Transepithelial Transport of Levofloxacin in the Isolated Perfused Rat Kidney

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Purpose. The transepithelial transport of levofloxacin was evaluated in the isolated perfused kidney to investigate its renal secretory mechanisms.

Methods. Levofloxacin was instantaneously administered into the renal artery together with inulin and Evans blue-labeled albumin, and the single-pass dilution curves of the renal venous and urinary outflow were determined in the absence or presence of various compounds. Kinetic parameters were computed based on non-compartment moment analysis.

Results. The ratio of fractional excretion to filtration fraction (FE/FF) for levofloxacin was 2.99 ± 0.18, indicating the involvement of tubular secretion. In the presence of cimetidine and quinolones, the FE/FF of levofloxacin was significantly decreased and the transepithelial mean transit time (T_{cell}) of levofloxacin was prolonged. The T_{cell} showed a negative correlation with renal secretion of levofloxacin, while the volume of distribution of levofloxacin showed no correlation.

Conclusions. Transport on the brush-border membrane plays a determining step in the renal secretion of levofloxacin, and cimetidine and quinolones interact with levofloxacin transport on the brush-border membrane.

KEY WORDS: quinolones; transport; renal secretion; isolated perfused kidney; moment analysis.

INTRODUCTION

Levofloxacin is frequently used to treat various bacterial infections. It is well absorbed from the intestine and distributed to tissues, and is mainly eliminated by renal excretion in humans (1). Renal excretion of levofloxacin in humans is the sum of glomerular filtration, tubular secretion, and tubular reabsorption processes (2). We evaluated the renal handling of levofloxacin in rats and found that its tubular secretion was inhibited by cimetidine (3). We also reported that levofloxacin was transported unidirectionally from basolateral to apical side in a kidney epithelial cell line, LLC-PK_1, and that levofloxacin had higher affinity for the transport system on the apical membrane, distinct from the H^+/organic cation antiport system (4). However, there is no information regarding how transport of levofloxacin on the luminal membrane contributes to the renal secretion of levofloxacin in the intact kidney.

The isolated perfused kidney is a useful system to investigate the transcellular transport of drugs in intact tissue, because nonrenal factors such as hepatic metabolism, binding to other tissues and hormonal regulation can be excluded (5,6). Therefore, although levofloxacin was metabolized to a considerable extent in rats (3), the isolated perfused rat kidney was considered to be a good model for the study of renal secretion mechanisms of levofloxacin in humans. The multiple indicator dilution technique is often used for investigation of the kinetic behavior of substrates and metabolites in eliminating organs (7,8). In addition, because the venous outflow and renal excretion curves following renal artery injection can be simultaneously determined in the kidney, the drug disposition on both the brush-border and basolateral membranes of the renal epithelial cells in the intact kidney could be estimated by non-compartment moment analysis (9,10). In this study, we investigated the transepithelial transport of levofloxacin using the isolated perfused rat kidney and non-compartment moment analysis to evaluate the interaction of levofloxacin with each membrane.

MATERIALS AND METHODS

Materials

[^{14}C]Levofloxacin (1.07 GBq/mmol), unlabeled levofloxacin, and grepafloxacin were kindly supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan) and Otsuka Pharmaceutical Co. (Tokyo, Japan), respectively. [^{3}H]Inulin (12.91 GBq/g) was purchased from Du Pont-New England Nuclear Research Products (Boston, MA). Cimetidine, tetraethylammonium (TEA) and *p*-aminohippurate (PAH) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Bovine serum albumin (BSA, Fr V, Reagent Grade) was purchased from Miles Inc. (Kankakee, IL). All other chemicals used were of the highest purity available.

Preparation of the Isolated Perfused Rat Kidney

The animal experiments were performed in accordance with the *Guidelines for Animal Experiments of Kyoto University.* Male Wistar albino rats weighing 270–350 g were used for the perfused kidney studies. The rat kidney was perfused as described previously (11), with some modifications. Briefly, the animals were anesthetized with sodium pentobarbital (50 mg/kg), and 100 mg of mannitol in isotonic saline was injected into the femoral vein. The right kidney was exposed, and the ureter was cannulated for urine collection using a PE-10 tube (Becton Dickinson, Parsippany, NJ). Heparin solution (1000 IU/kg) was injected into the femoral vein, and a venous cannula (polyethylene tubing, o.d. 2 mm, i.d. 0.8 mm, Hibiki, Tokyo, Japan) was placed in the vena cava just below the right renal vein. The renal artery was cannulated *via* the mesenteric artery using a 20 G needle, and the kidney was perfused without interrupting the renal blood flow. The rat kidney was equilibrated with constant perfusion at 16 ml/min. The perfusate consisted of Krebs-Henseleit bicarbonate buffer containing 5% BSA, 5 mM glucose, 3% mannitol, and 8 amino acids (0.5 mM methionine, 2 mM alanine, 5 mM glycine, 2 mM serine, 1 mM arginine, 2 mM proline, 1 mM isoleucine and 3 mM aspartic acid) (12), aerated with 95% O_2 + 5% CO_2 and was kept at 37 °C. An initial equilibration time of 10 min was allowed before the injection of radiolabeled levofloxacin. Glomerular filtration rate (GFR) was determined by inulin clearance

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together with the administration of levofloxacin. Fractional reabsorption of sodium and glucose, and GFR were used as indices to monitor the viability of perfused kidney. Perfusate flow rates were controlled in a similar range (14–19 ml/min) for all experiments. The perfusion experiments were performed under osmotic diuresis with 3% mannitol to collect a constant volume of urine (0.1 ml/min).

To investigate the interaction of levofloxacin with various compounds in renal secretion, the inhibitory experiments were performed under steady-state conditions. Each inhibitor (100–500 μM) was continuously added to the perfusate all the time to keep the drug concentration in the kidney constant.

Multiple Indicator Dilution Experiment

The renal handling of levofloxacin was investigated by measuring single-pass outflow of each indicator simultaneously injected into the renal artery. Evans blue-labeled albumin was used as a vascular space marker and [^{3}H]inulin was used as a marker of the extracellular space and glomerular filtration. The injection solution contained [^{14}C]levofloxacin (0.5 mM, 0.533 MBq/ml), [^{3}H]inulin (0.287 μg/ml, 3.7 MBq/ml) and Evans blue-labeled albumin (8.5 mg/ml Evans blue and 5% BSA) in Krebs-Henseleit bicarbonate buffer. After a bolus injection of 0.05 ml of this solution *via* the renal artery, venous effluent and urine samples were collected up to 15 min as described previously (13). At the end of the experiment, the kidney was removed, blotted and weighed, then homogenized with four volumes of saline. The fractional excretion (FE) of levofloxacin was computed by taking the ratio of the amount excreted in urine to the sum of recoveries in plasma, urine and tissue. The filtration fraction (FF) of levofloxacin was estimated from the fractional excretion of [^{3}H]inulin and the unbound fraction in the perfusate of levofloxacin (68.3 ± 2.5%).

Data Analysis

Moment analysis was performed according to the previously described procedure (9,10) with a slight modification. In this study, levofloxacin movement in the epithelial cell was assumed to be unidirectionally transported from the blood to the lumen side because levofloxacin reabsorption could be ignored due to relatively high urine flow rate. The mean transit time for venous outflow (MTT_v) and the urine outflow (MTT_u) were calculated by the trapezoidal integration from 0 to 30 sec and during a 15-min period, respectively, according to the following equations:

$$MTT_v = \int_0^{30} t \cdot C_{out}(t)\, dt \Big/ \int_0^{30} C_{out}(t)\, dt \quad (1)$$

$$MTT_u = \int_0^{15} t\, (dX_u/dt) dt \Big/ \int_0^{15} (dX_u/dt)\, dt \quad (2)$$

where $C_{out}(t)$ is the venous outflow concentration normalized by the dose, and the dX_u/dt is the urinary excretion rate normalized by the dose. Since the recovery of levofloxacin in the venous blood until 30 sec after the injection was more than 98% of that until 15 min, 30 sec was long enough to calculate the moment.

The availability for the tubular transport process of levofloxacin (F) was evaluated by the following equation:

$$F = F_{v,LVFX}/F_{GF} \quad (3)$$

where F_{GF} is the availability for the glomerular filtration process and was calculated from the extraction ratio of inulin (E_{GF}) and unbound fraction in the perfusate of levofloxacin (f_u) as $F_{GF} = 1 - f_u \cdot E_{GF}$. $F_{v,LVFX}$ is the organ availability of levofloxacin and was calculated as the ratio of the recovery from venous blood to the total recovery. The apparent tubular secretion intrinsic clearance (CL_{int}) was calculated from the availability:

$$CL_{int} = Q \cdot (1 - F)/f_u/F \quad (4)$$

where Q is the renal perfusate flow rate. The CL_{int} is the overall parameter including the rates of influx and sequestration. The mean time for secreted molecules to transfer across renal epithelial cells was described in terms of the mean residence time in renal epithelial cells, $\overline{T}_{cell}$, as follows:

$$\overline{T}_{cell} = MTT_{u,s} - MTT_{u,g} \quad (5)$$

The parameters $MTT_{u,s}$ and $MTT_{u,g}$ are MTT_u for the secreted fraction and filtrated fraction, respectively. This made it possible to determine the mean residence time in the renal tubular cells. The volume of distribution for noneliminated drugs such as albumin and inulin in tubules was model-independently calculated according to the following equation:

$$Vd = Q \cdot MTT_v \quad (6)$$

The steady-state equivalent volume of distribution for levofloxacin (Vd_{LVFX}) was calculated based on the well-stirred model:

$$Vd_{LVFX} = Q \cdot MTT_v + CL_{int} \cdot \overline{T}_{cell} \quad (7)$$

Analytical Methods

The radioactivity of [^{14}C]levofloxacin and [^{3}H]inulin were simultaneously measured using a liquid scintillation counter. Evans blue-labeled albumin in the perfusate was determined by spectrophotometry (610 nm). Sodium concentration was determined using a selective ion electrode (pH meter F-8 AT, Horiba, Kyoto, Japan). Glucose concentration was determined by the *o*-toluidine method (Glucose test-Wako, Wako Pure Chemical Industries, Osaka, Japan). Protein binding of levofloxacin in the perfusate was determined by the ultrafiltration method using a micropartition system (MPS-1, Amicon, Beverly, MA).

Statistical Analysis

Statistical comparisons were performed by the appropriate analysis of variance model; Dunnett's test for multiple comparisons was used if the variances of groups were similar. If not, a Dunnet-type test was applied after Kruskal-Wallis analysis. Differences were considered significant when $P < 0.05$.

RESULTS

Experimental Conditions of Kidney Perfusion

The perfusate flow rate, urine flow rate, GFR and fraction reabsorption of sodium and glucose in the present perfused kidney preparations are summarized in Table 1. Glomerular filtration rate was not significantly affected by any treatment. The fractional reabsorption of sodium and glucose in the control perfused kidney were more than 50% and 70%, respectively,

Table 1. Various Parameters in the Isolated Perfused Rat Kidney

	PFR (ml/min)[a]	UFR (ml/min)[a]	GFR (ml/min)[a]	FR_{Na+}[a]	FR_{Glu}[a]
Control (6)	16.7 ± 0.6	0.20 ± 0.02	0.29 ± 0.03	61.4 ± 2.8	83.1 ± 2.6
Levofloxacin, 100 μM (3)	15.9 ± 1.9	0.20 ± 0.01	0.28 ± 0.05	61.1 ± 5.7	85.2 ± 2.6
Levofloxacin, 500 μM (3)	15.7 ± 0.9	0.24 ± 0.05	0.30 ± 0.05	53.2 ± 3.6	82.8 ± 3.2
Grepafloxacin, 100 μM (3)	16.5 ± 0.5	0.21 ± 0.04	0.27 ± 0.03	56.8 ± 3.2	87.7 ± 2.6
Cimetidine, 500 μM (3)	17.1 ± 0.9	0.15 ± 0.02	0.26 ± 0.02	70.1 ± 1.6	84.3 ± 4.6
TEA, 300 μM (3)	18.3 ± 0.5	0.11 ± 0.02	0.26 ± 0.01	81.7 ± 4.7	96.0 ± 0.6
PAH, 300 μM (3)	16.4 ± 0.6	0.23 ± 0.05	0.42 ± 0.05	67.5 ± 5.3	90.4 ± 1.4

Note. Values are means ± S.E. Numbers in parentheses represent the number of experiments.
[a] PFR, perfusate flow rate; UFR, urine flow rate; GFR, glomerular filtration rate; FR_{Na+}, fractional reabsorption of Na^+; FR_{Glu}, fractional reabsorption of glucose.

and stable throughout the experiment. These indices of kidney function were not different from the control values in any experiment using inhibitors.

Renal Excretion of Levofloxacin in the Isolated Perfused Kidney

Figure 1 shows the typical venous and urinary outflow curves of Evans blue-labeled albumin, [^{3}H]inulin and [^{14}C]levofloxacin after a simultaneous bolus injection into the renal artery. Under control conditions, although there was little difference in the venous concentration induced by these substances (Fig. 1A), the urinary excretion rate of levofloxacin was markedly greater than that of inulin (Fig. 1C), indicating that secretion was predominant rather than reabsorption in the renal handling of levofloxacin. Unlabeled levofloxacin (500 μM) decreased the urinary excretion rate of levofloxacin to the level of inulin (Fig. 1D). The renal venous concentration of levofloxacin was hardly affected by unlabeled levofloxacin (Fig. 1B), because the urinary excretion of levofloxacin was less than 5% of the administered dose (data not shown).

Effects of Various Compounds on Levofloxacin Secretion

Previously, we reported that the transcellular transport of levofloxacin was inhibited by several quinolones, accompanied by the increased cellular accumulation in LLC-PK_1 cells (4). To investigate whether there is a specific quinolone transport system exists in the rat kidney, we examined the effects of quinolones on the renal secretion of levofloxacin. The FE/FF

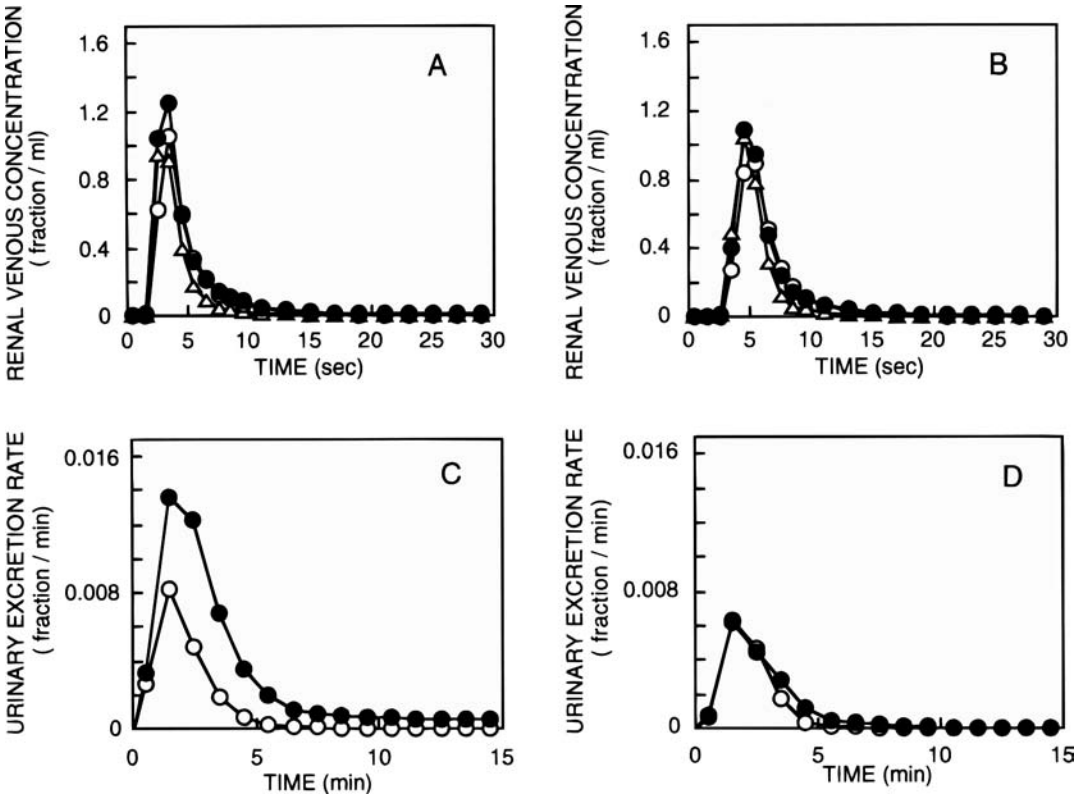

Fig. 1. Renal venous outflow and urinary excretion rate *vs.* time curves for levofloxacin, inulin and Evans blue-labeled albumin in the isolated perfused rat kidney. A and B show renal venous outflow curves, and C and D show urinary excretion rate *vs.* time curves in the absence (A and C) or presence (B and D) of unlabeled levofloxacin (500 μM) after a simultaneous bolus injection of [^{14}C]levofloxacin (●), [^{3}H]inulin (○) and Evans blue-labeled albumin (△).

of [^{14}C]levofloxacin was decreased in the presence of unlabeled levofloxacin in a concentration-dependent manner (Fig. 2). Likewise, the FE/FF of [^{14}C]levofloxacin was also significantly reduced in the presence of grepafloxacin (100 μM) (Fig. 2). Since levofloxacin is a zwitterion at physiological pH, we also examined the effects of cimetidine (500 μM), TEA (300 μM) and PAH (300 μM) on the renal secretion of levofloxacin. The FE/FF of levofloxacin was significantly decreased by cimetidine ($P < 0.05$), while TEA and PAH did not influence the FE/FF ratio of levofloxacin (Fig. 2).

Moment Analysis of Levofloxacin in the Isolated Perfused Rat Kidney

To clarify which membrane interacts with levofloxacin, we evaluated [^{14}C]levofloxacin excretion in the isolated perfused kidney by non-compartment moment analysis. There were no differences among MTT_v of albumin, inulin and levofloxacin under control conditions (data not shown). On the other hand, MTT_u of levofloxacin was greater than that of inulin because of the renal tubular secretion of levofloxacin. Table 2 shows $\overline{T}_{cell}$, CL_{int} and Vd_{LVFX} in the absence or presence of various compounds. The $\overline{T}_{cell}$ of [^{14}C]levofloxacin was prolonged to 2.5 times the control value by cimetidine, and was slightly increased in the presence of 500 μM unlabeled levofloxacin and 100 μM unlabeled grepafloxacin. The CL_{int} of [^{14}C]levofloxacin was significantly decreased in the presence of cimetidine, TEA, unlabeled levofloxacin (500 μM) and grepafloxacin (100 μM). Vd_{LVFX} was not affected by any treatment.

Relationship Between Tubular Secretion Fraction and $\overline{T}_{cell}$ or the Volume of Distribution

Based on the results of moment analysis, we evaluated the relationship between the tubular secretion fraction of levofloxacin and $\overline{T}_{cell}$ or Vd_{LVFX}. Tubular secretion fraction of levofloxacin was calculated by dividing the secreted amount by the total excreted amount. The $\overline{T}_{cell}$ for levofloxacin was inversely correlated with the secretion fraction ($r = -0.940$, Fig. 3A), and the Vd_{LVFX} was not affected by the secretion fraction (Fig. 3B).

Table 2. Non-Compartment Moment Parameters in the Isolated Perfused Rat Kidney

	$\overline{T}_{cell}$ (min)[a]	CL_{int} (ml/min)[a]	Vd_{LVFX} (ml)[a]
Control (6)	2.01 ± 0.14	0.58 ± 0.03	2.95 ± 0.08
Levofloxacin, 100 μM (3)	2.19 ± 0.28	0.49 ± 0.06	3.03 ± 0.08
Levofloxacin, 500 μM (3)	3.39 ± 0.10	0.20 ± 0.04*	2.48 ± 0.09
Grepafloxacin, 100 μM (3)	3.90 ± 0.34	0.26 ± 0.01*	2.81 ± 0.13
Cimetidine, 500 μM (3)	5.06 ± 0.99	0.13 ± 0.02*	2.36 ± 0.24
TEA, 300 μM (3)	2.78 ± 0.49	0.29 ± 0.06*	2.57 ± 0.20
PAH, 300 μM (3)	2.16 ± 0.18	0.58 ± 0.07	3.03 ± 0.08

Note. Values are means ± S.E. Numbers in parentheses represent the number of experiments.
[a] $\overline{T}_{cell}$, the mean transepithelial transit time of [^{14}C]levofloxacin; CL_{int}, the intrinsic clearance; Vd_{LVFX}, the volume of distribution.
* $P < 0.05$, significantly different from control.

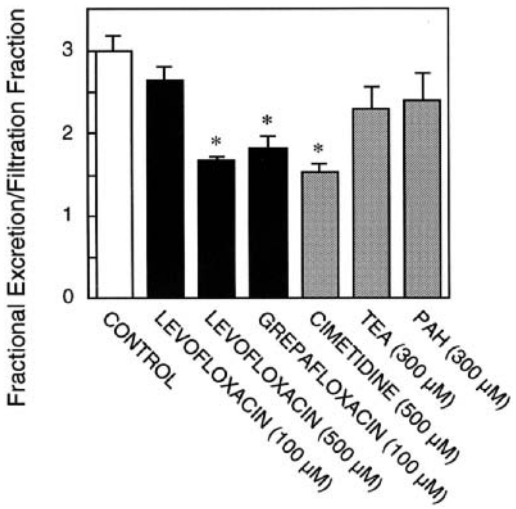

Fig. 2. Effects of various compounds on renal tubular secretion of levofloxacin in the isolated perfused rat kidney. The tubular secretion of levofloxacin was investigated in the absence or presence of inhibitors. The ordinate represents the ratio of fractional excretion to filtration fraction of [^{14}C]levofloxacin. The latter was estimated from the fractional excretion of [^{3}H]inulin and the plasma unbound fraction of levofloxacin. The values are means ± S.E. for at least three independent experiments. *$P < 0.05$, significantly different from control.

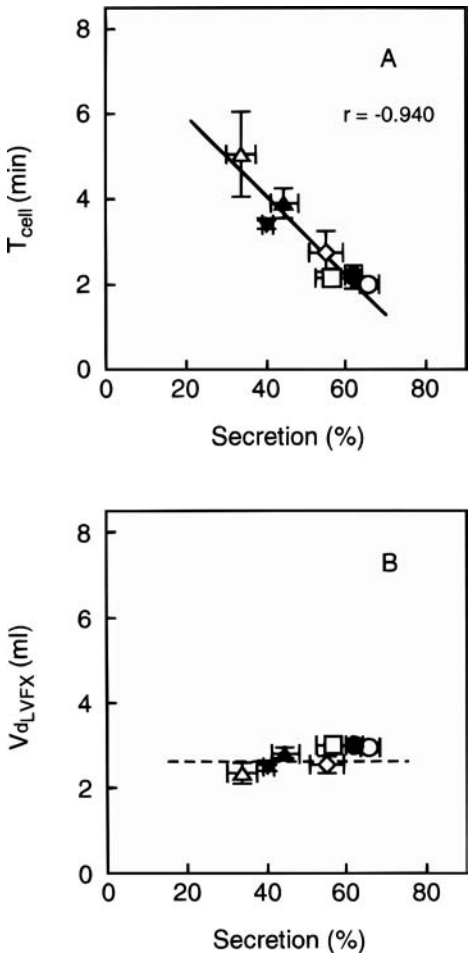

Fig. 3. The relationships between the tubular secretion fraction and $\overline{T}_{cell}$ (A), and Vd_{LVFX} (B). Each point represents the mean ± S.E. for at least three independent experiments. Key: control (○); 100 μM levofloxacin (●); 500 μM levofloxacin (▼); 100 μM grepafloxacin (▲); 500 μM cimetidine (△); 300 μM TEA (◇); 300 μM PAH (□).

DISCUSSION

Levofloxacin was actively secreted into urine in the isolated perfused rat kidney, because the FE/FF for levofloxacin was about 3.0 (Fig. 2). The presence of active secretion of levofloxacin was consistent with our results regarding *in vivo* clearance (3). Tubular secretion is a transcellular transport process including transmembrane transport across the basolateral membrane, movement in the cytosol, and transport across the brush-border membrane. Conventional analytical methods such as the clearance technique do not allow the membrane transport on both sides of the epithelial cells in a whole organ system to be evaluated separately. To solve this problem, the method using the isolated perfused kidney and moment analysis was developed and was applied to the renal handling of ionic substances (9,10). In this study, we evaluated the interaction of levofloxacin with both the basolateral and brush-border membranes using this method.

Basolateral membrane transport is essential for cellular distribution, and changes in the transport on the basolateral membrane should be reflected in the volume of distribution. As the Vd_{LVFX} was not affected by any treatment (Table 2) and there was no relationship between tubular secretion and the Vd_{LVFX} (Fig. 3), the basolateral transport of levofloxacin might not be a determining step in the renal secretion process of this drug. We previously reported that levofloxacin and grepafloxacin were transported by a specific active transport system in the rat kidney (14). However, as levofloxacin had relatively low affinity for this basolateral transport system (14), transport on the basolateral membrane might not be a determining step in the therapeutic concentration range. The $\overline{T}_{cell}$ is the mean transit time from blood to lumen in epithelial cells. If the efflux from the cell to lumen is decreased, the transit time should be prolonged. The $\overline{T}_{cell}$ of levofloxacin was prolonged in the presence of quinolones and cimetidine (Table 2), and there was a negative correlation between the tubular secretion and $\overline{T}_{cell}$ ($r = -0.940$, Fig. 3). These results indicated that the decrease in the secretion of levofloxacin was caused by selective inhibition of the transport from cells to the lumen across the brush-border membrane.

We previously suggested that levofloxacin was unidirectionally transported by a specific transport system on the apical membrane in LLC-PK_1 cells, distinct from the H^+/organic cation transport system (4). If the renal secretion of levofloxacin is mediated by the specific transport system for quinolones, levofloxacin transport should be affected by quinolones in the isolated perfused kidney. The FE/FF of levofloxacin was significantly inhibited by unlabeled levofloxacin (500 μM) and grepafloxacin (100 μM). Furthermore, moment analysis demonstrated that unlabeled quinolones interacted with levofloxacin on the brush-border membrane rather than the basolateral membrane (Table 2). These results suggested that specific transport systems for quinolones may exist on the brush-border membrane and play an important role in the renal secretion of levofloxacin.

Levofloxacin is a zwitterion at physiological pH, and the organic cation and/or anion transport systems may contribute to the renal transport of levofloxacin. The CL_{int} of levofloxacin was inhibited in the presence of cimetidine and TEA, organic cations, but was not inhibited by PAH, an organic anion. These results were consistent with those obtained by the *in vivo* clearance method in rats (3). Moreover, it has been reported that cimetidine reduced the renal clearance of levofloxacin and enoxacin in healthy subjects (15,16). These findings suggested that the organic cation transport system might contribute to the renal secretion of levofloxacin.

P-glycoprotein, which functions as an ATP-dependent drug-efflux pump, has been found on the brush-border membranes of proximal tubules of the kidney (17). We previously reported that levofloxacin was transported by human P-glycoprotein expressed in a kidney epithelial cell line (18). However, the renal secretion of levofloxacin was not influenced by cyclosporin A, a P-glycoprotein modulator, in the isolated perfused kidney (data not shown). These results suggested that transport mediated by P-glycoprotein might not be the main secretion mechanism of levofloxacin in the rat kidney.

In conclusion, levofloxacin was secreted into the urine in the isolated perfused rat kidney, and transport on the brush-border membrane was a determining step in the renal secretion process. Our findings suggested that the interaction on the brush-border membrane determines the extent of renal secretion of levofloxacin *in vivo*.

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