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Differences in the Hepatobiliary Transport of Two Quinolone Antibiotics, Grepafloxacin and Lomefloxacin, in the Rat

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ABSTRACT: The biliary excretion of grepafloxacin (GPFX) was compared with that of lomefloxacin (LFLX) in rats. The biliary clearances (Cl_{bile}^{plasma}) of GPFX was 2.9 times greater than LFLX based on the plasma concentration reached during constant intravenous (iv) infusion. The liver–plasma unbound concentration ratio, K_{pu}, of GPFX (1.7) was also higher than that of LFLX (0.7). The hepatic uptake clearance, assessed from an integration plot analysis, of GPFX was comparable with the hepatic blood flow rate, and 1.5 times that of LFLX, indicating that membrane transport in the uptake process is more efficient for GPFX. This was also supported by the difference between the uptake clearance of GPFX and LFLX in isolated rat hepatocytes. The bile–liver unbound concentration ratio of GPFX and LFLX was approximately 6 and 3, respectively, and the biliary clearance based on the unbound liver concentration of GPFX was 1.8 times that of LFLX. These results suggest that the concentrative transport of GPFX also across the canalicular membrane was more efficient than that of LFLX. Thus, the membrane transport activity via both sinusoidal and canalicular membranes determines the net excretion of each compound.

Key words: quinolone antibiotics; grepafloxacin; lomefloxacin; hepatobiliary transport

Introduction

Grepafloxacin (GPFX) is one of the new quinolone antibiotics (NQs). The ratio of the amount excreted into the bile to that into the urine is much higher for GPFX than that for other NQs [1]. The authors carried out an earlier quantitative analysis of their hepatobiliary transport and found that GPFX was actively taken up by a carrier-mediated transport system in a study using isolated rat hepatocytes [2]. The biliary excretion of GPFX was reduced in mutant Eisai-hyperbilirubinemic rats (EHBR) [3], which have an inherited deficiency in their biliary excretory transporter (canalicular multispecific organic anion transporter, cMOAT) [4–12], compared with normal rats. An ATP-dependent uptake of GPFX by bile canalicular membrane vesicles (CMV) from normal rats was observed, while almost none was observed by CMV from EHBR [13]. These results demonstrated that at least part of the GPFX transport across the bile canalicular membrane may be mediated by a primary active transport mechanism, cMOAT [13]. In those *in vitro* studies, the authors found that GPFX had a higher affinity for both transporters that mediate the hepatic uptake and biliary excretion compared with other NQs. In this study, in order to understand how differences in transport activity between GPFX and lomefloxacin (LFLX) in hepatic uptake and biliary excretion affect the net biliary excretion of these compounds, each process as well as the net biliary excretion of GPFX and LFLX were compared.

Materials and Methods

Chemicals

Unlabeled GPFX, LFLX, OPC-17203 (internal standard for HPLC analysis of GPFX) and ciprofloxacin (CPFX, internal standard for LFLX analysis) were synthesized by Otsuka Pharmaceutical Company (Tokyo, Japan). Collagenase was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were of reagent grade.

Hepatocyte Preparation

Hepatocytes were isolated from male Sprague–Dawley (SD) rats (Nihon Ikagaku, Tokyo) by the procedure of Baur *et al*. [14]. After isolation, the hepatocytes were suspended (2 mg protein/mL) in ice-cold albumin-free Krebs–Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.3). Cell

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viability was routinely checked by the trypan blue [0.4% (w/v)] exclusion test. The authors used over 90% as a viability criterion for the hepatocyte studies. Protein concentrations were determined by the method described by Bradford [15], using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

Hepatocyte Uptake Study of GPFX and LFLX

Uptake of GPFX or LFLX (50 and 1000 μM) was initiated by adding the ligand solution (0.5 mL) to the preincubated cell suspension (2 mg protein/mL, 0.5 mL) at 37°C for 5 min. At a designated time, the reaction was terminated by separating cells from medium using a centrifugal filtration technique [2,16]. The amounts of GPFX and LFLX in medium and cells were determined as described using HPLC [2]. The detection limit of the assay for GPFX in plasma, the medium and hepatocyte details were, respectively, 60 nM, 150 nM and 7.5 pmol/mg protein. The sensitivity for LFLX was similar to that for GPFX. The amount of drug taken up by hepatocytes at 50 μM and 1000 μM was over 5 nmol/mg protein. Therefore, the GPFX and LFLX concentrations in plasma and hepatocytes in this study could be determined with sufficient accuracy by this HPLC assay. The time-dependence of this uptake was plotted as an uptake value (μL/mg protein) obtained by dividing the amount taken up by the concentration in the medium.

Hepatic Uptake Study of LFLX In Vivo

Under ether anesthesia, LFLX was administered to male SD rats, weighing approximately 250–300 g, via the femoral vein at a dose of 5 mg/kg/2 mL saline (14.5 μmol/kg). Blood samples were then collected from the femoral artery at designated times over 2 or 3 min with a heparinized syringe and a portion of liver was collected by biopsy at 30 s or 1 min. The liver samples were immediately cooled in liquid nitrogen after excision to prevent further metabolism. The rats were killed by severing the main artery and vein at 2 or 3 min and the whole liver excised immediately. A portion of the tissue was weighed and stored at −30°C until required for assay. Liver samples were added to nine volumes 75% methanol (w/w) and homogenized. An internal standard (CPFX, 500 ng) was added to the homogenate (50 μL) and, following dilution with methanol (200 μL), samples were centrifuged in a tabletop microfuge ($10000 \times g$, Beckman Instruments Inc., Fullerton, CA). The resulting supernatants (10 μL) were subjected to HPLC. Plasma samples (25 μL) were obtained by centrifugation of blood and the internal standard (100 ng) added together with methanol (200 μL) to precipitate proteins. After centrifugation, the supernatants (10 μL) were subjected to HPLC. The HPLC conditions and calculation method were as described for the cell uptake study. The data for GPFX were taken from the results of Sasabe *et al*. [2].

When the hepatic uptake was measured over a short period, during which efflux, excretion and metabolism were negligible, the uptake rate of the drug can be described by the following equation:

$$\frac{X_t}{C_P} = \mathrm{Cl}_{\mathrm{uptake}}^{\mathrm{plasma}} \times \frac{\mathrm{AUC}_{(0-t)}}{C_P} + V_E \quad (1)$$

where $\mathrm{Cl}_{\mathrm{uptake}}^{\mathrm{plasma}}$ is the hepatic uptake clearance based on the plasma concentration of the drug, X_t is the amount of unchanged drug in the liver at time t, and C_P is the plasma concentration of unchanged drug. $\mathrm{AUC}_{(0-t)}$ represents the area under the plasma concentration–time curve from 0 to t and V_E represents the distribution volume in the liver at time 0. V_E was obtained from the y intercept of the integration plot and includes the distribution volume in plasma resident within the liver as well as the distribution volume in the liver of the drug rapidly equilibrating with that in plasma. Therefore, the $\mathrm{Cl}_{\mathrm{uptake}}^{\mathrm{plasma}}$ value can be obtained from the initial slope of a plot of X_t/C_P versus $\mathrm{AUC}_{(0-t)}/C_P$, designated as the integration plot [2,17,18]. The $\mathrm{Cl}_{\mathrm{uptake}}^{\mathrm{blood}}$ for the concentration in whole blood is calculated by dividing the $\mathrm{Cl}_{\mathrm{uptake}}^{\mathrm{plasma}}$ by the R_B of the drug, where R_B is the blood-to-plasma concentration ratio (GPFX, 1.37 and LFLX, 0.959) [19,20].

In Vivo Study at Steady-State

Male SD rats and EHBR (SLC Japan, Shizuoka) weighing approximately 250–300 g were used throughout the experiments. Under light ether anesthesia, the femoral artery and vein were cannulated with a polyethylene catheter (PE-50) for blood sampling and for the injection of GPFX, respectively. The bile duct was cannulated with a polyethylene catheter (PE-10) for bile collection. The rats received constant infusions of GPFX or LFLX at a dose of 15 μg/min/kg (41.9 and 42.7 nmol/min/kg, respectively) following bolus iv administration of 5 mg/kg (13.9 and 14.2 μmol/kg, respectively). Since rats were anesthetized throughout the experiment, they were kept warm by electric bulb. Bile was collected in preweighed test tubes at 20 min intervals throughout the experiment. Bile flow rates did not change throughout the experiment during the infusion of GPFX. Blood samples (approximately 200 μL) were collected at 70, 90, 120 and 150 min after starting the infusion. Plasma was prepared by centrifuging the blood samples ($10000 \times g$). The rats were killed after 160 min and the entire liver and kidneys excised immediately. The tissues were weighed and stored at −30°C until required for assay. Portions of liver and kidney were added to nine volumes 50% methanol (v/v) and homogenized. To the ho-

mogenate (50 μL) an internal standard (OPC-17203, 100 ng for GPFX or CPFX, 500 ng for LFLX) was added followed by centrifugation in the tabletop microfuge after dilution with 50% methanol (200 μL). The resulting supernatant fractions (20 μL) were subjected to HPLC to determine the concentration of these drugs. The internal standard (OPC-17203, 100 ng or CPFX, 500 ng) was added to plasma (25 μL) together with 50% methanol (200 μL) to precipitate proteins. Bile and urine (10 μL) were diluted with 25% acetonitrile (400 μL). After centrifugation, the supernatants (20 μL) were subjected to HPLC.

The steady-state plasma concentration C^{ss}_{plasma} was assessed as the mean plasma concentration after 70, 90, 120 and 150 min, while the steady-state liver (C^{ss}_{liver}) and kidney (C^{ss}_{kidney}) concentrations were the tissue concentrations after 160 min. Total body clearance (Cl_{total}) was obtained by dividing the infusion rate by C^{ss}_{plasma}. The biliary clearances (Cl^{plasma}_{bile}) of GPFX and LFLX gave the mean clearance value calculated by dividing the biliary excretion rates by C^{ss}_{plasma}. Cl^{liver}_{bile} was the biliary clearance based on the liver concentration, which was obtained by dividing the biliary excretion rates by C^{ss}_{liver}. The K_p value represented the ratio of C^{ss}_{liver} to C^{ss}_{plasma}.

Intracellular Binding of GPFX and LFLX in Liver

The intracellular binding of GPFX and LFLX in liver was obtained by extrapolating to binding for 100% (w/v) homogenate from that for 8.4, 17 and 33.3% liver homogenates, which were determined by ultrafiltration. Rat liver homogenate of 33.3% (w/v) was prepared by a teflon homogenizer in isotonic phosphate buffer (PBS, pH 7.4). This homogenate was then serially diluted by PBS to make 17 and 8.4% homogenates. Initially, the 33, 17 and 8.4% blank homogenate was placed in the filtration tubes (Centrifree MPS-III, Amicon, Tokyo), and then these blank homogenates were centrifuged to obtain the filtrate ($2000 \times g$ for 30 min). The filtrate obtained above was applied to the other MPS-III tube and then centrifuged. This pretreatment with MPS-III reduced the nonspecific adsorption of drugs to the membrane filter. After incubation at 37°C for 3 min, homogenate samples with GPFX or LFLX (20 and 40 μM) were placed in MPS-III tubes, which was pretreated with filtrate from the same dilution homogenate. Then, the homogenate was centrifuged ($2000 \times g$ for 10 min) to give a filtrate containing unbound compound. The concentrations in homogenate (C_{total}) and filtrate (C_{free}) were determined as described, using HPLC. The concentration bound to tissue (C_{bound}) was calculated by subtracting C_{free} from C_{total}. After plotting C_{bound}/C_{free} against the homogenate concentration, a straight line was obtained. The C_{bound}/C_{free} at 100% homogenate concentration was then extrapolated. The free fraction (f_T) was then calculated according to the following equation:

$$f_T = \frac{1}{1 + Y} \tag{2}$$

where Y is the C_{bound}/C_{free} at 100% homogenate thus estimated. The concentration of the parent compound in liver homogenate (C_{total}) was determined after incubating with drug. The authors have checked that no glucuronidation of GPFX, which is a major metabolic pathway, took place *in vitro* in the homogenate. Therefore, metabolism during incubation and ultrafiltration can be ignored.

Results

Hepatic Uptake of LFLX In Vivo

After a single iv administration at a dose of 5 mg/kg to rats, the plasma concentrations of LFLX were higher than those of GPFX. The liver concentration of LFLX at 0.5 min after dosing was lower than that of GPFX, while that of the two NQs was similar at 1 min. The hepatic uptake clearances of LFLX were calculated from an integration plot based on these plasma and liver concentration profiles (Figure 1(b)). These uptake clearance thus calculated for LFLX were 0.708 mL/min/g liver, which was slightly smaller than that of GPFX (1.03 mL/min/g liver) [2]. Taking the liver weight (44 g/kg BW) and the R_B [19,20] into consideration, the uptake clearance of LFLX and GPFX based on the blood concentrations was calculated and found to be similar (32.5 and 33.1 mL/min/kg, respectively) for both. This uptake clearance was close to the hepatic blood flow (40 mL/min/kg) assessed from the hepatic uptake clearance of [^{3}H]-TCA, assuming that the TCA uptake is blood flow limited [2].

Uptake of GPFX and LFLX by Isolated Rat Hepatocytes

The uptake profiles of GPFX and LFLX by isolated rat hepatocytes are shown in Figure 2. Their hepatocellular uptake at 50 μM were linear up to 1 min and reached equilibrium at 2 min (Figure 2). The uptake rates of GPFX and LFLX were 77.0 ± 10.2 and 27.9 ± 3.9 μL/min/mg, respectively. GPFX uptake was approximately three times that of LFLX uptake. The uptake rates at steady-state was approximately 130 and 50 μL/mg protein for GPFX and LFLX, respectively (Figure 2). In the case of the 1000 μM substrate concentration, the uptake rates of GPFX and LFLX were 34.2 ± 4.7 and 16.9 ± 1.3 μL/min/mg, respectively, and were smaller than those at 50 μM, indicating a saturable uptake process.

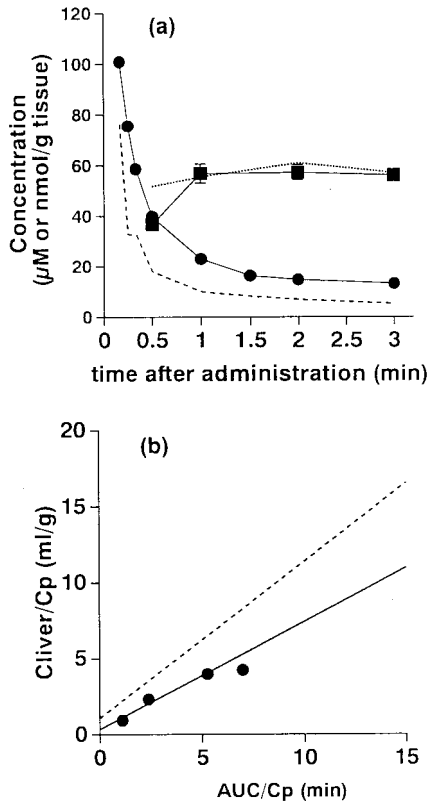

Figure 1. Time profiles (a) of plasma and liver concentrations after a single iv administration of LFLX and GPFX to rats and (b) integration plot for estimating the hepatic uptake clearance. (a) LFLX concentrations in plasma (●) and liver (■) after iv LFLX administration (14.5 μmol/kg) were determined in present study, while GPFX data were determined previously [2]. The dashed line and dotted line show the concentrations of GPFX (14.2 μmol/kg) in plasma and liver, respectively. (b) Initial slope (0.708 mL/min/g liver) for LFLX (●) represents hepatic uptake clearance. The dashed line shows the plot for GPFX (1.03 mL/min/g liver). Each plot and bar represents the mean ± S.E. ($n = 4$)

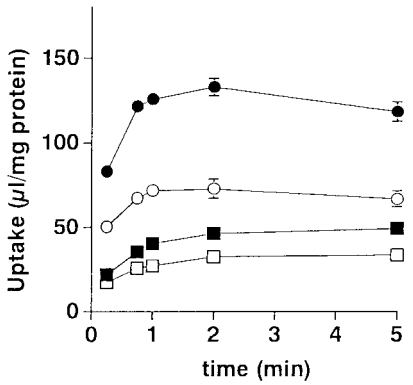

Figure 2. Uptake profiles of GPFX and LFLX by isolated rat hepatocytes. Uptake of GPFX and LFLX was measured by incubating isolated rat hepatocytes in Krebs–Henseleit buffer (pH 7.3) containing GPFX 50 μM (●) and 1000 μM (○) or LFLX 50 μM (■) and 1000 μM (□) at 37°C for 0.25, 0.75, 1, 2 and 5 min. GPFX and LFLX in the medium and cells were determined using HPLC. The ordinate value represents the cellular uptake divided by extracellular concentration. Each plot and bar represents the mean ± S.E. of 6–7 determinations from three different experiments

Intracellular Protein Binding of GPFX and LFLX in Liver

The free fraction (f_T) of GPFX and LFLX in liver cells was, respectively, 0.117 and 0.222 at 20 μM and 0.0885 and 0.308 at 40 μM (Table 1). The hepatic protein binding was larger for GPFX than that for LFLX.

Plasma Concentration Profiles at Steady-state

Plasma concentrations of both parent drugs reached equilibrium at 60 min during the constant infusion of GPFX and LFLX (Figure 3(a)) to rats. The Cl_{total} of GPFX was greater than that of LFLX (Table 1). The K_p value of GPFX and LFLX in liver at steady-state was 11.3 and 2.39, respectively, showing a marked difference (Table 1). The Cl_{total} values of GPFX and LFLX in EHBR were almost identical with those at the same infusion rate in normal rats (Figure 3(b), Table 1).

Biliary Excretion Profiles at Steady-state

The biliary excretion of GPFX at steady-state was greater than that of LFLX (Figure 4(a)). The Cl_{bile}^{plasma} of GPFX (0.735 mL/min/kg) was 2.9-fold that of

Table 1. Pharmacokinetic parameters of GPFX and LFLX at steady-state during constant infusion of GPFX (41.9 nmol/min/kg) of LFLX (42.7 nmol/min/kg) to normal rats and EHBR

		Cl_{plasma}^{ss} [a] (μM)	Cl_{total} [b] (mL/min/kg)	Cl_{bile}^{plasma} [c] (mL/min/kg)	Cl_{bile}^{liver} [d] (mL/min/kg)	$Cl_{bile}^{liver,u}$ [e] (mL/min/kg)	K_P	f_P [f]	f_T [g]
GPFX	Normal	3.75 ± 0.12	11.2 ± 0.3	0.7350 ± 0.096	0.0658 ± 0.0109	0.748 ± 0.124	11.3 ± 0.8	0.593	0.0885
	EHBR	3.12 ± 0.09	13.5 ± 0.4	0.5880 ± 0.029	0.0448 ± 0.0041		11.8 ± 0.3		
LFLX	Normal	7.95 ± 1.77	5.94 ± 0.98	0.2530 ± 0.041	0.0935 ± 0.0215	0.421 ± 0.097	2.39 ± 0.41	0.722	0.222
	EHBR	7.52 ± 0.49	5.74 ± 0.40	0.0934 ± 0.0141	0.0384 ± 0.0074		2.61 ± 0.20		

Each data represents the mean ± S.E. (normal rats $n = 4$, EHBR $n = 3$).
[a] Plasma concentration at steady-state (mean of plasma concentration at 70, 90, 120 and 150 min).
[b] Total body clearance obtained by dividing infusion rate by Cl_{plasma}^{ss}.
[c] Biliary clearance based on the plasma concentration.
[d] Biliary clearance based on the liver concentration.
[e] Biliary clearance based on the concentration of unbound drug in liver.
[f] Plasma unbound fractions were taken from Reference Akiyama *et al.* [19] and Okezaki *et al.* [20].
[g] The tissue unbound fractions of GPFX and LFLX were determined at concentrations of 40 and 20 μM in liver, respectively.

LFLX (0.253 mL/min/kg) (Table 1). The biliary clearance ($Cl_{bile}^{liver,\,u}$) based on the unbound drug concentration in the liver was 0.748 and 0.421 mL/min/kg for GPFX and LFLX, respectively (Table 1). The Cl_{bile}^{liver} of GPFX and LFLX in EHBR was 0.0448 and 0.0384, respectively, i.e. smaller than that in normal rats for each compound (Table 1).

Discussion

The systemic clearance of most quinolone antibiotics (NQs) is mainly governed by urinary excretion of parent forms; however, by contrast, some NQs are excreted into the bile and/or subject to metabolism, followed by urinary excretion of these metabolites. The former type includes OFLX and LFLX [21,22], while SPFX [23] and GPFX [19] belong to the latter. The efficiency in net biliary excretion is generally governed by several factors: the first is hepatic uptake ability, the second is the efflux from the liver to the blood compartment, and the third is sequestration inside the cells including intracellular metabolism and excretion into bile across the bile canalicular membrane. Therefore, in order to understand the mechanism that determines the degree of biliary clearance, the efficiency of each process should be independently assessed. With this in mind, the authors selected two NQs, GPFX and LFLX, and kinetically analyzed their hepatic uptake based on their plasma and liver concentration profiles. Moreover, achieving a steady-state by constant infusion, they investigated their net biliary excretion as well as the transport activity across the bile canalicular membrane *in vivo*.

It is likely that the ether anesthesia used in the present study had an effect on blood flow. The authors also determined the hepatic blood flow from the uptake clearance of taurocholate (TCA), and found it to be 40 mL/min/kg under anesthesia. This value was less than the reported values for rat hepatic blood flow, 50–60 mL/min/kg [24,25]. This difference might be due to the effect of ether anesthesia since Tsuji *et al*. reported that the blood flow was reduced by 20–30% following anesthesia with ether [26]. Taking account of the fact that GPFX is so rapidly taken up into liver cells that the uptake is nearly blood flow limited (Figure 1), it is possible that its hepatic uptake might also be reduced by anesthesia.

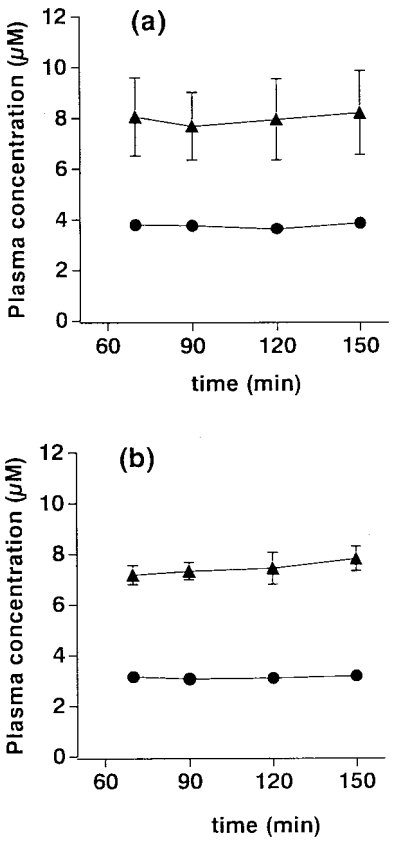

Figure 3. Plasma concentration profiles of GPFX and LFLX during constant infusion to normal rats (a) and EHBR (b). The infusion rate of GPFX (●) and LFLX (▲) was 13.9 and 14.2 nmol/min/kg, respectively. Each plot and bar represents the mean ± S.E. ($n = 4$)

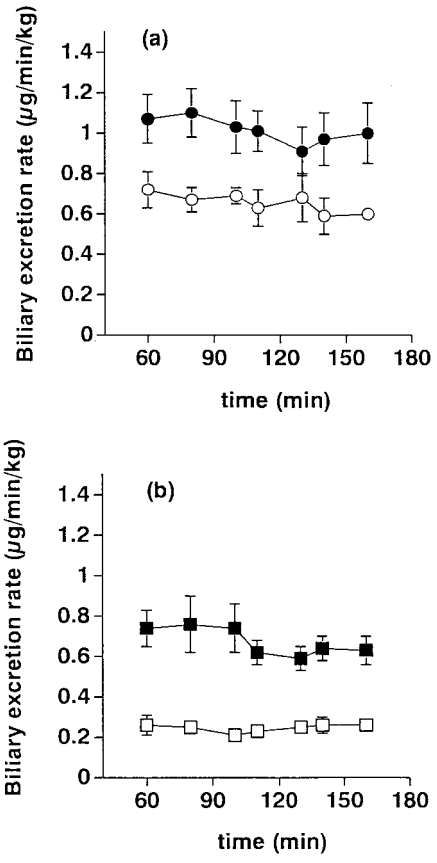

Figure 4. Biliary excretion rates of GPFX (a) and LFLX (b) during constant infusion to normal rats (●, ■) and EHBR (○, □). The infusion rate of GPFX and LFLX was 13.9 and 14.2 nmol/min/kg, respectively. Each plot and vertical bar represents the mean ± S.E. (normal rat $n = 4$, EHBR $n = 3$)

The Cl_{bile}^{plasma} of GPFX at steady-state was approximately three times that of LFLX (Table 1). Considering the physiological model, the Cl_{bile}^{plasma} can be written as a hybrid parameter consisting of several clearances: The mass balance equations in the liver (X_T) and bile (X_{bile}) compartment can be described by the following equations:

$$\frac{dX_T}{dt} = Cl_{uptake}^{plasma} \times C_P - (Cl_{efflux,\,app}^{liver,\,u} + Cl_{met}^{liver,\,u} + Cl_{bile}^{liver,\,u}) \times f_T \times C_T \quad (3)$$

$$\frac{dX_{bile}}{dt} = Cl_{bile}^{plasma} \times C_P = Cl_{bile}^{liver,\,u} \times f_T \times C_T \quad (4)$$

where Cl_{uptake}^{plasma} represents the hepatic uptake clearance based on the plasma concentration, which is obtained by integration plot analysis. C_P and C_T represent the concentration in the plasma and tissue (liver) compartment, respectively, and f_T is the unbound fraction in the tissue. $Cl_{efflux,\,app}^{liver,\,u}$ and $Cl_{met}^{liver,\,u}$ are the apparent efflux clearance from liver into circulating plasma and the metabolic clearance in the liver, respectively, both parameters also being based on the unbound hepatic concentrations.

At steady-state, Equation (3) equals zero. Therefore, Equation (5) can be obtained by rearranging Equations (3) and (4):

$$Cl_{bile}^{plasma} = Cl_{uptake}^{plasma} \times \frac{Cl_{bile}^{liver,\,u}}{Cl_{efflux,\,app}^{liver,\,u} + Cl_{met}^{liver,\,u} + Cl_{bile}^{liver,\,u}} \quad (5)$$

The Cl_{uptake}^{plasma} of GPFX was approximately 1.5 times that of LFLX (Figure 1), suggesting that a difference between the efficiency of the uptake process for GPFX and LFLX might be at least one of the factors that determines the degree of net biliary excretion of these two compounds. This difference in uptake ability of the two drugs was also confirmed by the uptake study with isolated rat hepatocytes (Figure 2). The authors have reported that an active transport system is involved in the hepatic uptake of GPFX. This was verified by kinetic modeling showing that this transport activity observed *in vitro* can account for the hepatic uptake *in vivo* [2]. Furthermore, it is argued that NQs, including both GPFX and LFLX, might be taken up into liver cells via the same transporter and the affinity of GPFX was higher than that of LFLX. Therefore, such a difference in the affinity for the transporter might be one of the factors that determines the uptake ability.

However, the above mentioned three times difference in the Cl_{bile}^{plasma} of the two compounds cannot be completely accounted for by a difference in the efficiency of the uptake process (Cl_{uptake}^{plasma}). Thus, the difference in the biliary excretion process through the canalicular membrane was also investigated *in vivo*. The $Cl_{bile}^{liver,\,u}$ of GPFX at steady-state was approximately 1.8 times that of LFLX (Table 1). This indicates that GPFX has a higher degree of membrane penetration through the bile canalicular membrane. Thus, based on Equation (3), the difference between the Cl_{bile}^{plasma} of GPFX and LFLX might also be due, at least partially, to the difference between these $Cl_{bile}^{liver,\,u}$ values. The ratio of the concentration in the bile to the unbound concentration in the liver was 6 and 3 for GPFX and LFLX, respectively. This ratio indicated that both GPFX and LFLX were concentratively excreted into the bile and showed that some active transport could be involved in the biliary excretion of the two drugs through the canalicular membrane. The present result showed a reduction in the biliary clearance of GPFX and LFLX in EHBR (Table 1). An ATP-dependent uptake of GPFX was observed by CMV from normal rats while no ATP-dependent uptake was observed by CMV from EHBR [13]. These results support the hypothesis that the biliary excretion of these NQs is, at least partially, mediated by cMOAT. Moreover, GPFX had a stronger inhibitory effect on the ATP-dependent uptake of DNP-SG, a typical substrate for cMOAT than LFLX [13]. It thus appears that GPFX and LFLX have a different transport ability with respect to the excretion process in which cMOAT plays a role. However, it should be noted that no difference between normal rats and EHBR was observed in the plasma concentration profiles at steady-state (Figure 3); this might be due to the small contribution of biliary clearance to total body clearance (Table 1). Based on Equation (3), the value of $Cl_{efflux,\,app}^{liver,\,u} + Cl_{met}^{liver,\,u}$ of GPFX and LFLX can be calculated from the values of Cl_{bile}^{plasma}, $Cl_{bile}^{liver,\,u}$ and Cl_{uptake}^{plasma}. These were respectively, 45.5 and 51.5 mL/min/kg. These were larger than the respective $Cl_{bile}^{liver,\,u}$ values, indicating that the greater part of the drug taken up into the liver returns to the circulating plasma or is metabolized, and the fraction excreted might be small.

If cMOAT is responsible for GPFX excretion, the K_p value should be slightly greater in EHBR than in normal rats. However, only a minimal K_p difference was observed between EHBR and in normal rats (Table 1). Theoretically, the K_p value depends on the ratio of free fractions in plasma (f_p) and liver cells (f_T), the ratio of the uptake into the liver and elimination (the sum of the efflux, metabolism and biliary excretion clearances) from liver. In these experiments, the glucuronide formation rate (approximately 25 nmol/min/kg), which was assessed as the sum of the biliary and urinary excretion rate of glucuronide was much higher than the biliary excretion rate of the parent compound (approximately 2.0 nmol/min/kg) at steady-state for GPFX [27]. Therefore, $Cl_{met}^{liver,\,u}$ should be much greater than $Cl_{bile}^{liver,\,u}$. Moreover, cMOAT might be partly responsible for the biliary excretion of GPFX, although another transporter(s) might also contribute since the biliary excretion of GPFX was reduced, but still

present in EHBR [13]. Therefore, the K_p difference between normal rats and EHBR should be small, if any.

The uptake of GPFX and LFLX by isolated hepatocytes at steady-state was approximately 130 and 50 μL/mg protein, respectively (Figure 3). The intracellular volume of hepatocytes is reported to be 4.3 μL/mg protein [28], so the cell–medium concentration ratio (C/M ratio) should be approximately 30 and 12, respectively. This C/M ratio *in vitro* can be directly compared with the ratio of total (the sum of bound and unbound) concentration in the liver to the unbound concentration in plasma *in vivo*, which is equivalent to the K_p/f_p value. The fact that C/M is greater for GPFX than LFLX is compatible with that of K_p/f_p for GPFX *in vivo* being greater than LFLX (18.8 and 3.3, respectively; Table 1). However, the absolute values for the C/M ratio were greater than the K_p/f_p for each compound. Adsorption to the surface of isolated hepatocytes might be one reason. There seems to be a y intercept for both compounds in the time profile of uptake by isolated hepatocytes (Figure 2). Such a y intercept may represent nonspecific adsorption to the surface of cells. Assuming thus, such adsorption of GPFX and LFLX can be extrapolated to be 64 and 15 μL/mg protein, respectively, as assessed by the y intercept in the time profile (Figure 2). Subtracting the adsorption thus obtained from the uptake at steady-state, the C/M ratio was calculated to be approximately 15 and 8, respectively, which was closer to the K_p/f_p *in vivo*. The other possibility is that the smaller *in vivo* uptake might be due to the presence of an unstirred water layer in the liver interstitial space *in vivo*. The authors have previously compared the uptake clearance in a rat liver perfusion system with that in isolated rat hepatocytes, for 15 compounds with various degrees of permeability through the sinusoidal membrane. For compounds with a lower membrane permeability, the uptake clearance was almost comparable for each system while for the compounds with a higher membrane permeability, the uptake clearance in the perfusion system was lower than that in the isolated hepatocytes [29,30]. This can be explained if it is considered that the rate limiting step for the uptake of such highly membrane permeable compounds *in vivo* is diffusion through the interstitial space because of the existence of an unstirred water layer in this space. The uptake clearance of GPFX and LFLX by isolated hepatocytes was, respectively, 77.0 and 27.9 μL/min/mg protein, which is close to the values for compounds showing diffusion-limited uptake. Therefore, diffusion in the interstitial space might be a rate limiting step for these compounds *in vivo*, resulting in the lower K_p/f_p value compared with that in isolated hepatocytes.

The biliary clearance value at steady-state (0.735 mL/min/kg) differed from that previously reported in the iv bolus study (1.79 mL/min/kg) [13]. At present, the mechanism of the discrepancy between the two biliary excretion clearances cannot be clearly explained. The plasma concentration of GPFX is maintained at 3.75 μM at steady-state while that after iv bolus administration was higher ($\sim$10 μM) during the initial phase and $\sim$3 μM after 10 min until 2 h [13]. Therefore, such a discrepancy in GPFX concentration may result in a difference in biliary clearance. However, these concentrations are still lower than the K_m (173 μM) [2] for the uptake transporter of GPFX. Additionally, the liver concentration of GPFX (42 μM) at steady-state should be much lower than the inhibitory constant, K_i (1.89 mM) for cMOAT [13], and so the authors believe that the carrier-mediated transport systems should not be saturated at steady-state and in the iv bolus studies. It might be due to cytotoxicity due to the high hepatic concentration of drug for the longer period of deep anesthesia in the infusion study ($\sim$3 h), compared with the iv bolus study ($\sim$2 h). Another possible explanation might be a difference between the analytical methods in the two experiments (HPLC in the steady-state study and TLC in the previous iv bolus study). The TLC results might include unknown minor metabolite(s) with structures resemble to the parent compound and R_f values identical to that of the standard for GPFX.

Considering such a discrepancy in the absolute values of biliary clearance, the authors attempted to use the same experimental systems in the present study to compare the hepatobiliary transport of GPFX and LFLX. To compare the hepatic uptake, they performed an integration plot analysis for both compounds (iv bolus study, Figure 1). To compare the net biliary excretion, they performed an iv infusion study (Figure 4). Therefore, the present conclusion concerning the difference in hepatobiliary transport of the two compounds should still be proposed in the present analysis.

In this comparison of the hepatobiliary transport of GPFX and LFLX, it was found that the net biliary clearance of GPFX was greater than that of LFLX. This phenomenon is, at least partially, due to the different transport activity stemming from the differential recognition by active transport systems on sinusoidal membrane in the hepatic uptake process and by the primary active transporter (cMOAT) on the canalicular membrane in the biliary excretion process. In both uptake and excretion processes, GPFX is more concentratively taken up into cells and excreted into bile, compared with LFLX.

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