; $NaHCO_3$ 25 mM; glucose, 10 mM; KCl, 3 mM; $CaCl_2$; 1.4 mM; $MgSO_4$, 1.2 mM; $K_2S\ O_4$, 0.4 mM; and 4-(2-hydroxyethyl)-*n*-piperazineethanesulfonic acid, 10 mM, pH 7.3, equilibrated with 95% O_2-5% CO_2 gas at 37°C. After preincubation for 1 min at 37°C, the choroid plexus was transferred to the incubation medium containing radio-labeled ligands with or without several inhibitors. The final concentrations of [^{14}C] FLRX and [^{3}H] benzylpenicillin were 25 μM and 0.040 μM, respectively. To examine the effect of an inwardly directed Na^+ gradient, we replaced the Na in the incubation medium with N-methyl-D-glucamine.

Total ^{3}H and ^{14}C radioactivity was determined in a liquid scintillation spectrophotometer (model LS6000SE, Beckman Instruments Inc., Berkeley, CA). The counting efficiency and crossover correction were determined by the external standard technique. The tissue-to-medium concentration ratio (T/M ratio) of [^{14}C] FLRX and [^{3}H] benzylpenicillin was calculated using [^{3}H] water and [^{14}C] butanol as cell water space markers, respectively, and was corrected for the adherent water space (7). The concentrations of [^{3}H] water and [^{14}C] butanol in incubation medium were 67 kBq/ml and 15 kBq/ml, respectively.

Kinetic Studies

For kinetic studies on the transport of FLRX, the Michaelis constant (K_m), the maximum velocity (V_{max}) and the P value representing the passive diffusion and the nonspecific adsorption to the cell surface of isolated choroid plexus were calculated by equation 1 using nonlinear least-squares analysis (9):

$$\frac{\text{T/M of [}^{14}\text{C]FLRX at 3 min}}{\text{3 min}} = \frac{V_{max,FLRX}}{K_{m,FLRX} + C} + P_{FLRX} \qquad (1)$$

where C is the concentration of FLRX in the incubation medium. We also examined the inhibitory effect of benzylpenicillin and probenecid on the accumulation of [^{14}C] FLRX. The inhibition constant (K_i) of benzylpenicillin and probenecid was calculated from Equation 2:

$$\frac{\text{T/M of [}^{14}\text{C]FLRX}_{\text{at 3 min}}}{\text{3 min}} = \frac{V_{max,FLRX}}{K_{m,FLRX}\cdot(1 + I/K_i) + C} + P_{FLRX} \qquad (2)$$

where I denotes the concentration of inhibitors. To determine the K_i values, $V_{max,FLRX}$, $K_{m,FLRX}$, and P_{FLRX} values were fixed to those determined by equation 1.

Furthermore, we examined the inhibitory effect of FLRX on the accumulation of [^{3}H] benzylpenicillin. The K_i of FLRX was calculated by Equation 3:

$$\frac{\text{T/M of [}^{3}\text{H]benzylpenicillin}_{\text{at 3 min}}}{\text{3 min}} = \frac{V_{max,PCG}}{K_{m,PCG}\cdot(1 + I/K_i) + C} + P_{PCG} \qquad (3)$$

where I and C represents the concentration of unlabeled FLRX and [^{3}H] benzylpenicillin (0.040 μM) in incubation medium, respectively. $K_{m,PCG}$, $V_{max,PCG}$ and P_{PCG} denote the Michaelis constant, the maximum velocity, and the permeability for the passive diffusion of the accumulated benzylpenicillin in the choroid plexus, respectively (7). Previously reported values ($V_{max,PCG}$ of 84 pmol · min^{-1} · μl $tissue^{-1}$, $K_{m,PCG}$ of 58 μM and P_{PCG} of 0.19 min^{-1} · μl $tissue^{-1}$, (7)) were substituted in equation 3 to determine the K_i values of FLRX.

Data Analysis

The results of the kinetic analysis are expressed as means ± calculated s.d. except when noted otherwise. Statistical analysis was performed by Dunnett's test.

RESULTS

Accumulation of [^{14}C] FLRX by the Rat Choroid Plexus

The time course for the accumulation of [^{14}C] FLRX by the isolated choroid plexus is shown in figure 1. The accumulation was almost linear for 3 min after initiation of the experiment. This accumulation was reduced completely in the presence of unlabeled FLRX (1 mM).

The concentration dependence of the accumulation of [^{14}C] FLRX is shown in figure 2. The T/M ratio of [^{14}C] FLRX decreased as the concentration of unlabeled FLRX in the incubation medium increased. The calculated values of $K_{m,FLRX}$, $V_{max,FLRX}$, and P_{FLRX} were 664 ± 56 μM, 240 ± 30 pmol · min^{-1} · μl $tissue^{-1}$ and 0.424 ± 0.026 min^{-1} · μl $tissue^{-1}$, respectively.

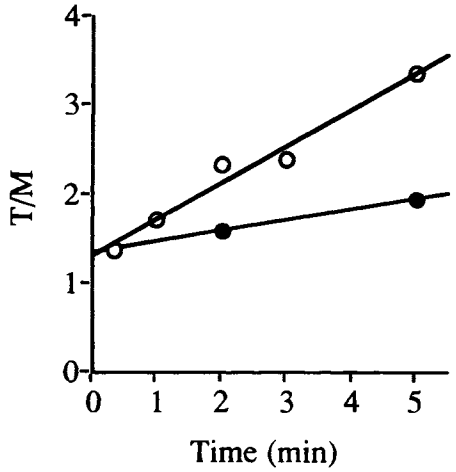

Fig. 1. Time-course for the accumulation of [^{14}C] fleroxacin (expressed as a T/M ratio) by rat choroid plexus. Choroid plexus was incubated with or without unlabeled fleroxacin (1 mM) at 37 °C in medium containing [^{14}C] fleroxacin (25 μM). Values shown represent the mean of four independent experiments. The s.e. is contained within the limits of the symbol. ○, without unlabeled fleroxacin; ●, with unlabeled fleroxacin (1 mM).

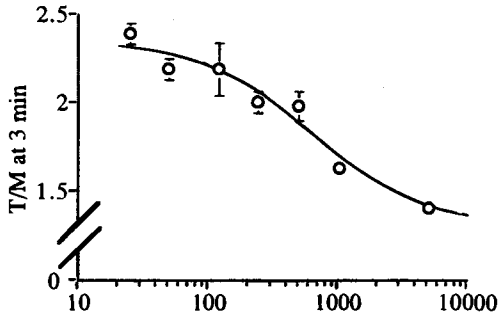

Fig. 2. Concentration dependence of the T/M ratio of [^{14}C] fleroxacin. Choroid plexus was incubated for 3 min at 37 °C in medium containing [^{14}C] fleroxacin (25 μM). Each point and vertical bar represents the mean ± s.e. of four independent experiments. Where vertical bars are not shown, the s.e. is contained within the limits of the symbol. The curve was calculated by nonlinear least-squares analysis (see text).

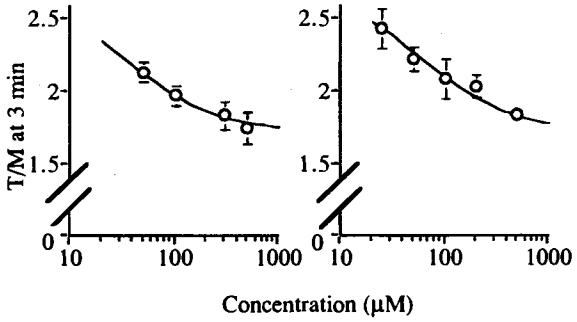

Fig. 4. Effect of benzylpenicillin and probenecid on the accumulation of [^{14}C] fleroxacin (expressed as a T/M ratio). Choroid plexus was incubated with benzylpenicillin (left panel), and probenecid (right panel) for 3 min at 37 °C in medium containing [^{14}C] fleroxacin (25 μM). Each point and vertical bar represents the mean ± s.e. of four independent experiments. Where vertical bars are not shown, the s.e. is contained within the limits of the symbol. Curve was calculated by nonlinear least-squares analysis (see text).

The accumulation of [^{14}C] FLRX was significantly inhibited by metabolic inhibitors (rotenone, 30 μM and FCCP, 100 μM) and sulfhydryl reagent (PCMBS, 100 μM), respectively (Fig. 3). Na^+ replacement by N-methyl-D-glucamine had no effect and ouabain produced no inhibition (Fig. 3).

Kinetics of the Accumulation of [^{14}C] FLRX

Both benzylpenicillin and probenecid inhibited the accumulation of [^{14}C] FLRX by the isolated choroid plexus in a dose-dependent manner (Fig. 4). The K_i values calculated from equation 2 were 29.3 ± 6.9 μM and 50.7 ± 10.2 μM for benzylpenicillin and probenecid, respectively (Table 1).

Unlabeled FLRX also inhibited the accumulation of [^{3}H] benzylpenicillin in a dose-dependent manner (Fig. 5). The K_i value of FLRX for the accumulation of benzylpenicillin was 384 ± 32 μM (Table 1).

Table 1. Relationship Between the K_i (or K_m) Values for the Accumulation of [^{3}H] Benzylpenicillin and [^{14}C] Fleroxacin by the Isolated Rat Choroid Plexus

	[^{3}H] Benzylpenicillin		[^{14}C] Fleroxacin	
	K_m (μM)	K_i (μM)	K_m (μM)	K_i (μM)
Fleroxacin		384 ± 32	664 ± 56	
Probenecid		74.0 ± 16.4[1]		50.7 ± 10.2
Benzylpenicillin	58[a]			29.3 ± 6.9

[a]The data are taken from our previous paper (6, 7).
Note: The values were calculated by nonlinear least-squares analysis. Data shows the mean ± calculated s.d. of four independent experiments.

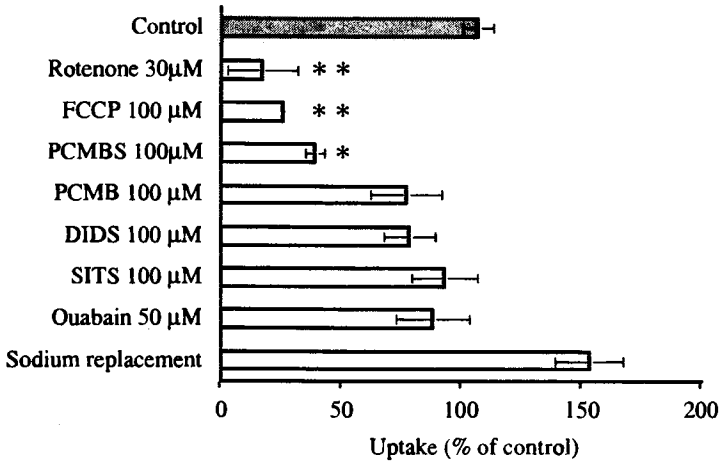

Fig. 3. Effect of metabolic inhibitors, sulfhydryl reagents, anion exchange inhibitors and ouabain on the saturable accumulation of [^{14}C] fleroxacin (expressed as % of control). Choroid plexus was incubated with or without inhibitors for 3 min at 37°C in medium containing [^{14}C] fleroxacin (25 μM). N-methyl-D-glucamine was used to replace sodium. Each bar represents the mean ± s.e. of four independent experiments. * $p < 0.05$, ** $p < 0.01$, by Dunett's test.

DISCUSSION

In a previous study, we characterized the transport properties of organic anions in the rat choroid plexus using β-lactam antibiotics as model compounds (6–8). We reported that these compounds are actively transported in the choroid plexus *via* a sodium-independent anion exchanger (6–8). The aim of the present study is to characterize the transport properties of quinolone antibiotics (zwitterionic compounds) in the choroid plexus. The trifluorinated quinolone, FLRX, was used as a model ligand. The inhibitory effect of the metabolic inhibitors (FCCP and rotenone) on the accumulation of [^{14}C] FLRX indicate that the accumulation of FLRX is due to active transport. The inhibitory effect of a sulfhydryl reagent, PCMBS, on the accumulation of FLRX also suggests a contribution of a proteonic carrier system. Furthermore, the potent anion transport inhibitor, probenecid, and typical ligand of anion transporter, benzylpenicillin, also reduced the uptake of FLRX in a dose-dependent manner (Fig. 4).

FLRX was accumulated by the isolated choroid plexus *via* a saturable transport process with a Michaelis constant of 664 μM (Table 1). Accumulation of FLRX was inhibited competitively by both benzylpenicillin and probenecid with an inhibition constant of 29 μM and 51 μM, respectively (Fig. 4, Table 1). These values were comparable with the Michaelis constant of benzylpenicillin transport in the choroid plexus (58 μM, (7)) and the inhibition constant of probenecid on benzylpenicillin transport (74 μM, (6)), respectively. Furthermore, FLRX inhibited competitively the accumulation of benzylpenicillin with an inhibition constant of 384 μM (Table 1), a value comparable with the Michaelis constant (664 μM) of the saturable accumulation of FLRX. A difference between the calculated K_m and K_i values for benzylpenicillin and FLRX (Table 1) might be accounted for by paucity of the experimental data, in that much more inhibitor concentrations around respective K_i values should be used. However, these results, together with the finding that an inwardly directed Na^+ gradient did not reduce the accumulation of FLRX, suggest that FLRX is transported *via* a sodium-independent anion exchange system in the rat choroid plexus which is responsible for the transport of benzylpenicillin (10).

Since the characteristics of the transport system in the choroid plexus are reported to resemble that in the kidney (11), the present results should be discussed in relation to renal transport. FLRX has a carboxyl group (pKa_1: 5.5) and piperazinyl moiety containing a nitrogen atom (pKa_2: 8.1) in its structure (12). The proton dissociation constants allowed us to calculate that approximately 80% of the FLRX molecules are present in zwitterionic form at physiological pH 7.4 (12). Renal studies of FLRX in laboratory animals and humans demonstrated that FLRX is actively secreted into the tubules and co-administration of probenecid decreases its renal clearance (13), suggesting that an anion transport mechanism may be responsible for the renal transport of FLRX. On the other hand, Okano *et al.*(14) examined the transport of another fluorinated quinolone, ofloxacin, using renal brush-border membrane vesicles and found that uptake of tetraethylammonium was inhibited by ofloxacin in a dose-dependent manner. This result suggests interaction between ofloxacin and the cation transport system on the renal brush-border membrane. We also examined the transport of tetraethylammonium in choroid plexus (15), and suggested a small contribution by the organic cation transport system to the ligand transport. Further investigation is required to determine the contribution of this cation transport system for FLRX transport.

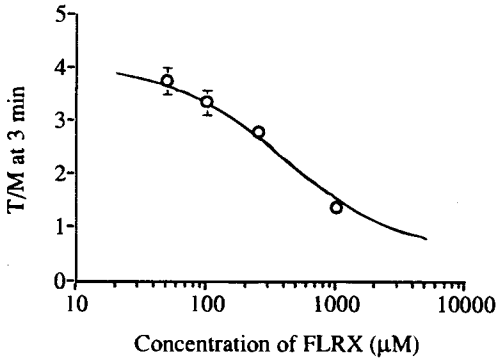

Fig. 5. Effect of fleroxacin on the accumulation of [^{3}H] benzylpenicillin (expressed as a T/M ratio). Choroid plexus was incubated with fleroxacin for 3 min at 37 °C in medium containing [^{3}H] benzylpenicillin (0.040 μM). Each point and vertical bar represents the mean ± s.e. of four independent experiments. Where vertical bars are not shown, the s.e. is contained within the limits of the symbol. Curve was calculated by nonlinear least-squares analysis (see text).

Previous studies from this laboratory demonstrated that the isolated choroid plexus can be a useful tool to predict the *in vivo* elimination clearance of organic anions from the CSF. Ogawa *et al.*(6) carried out a kinetic investigation of the CSF transport of benzylpenicillin in rats both *in vitro* and *in vivo*. *In vivo*, benzylpenicillin was eliminated from CSF *via* a saturable process after intracerebroventricular (icv) administration ($K_{m\ in\ vivo}$ = 43 μM, $V_{max\ in\ vivo}$ = 619 pmol/min/rat). The kinetic parameters determined *in vivo* were comparable with those determined *in vitro*; benzylpenicillin was accumulated by the isolated choroid plexus *via* a saturable process ($K_{m\ in\ vitro}$ = 58 μM, $V_{max\ in\ vitro}$ = 504 pmol/min/rat; (7)). Furthermore, some other organic anions, such as probenecid, ampicillin, cefodizime, cefotaxime and ceftriaxone, all reduced the transport of benzylpenicillin in a dose-dependent manner both *in vitro* and *in vivo* (6). A good correlation was observed between the log inhibition constant (K_i) values obtained for these ligands *in vivo* and *in vitro* (6). Based on these findings, we concluded 1) that the choroid plexus is the predominant site for the elimination of β-lactam antibiotics from the CSF and 2) that the isolated choroid plexus can be a useful tool for predicting the *in vivo* elimination clearance for β-lactam antibiotics from the CSF(6). If we consider the fact that FLRX is accumulated by the choroid plexus *via* a mechanism shared by benzylpenicillin, *in vitro* uptake of FLRX might be responsible for the *in vivo* elimination.

Previous studies from this and other laboratories have also demonstrated that the affinity of compounds for the transport system on the choroid plexus is an important factor in determining their disposition in the CSF, i.e., β-lactam antibiotics with very low affinity for this transport system, such as imipenem and ceftriaxone, are not eliminated rapidly from the CSF and attain high CSF-to-serum concentration ratios (7, 8, 16–18). The K_m of FLRX for the choroid plexus transport system is about 10 times larger than that of benzylpenicillin (Table 1) and, therefore, it is expected that the elimination of FLRX from the CSF may not be as great as that of benzylpenicillin. To examine this hypothesis, the *in vivo* elimination clearance of FLRX was extrapolated from the *in vitro* data based on the previously described method (6). The uptake clearance (V_{max}/K_m) of FLRX by the isolated choroid plexus over a linear range can be calculated as 0.361

$\mu l \cdot min^{-1} \cdot \mu l$ tissue $^{-1}$, based on the kinetic constants (K_m = 664 μM, V_{max} = 240 pmol $\cdot$ $min^{-1} \cdot \mu l$ $tissue^{-1}$ see "Results"). Considering the tritiated water space of the rat choroid plexus (6 μl, (6)) as well as the clearance value determined *in vitro*, the *in vivo* elimination clearance was calculated to be 2.17 $\mu l \cdot min^{-1}$. This value is comparable with the bulk flow rate of CSF (2.9 $\mu l \cdot min^{-1}$, (6)), indicating that the active transport of FLRX across the choroid plexus possesses a comparable functional significance with the convective flow of the CSF in removing FLRX from the CSF.

In ventriculocisternal perfusion studies, however, Spector and Lorenzo reported that there was no active transport of salicylate from CSF to blood (19), although salicylate was accumulated by the choroid plexus *in vitro* (20). We cannot exclude at present the possibility that FLRX is not transported from the CSF to circulating blood *via* the choroid plexus. The low CSF/plasma concentration ratio of FLRX after systemic administration (3–5) might also be accounted for by assuming the presence of an efflux transport system across the blood-brain barrier.

In a previous study from this laboratory, Matsushita *et al.* (21) demonstrated the presence of a putative efflux transport system for β-lactam antibiotics at the blood-brain barrier. Matsushita *et al.* (21) injected [^{14}C] cefodizime, cephalosporin antibiotic which is not metabolized, to rats intravenously (iv) and determined the concentration profiles in plasma and brain. We analyzed these data and found that the permeability surface area (PS) product for efflux (brain interstitial fluid (ISF) to plasma; PS_{eff} = 2.4 $\times$ 10^{-4} ml/sec/g brain) was higher than that for influx defined for the unbound plasma concentration (plasma to ISF; PS_{inf} = 5.2 $\times$ 10^{-5} ml/sec/g brain; (21)). Moreover, using quantitative brain microdialysis in rats, we have demonstrated 1) that the brain ISF concentration of FLRX is lower than the serum unbound concentration after iv administration and 2) that the brain ISF concentration of FLRX is approximately 2 times lower than in the CSF at steady-state (5). These results suggest the presence of an asymmetric transport system for organic anions across the blood-brain barrier. Collectively, it is plausible that such an asymmetric transport system might also be responsible for maintaining low CNS concentration of FLRX after iv administration (3–5).

In conclusion, the transport of FLRX in the rat choroid plexus was mediated by a sodium-independent active transport system which is shared by other organic anions such as benzylpenicillin. This transport system may have a functional significance in that it lowers the CSF level of FLRX.

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