Hepatobiliary Transport Governs Overall Elimination of Peptidic Endothelin Antagonists in Rats

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ABSTRACT

The overall disposition and hepatobiliary transport of BQ-123, an anionic cyclopentapeptide, and three analogs were examined in rats in vivo. Total body clearance (CL<sub>total</sub>) and biliary excretion clearance (CL<sub>bile, p</sub>) exhibited 4- to 8-fold differences between the compounds, with those for BQ-485 and compound A having the highest and lowest values, respectively. The CL<sub>bile, p</sub> values of BQ-485, BQ-123, and BQ-518 were almost equal to the CL<sub>total</sub> suggesting that hepatobiliary transport is the major elimination pathway for these compounds. Hepatic uptake clearance (CL<sub>uptake, vivo</sub>) and biliary excretion clearance (CL<sub>bile, p</sub>/f<sub>T</sub>), which was defined for the hepatic unbound concentration, were separately determined to examine the hepatic uptake and excretion processes, respectively. Both the CL<sub>uptake, vivo</sub> and CL<sub>bile, p</sub>/f<sub>T</sub> of BQ-485 were higher than those of BQ-123, whereas the corresponding values for BQ-518 were similar to those for BQ-123. The CL<sub>uptake, vivo</sub> and CL<sub>bile, p</sub>/f<sub>T</sub> of compound A were, respectively, approximately two thirds and one half those of BQ-123, suggesting that the lower CL<sub>bile, p</sub> value is due to the low efficiency of both the uptake and excretion processes. The CL<sub>uptake, vivo</sub> of these four peptides in vivo was similar to the extrapolated values based on the carrier-mediated transport activity previously assessed in vitro in isolated rat hepatocytes. The primary active transport previously assessed in an in vitro study in canalicular membrane vesicles was also highest for BQ-485 and lowest for compound A, similar to CL<sub>bile, p</sub>/f<sub>T</sub> in vivo. Thus, the transporters on both the sinusoidal and canalicular membranes determine the efficiency of the peptide overall elimination from the circulation.

A variety of small peptides have been recently developed as therapeutic agents; these include endothelin antagonists (Ni-rei et al., 1993; Nishikibe et al., 1993), renin inhibitors (On-detti et al., 1981), somatostatin analogs (Lambert et al., 1985), and thrombin inhibitors (Eckhardt et al., 1996). The metabolic stability of these peptides to the action of peptidases has been improved by the introduction of unusual amino acids and other modifications. However, such peptidemimetic compounds do not generally remain long in circulating plasma in vivo due to their rapid biliary excretion. Pharmacokinetic studies in rats have shown that octreotide and angiopeptin, both somatostatin analogs (Cathaperman et al., 1991; Lemaire et al., 1989), and ditekiren, a renin inhibitor (Greenfield et al., 1989), are efficiently taken up by the liver and subsequently excreted as intact peptides in bile.

Biliary excretion is one of the principal elimination mechanisms for xenobiotics, including therapeutic agents. Carrier-mediated transport systems have been identified for uptake on the sinusoidal membrane and for biliary excretion on the canalicular membrane (Keppler et al., 1997; Lomri et al., 1996; Meier et al., 1997; Müller et al., 1997; Yamazaki et al., 1996). These transporters have also been reported to be involved in the biliary excretion of small peptides. For example, carrier-mediated transport systems on the sinusoidal membrane have been reported for peptides, including cholecystokinin (Gores et al., 1986, 1989), renin inhibitors (Bertrams et al., 1991a, b), and somatostatin analogs (Ziegler et al., 1988, 1991). Transporters on the bile canalicular membrane for small peptides, including a renin inhibitor EMD-51921 (Ziegler et al., 1994), ditekiren (Takahashi et al., 1997), and octreotide (Yamada et al., 1996), are primary active transport systems that use ATP hydrolysis as their driving force.

BQ-123, an anionic cyclopentapeptide endothelin ET<sub>A</sub> receptor antagonist, also has pharmacokinetic characteristics similar to those of the other small peptides described above (Nakamura et al., 1996; Shin et al., 1996). Its elimination from plasma in rats after i.v. injection is rapid, with the

ABBREVIATIONS: CMV, canalicular membrane vesicle; cMOAT, canalicular multispecific organic anion transporter; HPLC, high-performance liquid chromatography; f<sub>u</sub>, the plasma unbound fraction; R<sub>b</sub>, blood-to-plasma concentration ratio; f<sub>T</sub>, unbound fraction in the liver; PS<sub>swi</sub>, permeability surface area product across the isolated hepatocytes; PS<sub>i</sub>, the influx clearance across basolateral membrane; PS<sub>e</sub>, the efflux clearance across the basolateral membrane.
early-phase $T_{1/2}$ being approximately 4 min (Nakamura et al., 1996). Within 1 h after injection, 86% of the dose is excreted in bile in its intact form (Nakamura et al., 1996). We previously reported that active transport systems on both sinusoidal (Nakamura et al., 1996; S. Akhteruzzaman, Y. Kato, H. Kouzuki, H. Suzuki, A. Hisaka, B. Stieger, P. J. Meier and Y. Sugiyama, submitted for publication) and canalicular (Shin et al., 1997; Akhteruzzaman et al., 1999) membranes are involved in the biliary excretion of BQ-123 in rats. The uptake of BQ-123 by hepatocytes can be inhibited by anionic compounds such as the bile acid, taurocholate, and an organic anion, dibromosulfophthalein (Nakamura et al., 1996). The excretion of BQ-123 on bile canalicular membrane is mainly mediated by canalicular multispecific organic anion transporter (cMOAT) (Shin et al., 1997).

A series of BQ-123 derivatives were synthesized in the present study with the aim of producing a long-lasting endothelin antagonist. These derivatives include compound A, which also has a cationic moiety (Fukami et al., 1996); in light of the previous studies, this might be important for recognition by transporters on both sinusoidal and canalicular membranes. BQ-485, an anionic linear peptide (Itoh et al., 1996), and BQ-518, where the $\psi$-Val in BQ-123 has been substituted with $\psi$-thiophenolglycine (d-Thg) (Fukami et al., 1995), have also been synthesized. The purpose of the present study was to examine quantitatively the contribution of transport activity on sinusoidal and canalicular membranes to the net biliary excretion of these compounds. To this end, each transport activity was separately determined in vivo: integration plot analysis was performed for hepatic uptake clearance, and biliary excretion clearance, as defined in terms of the hepatic unbound concentration, was determined in a steady-state infusion.

### Materials and Methods

**Chemicals and Reagents.** BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]), BQ-485 (perhydroazepino-N-carboxy-L-Tyr-D-Trp-D-Trp), BQ-518 (cyclo[D-Trp-D-Asp-L-Pro-D-Thg-L-Leu]), and compound A (cyclo[D-Trp-D-Asp-L-Hyp-L-Arg]-D-Val-L-Leu) were synthesized at the Tsukuba Research Institute of Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan). [Prolyl-3,4(n)-3H]BQ-123 (31.0 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). All other chemicals and reagents were commercial products of analytical grade.

**Animals.** Male Sprague-Dawley rats, weighing approximately 250 to 300 g, were purchased from Nisseizai (Tokyo, Japan). This study was carried out in accordance with the “Guide for the Care and Use of Laboratory Animals” as adopted and promulgated by the National Institutes of Health.

**Steady-State Infusion Study.** With the animals under light ether anesthesia, both the femoral artery and vein were cannulated with a polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ) for blood sampling and drug infusion, respectively. The bile duct was also cannulated with a polyethylene catheter (PE-10; Clay Adama) for bile collection. After dissolving in saline, each peptide was infused over a period of 60 min. Bile was collected in preweighed test tubes at 10-min intervals. The plasma was prepared by the centrifugation of the bile samples (Microfuge E; Beckman, Fullerton, CA). At the end of the infusion, the liver was excised and weighed. The concentration of the drug in these samples was determined by high performance liquid chromatography (HPLC) method as described below. Based on the results obtained from the infusion study, pharmacokinetic parameters were calculated according to the following equations:

\[
CL_{tot} = \frac{I}{C_{pss}} \quad (1)
\]

\[
CL_{bile, h} = \frac{V_{bile}}{C_{pss}} \quad (2)
\]

\[
CL_{bile, p} = \frac{V_{bile}}{C_{pss}} \quad (3)
\]

where $I$, $C_{pss}$, $V_{bile}$, $C_{bile}$, $CL_{tot}$, $CL_{bile, p}$, and $CL_{bile, h}$ represent infusion rate (nmol/min/kg), plasma concentrations at steady-state ($\mu$M), biliary excretion rate at steady-state (nmol/min/kg), hepatic concentration at steady-state ($\mu$M), total body clearance (ml/min/kg), biliary excretion clearance (ml/min/kg) defined with respect to plasma concentration, and biliary excretion clearance (ml/min/kg), defined with respect to hepatic concentration, respectively. $C_{pss}$ was determined as the mean values of the plasma concentrations at 50 and 60 min. $V_{bile}$ was determined as the biliary excretion rate from 50 to 60 min. $C_{bile}$ was determined as the hepatic concentration at 60 min. To calculate $C_{bile}$, the specific gravity of the liver assumed to be unity. Thus, the amount in the liver (nmol/g liver) can be regarded as the hepatic concentration ($\mu$M), and the units of $CL_{bile, h}$ should be ml/min/kg.

**Integration Plot Analysis for Determination of In Vivo Hepatic Uptake Clearance ($CL_{uptake, \text{vivo}}$).** After i.v. bolus injection (500 nmol/kg b.w.t.) via femoral vein, blood and bile samples were collected from the femoral artery and bile duct, respectively, for 3 min. During this period, a section of liver sample (100 mg) was resected at 30 s, 1.5 min, and 3 min by a biopsy technique. The concentration of the drug in the samples was determined by HPLC as described below. The plasma concentration-time curve was fitted to the following exponential equation by a nonlinear iterative least-squares method by use of a MULTI program (Yamaoka et al., 1981).

\[
C_p = A \exp(-\alpha t) + B \exp(-\beta t) \quad (4)
\]

where $C_p$ is the plasma concentration, $\alpha$ and $\beta$ are the apparent rate constants, $A$ and $B$ are the corresponding zero time intercept, and $t$ is time. The area under the plasma concentration-time curve was calculated as:

\[
AUC = \frac{A}{\alpha} \left[1 - \exp(-\alpha t)\right] + \frac{B}{\beta} \left[1 - \exp(-\beta t)\right] \quad (5)
\]

Because the biliary excretion of the endothelin antagonists was rapid and at least a small portion of the peptide, once taken up by hepatocytes, was recovered in bile, the amount of peptide after uptake by the liver ($X_{\text{liver}}$) was calculated from the following equation:

\[
X_{\text{liver}} = X_{\text{liver, app}} + X_{\text{bile}} \quad (6)
\]

where $X_{\text{liver, app}}$ and $X_{\text{bile}}$ represent the amount of peptide in the liver and that recovered in bile, respectively. The integration plot was obtained by plotting $X_{\text{liver}}/C_p$ against area under the plasma concentration-time curve/C. The initial slope of the line represents the $CL_{uptake, \text{vivo}}$.

**HPLC Analysis of Endothelin Antagonists in Plasma, Bile, and Liver Samples.** Each plasma and bile sample was mixed with 4 volumes of ethanol and was centrifuged (2000 g, 2 min). The concentration of peptides in supernatant was determined by a Polytron homogenizer (T25-S1; IKA Japan Co. Ltd., Yokohama, Japan) in 4 volumes of ethanol and then centrifuged. The concentration of the drug in the supernatant was determined by HPLC. The HPLC analysis was performed according to a published method (Nakamura et al., 1996) using...
a Spherisorb S3 ODS2 (4.6 × 150 mm) column (Tosoh, Japan). The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid and 35% (v/v) acetonitrile for BQ-123, BQ-518, and compound A and 55% acetonitrile for BQ-485. A flow rate of 0.8 ml/min (BQ-485) and 1.0 ml/min (BQ-123, BQ-518, and compound A) and an injection volume of 50 μl were used for all experiments. The fluorescent detector was operated at an excitation wavelength of 287 nm and an emission wavelength of 348 nm. The detection limit was 30, 25, 30, and 20 pg for BQ-123, BQ-485, BQ-518, and compound A, respectively.

**Determination of Plasma Protein Binding.** The plasma protein binding of the endothelin antagonists were determined by ultrafiltration. Each peptide dissolved in phosphate buffer (50 mM, pH 7.4) was diluted 10 times with rat plasma to give the final concentrations that were close to the steady-state plasma concentration present in the in vivo infusion study (0.370, 0.371, 0.595, and 1.54 μM for BQ-123, BQ-485, BQ-518, and compound A, respectively). The mixture was incubated at 37°C for 30 min to ensure binding equilibrium. After incubation, 40 μl of aliquot was taken for the determination of total plasma concentration. Next, the plasma was placed in an ultrafiltration apparatus (Centricon; Amicon, Inc., Beverly, MA) with a molecular mass cutoff of 13 kDa and centrifuged at 3000 rpm (TOMY RL-100, Tokyo, Japan) for 10 min. After centrifugation, the concentration in filtrate was also determined by HPLC as the unbound concentration. The plasma unbound fraction (f_u) was calculated by dividing the unbound concentration by the total plasma concentration. The recoveries of BQ-123, BQ-485, BQ-518, and compound A, respectively, were calculated according to the following equation:

\[ R = \frac{C_{u,100\text{%}}}{C_{u,100\text{%}} \text{filtrate blank}} \]

where \( R \) is the recovery of the peptide, \( C_{u,100\text{%}} \) is the unbound concentration at 100% homogenate, and \( C_{u,100\text{%}} \text{filtrate blank} \) is the concentration in the filtrate blank.

**Determination of Blood-to-Plasma Distribution.** Each peptide was dissolved in phosphate buffer (50 mM, pH 7.4) and diluted 10 times with rat plasma to give the final concentration described above and incubated at 37°C for 30 min. To determine the concentration in whole blood, 50 μl of the blood was transferred into an Eppendorf tube immediately after incubation. The concentration was determined by HPLC as the unbound concentration. The plasma unbound fraction (f_u) was calculated by dividing the unbound concentration by the total plasma concentration. The recoveries of BQ-123, BQ-485, BQ-518, and compound A were 94%, 91%, 90%, and 74% for BQ-123, BQ-485, BQ-518, and compound A, respectively. The binding was normalized with respect to the filter blank.

**Determination of Red Blood Cell Distribution.** Each peptide was dissolved in phosphate buffer (50 mM, pH 7.4) and diluted 10 times with rat whole blood to give the final concentration described above and incubated at 37°C for 30 min. To determine the concentration in whole blood, 50 μl of the blood was transferred into an Eppendorf tube immediately after incubation. The concentration was determined by the same HPLC method as used for plasma. After incubation, the blood was centrifuged at 3000 rpm (TOMY RL-100) for 10 min at 4°C to obtain the plasma. The concentration in plasma was determined by the HPLC method as described above. Blood-to-plasma concentration ratio (R_b) was calculated by dividing the concentration in whole blood by the plasma concentration.

**Determination of Tissue Binding of Endothelin Antagonists in Liver.** Rat liver homogenate of 33.3% (w/v) was prepared using a Teflon homogenizer (Iuchi, Japan) in PBS (pH 7.4). This homogenate was serially diluted by PBS to make 16.6% and 8.3% homogenates. Each compound was then dissolved in 1 ml of these homogenate to give the concentration near to the steady-state hepatic concentration (2 μM for BQ-123, BQ-485, and compound A and 4 μM for BQ-518). Then, the mixture was incubated for 3 min at 37°C. After incubation, an aliquot was taken, and the concentration was determined by HPLC. This was designated as total concentration (C_t). Then, 600 μl of the mixture was placed in an ultrafiltration apparatus (Centricon) and was centrifuged at 3000 rpm (TOMY RL-100) for 10 min. After centrifugation, the free concentration (C_f) in the filtrate was also determined by the HPLC method. The bound concentration in the tissue (C_b) was calculated by subtracting C_f from C_t. After plotting C_f/C_t against the homogenate concentration, a straight line was obtained. The C_f/C_t at 100% homogenate concentration was then extrapolated, and nonspecific adsorption was subtracted from the extrapolated value. The unbound fraction in the liver (f_u) was then calculated according to the following equation:

\[ f_u = \frac{1}{1 + Y} \]

where Y is the estimated C_f/C_t at 100% homogenate.

**Extrapolation of Hepatic Uptake Clearance Based on In Vitro Data obtained in Isolated Rat Hepatocytes.** The permeability-surface area product across the isolated hepatocytes (PS_cell) was calculated according to the following equation using previously obtained data on the peptide in vitro uptake characteristics (S. Akteruzzaman, Y. Kato, H. Kouzuki, H. Suzuki, A. Hisaka, B. Stieger, P. J. Meier and Y. Sugiyama, submitted for publication):

\[ PS_{\text{cell}} = \frac{V_{\text{max}}}{K_m} \text{(Na}^+\text{-dependent)} + \frac{V_{\text{max}}}{K_m} \text{(Na}^-\text{-independent)} + P_{\text{dif}} \text{(Na}^+\text{-dependent)} + P_{\text{dif}} \text{(Na}^-\text{-independent)} \]

where \( V_{\text{max}} \) is the maximum uptake rate, \( K_m \) is the Michaelis-Menten constant, and \( P_{\text{dif}} \) is the nonspecific uptake clearance. Using the calculated PS_{cell} value from the in vitro data, the in vivo uptake clearance was predicted according to the following equation:

\[ CL_{\text{uptake, in vitro}} = \frac{Q_p f_u PS_{\text{cell}}}{Q_p f_u PS_{\text{cell}} + 1 - H_t R_b} \]

where \( CL_{\text{uptake, in vitro}} \) is the hepatic uptake clearance that is predicted from in vitro data, \( Q_p \) is the hepatic plasma flow rate, and \( H_t \) is the hematocrit. \( Q_p \) was 34.8 ml/min/kg, which was determined by the infusion study of taurocholic acid in our previous study (M. Kato, Y. Kato, T. Nakamura and Y. Sugiyama, submitted for publication). \( H_t \) was assumed to be 0.45.

**Results**

**Plasma Concentration and Biliary Excretion Profiles of Endothelin Antagonists during Constant Infusion.** The plasma concentration of BQ-123, BQ-485, BQ-518, and compound A reached steady-state 50 min after the beginning of the i.v. infusion (Fig. 1). The CL_total was the highest for BQ-485, followed by BQ-123, BQ-518, and compound A, which had a value about 4-fold smaller than that of BQ-485 (Table 1). The biliary excretion profile was also examined during the i.v. infusion (Fig. 2). The biliary excretion rate of BQ-123, BQ-485, and BQ-518 was close to the infusion rate (10 µg/min/kg) at steady-state (Fig. 2, Table 1),
indicating very little metabolism of these three compounds. The $V_p$ of compound A was approximately 40% of the infusion rate (Fig. 2, Table 1). However, we found that compound A is hydrolyzed under the conditions presented by the physiological buffer, with the $T_{1/2}$ being about 180 min. Therefore, such degradation might also occur in vivo. The $CL_{bile, p}$ of BQ-485 was the highest, followed by that of BQ-123, BQ-518, and compound A, with an approximately 8-fold difference between BQ-485 and compound A (Table 1). The $CL_{bile, h}$ was also greatest for BQ-485, and there was an approximately 4-fold difference between BQ-485 and compound A (Table 1). This $CL_{bile, h}$ was defined in terms of the total (sum of unbound and bound) substrate concentration in the liver. The $CL_{bile, h}/f_T$, which was defined in terms of the unbound substrate concentration in the liver, was calculated based on the data for $CL_{bile, h}$ and $f_T$ (Table 1). The difference of $CL_{bile, h}/f_T$ for compound A was the lowest and approximately half that of BQ-123 (Table 1).

Integration Plot for Estimation of $CL_{uptake, vivo}$. The $CL_{uptake, vivo}$ was highest for BQ-485 and lowest for compound A (Fig. 3, Table 1). The $CL_{uptake, vivo}$ values for BQ-123 and BQ-518 were similar. The $CL_{uptake, vivo}$ for compound A was approximately half that of BQ-123 (Table 1).

Estimation of $f_u$, $R_b$, and $f_T$. The $f_u$, $R_b$, and $f_T$ were determined and shown in Table 1. The $f_u$ was much lower for BQ-485 than that for other three compounds (Table 1).

Extrapolation of Hepatic Uptake Clearance from In Vitro Isolated Rat Hepatocyte Data. Based on the extrapolation from the kinetic parameters previously obtained in vitro using isolated rat hepatocytes, the $CL_{uptake, vitro}$ was calculated and compared with the $CL_{uptake, vivo}$ observed in the present study (Fig. 4). The $CL_{uptake, vitro}$ obtained in this way from in vitro data was close to the $CL_{uptake, vivo}$ (Fig. 4).

Discussion
It is established that peptidemimetic drugs, such as renin inhibitors, somatostatin analogs, and endothelin

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$V_{ss}$ ($\mu$g/min/kg)</th>
<th>$C_{ss}$ (nmol/g liver)</th>
<th>$Cl_{ss}$ ($\mu$l/min/kg)</th>
<th>$F$ (% of total uptake clearance)</th>
<th>$CL_{uptake, vitro}$ ($\mu$l/min/kg)</th>
<th>$CL_{uptake, vivo}$ ($\mu$l/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BQ-123</td>
<td>16.3</td>
<td>0.52</td>
<td>2.38</td>
<td>15.9</td>
<td>15.2</td>
<td>15.8</td>
</tr>
<tr>
<td>BQ-485</td>
<td>15.9</td>
<td>0.63</td>
<td>2.29</td>
<td>15.9</td>
<td>15.2</td>
<td>18.2</td>
</tr>
<tr>
<td>BQ-518</td>
<td>15.3</td>
<td>0.67</td>
<td>2.46</td>
<td>15.3</td>
<td>15.2</td>
<td>15.8</td>
</tr>
</tbody>
</table>

$V_{ss}$: Steady-state plasma concentration.
$C_{ss}$: Steady-state hepatic concentration.
$Cl_{ss}$: Biliary excretion clearance at steady-state.
$F$: Fraction of drug excreted in bile.
$R_b$: Red blood cell-to-plasma concentration ratio.
$f_T$: Unbound fraction in the liver.

Fig. 2. Biliary excretion profile of endothelin antagonists during i.v. infusion. Each point and vertical bar represents the mean ± S.E. of three animals. Infusion rate was 10 $\mu$g/min/kg b.wt.: □, BQ-123; ◯, BQ-485; ○, BQ-518; △, compound A.
antagonists, are often efficiently excreted into the bile in an unchanged form, resulting in their low bioavailability (Bertrens et al., 1991a,b; Greenfield et al., 1989; Nakamura et al., 1996). However, the present study shows that both the \( CL_{\text{total}} \) and \( CL_{\text{bile, p}} \) differ between various endothelin antagonists. Compound A has a 4-fold lower \( CL_{\text{total}} \) than that of BQ-485 (Table 1). \( CL_{\text{bile, p}} \) also differs between each compound, with an 8-fold difference between compound A and BQ-485 (Table 1). The inhibition constant of compound A for binding to ETA is in the nanomolar range, as in the case of BQ-485 and BQ-123 (22, 3.4, and 13 nM for BQ-123, BQ-485, and compound A, respectively) (Fukami et al., 1996; Itoh et al., 1993; Moreland et al., 1994). This means that it is possible to construct a small peptide, like compound A, that exhibits relatively lower hepatic extraction but still has potent antagonist activity. The order of the absolute value of \( CL_{\text{bile, p}} \) (BQ-485 > BQ-123 > BQ-518 > compound A) was the same as that of \( CL_{\text{total}} \) (Table 1). In addition, the biliary excretion ratio for BQ-485, BQ-123, and BQ-518 was almost equal to unity (Fig. 2). Therefore, the hepatobiliary transport of these endothelin antagonists determines the efficiency of their overall elimination from the body.

We previously reported that both the uptake and excretion processes of these four endothelin antagonists by sinusoidal and canalicular membranes, respectively, are mediated by active transport systems (S. Akhteruzzaman, Y. Kato, H. Kouzuki, H. Suzuki, A. Hisaka, B. Stieger, P. J. Meier and Y. Sugiyama, submitted for publication; Akhteruzzaman et al., 1999). To understand the rate-determining process in the net biliary excretion of these endothelin antagonists, we attempted in this study to separately determine the clearance for the uptake process from the circulation into hepatocytes and the excretion process from the hepatocytes into bile. The difference in \( CL_{\text{uptake, vivo}} \), which represents the clearance for the uptake process, between each peptide (Table 1) suggests that the efficiency of hepatic uptake is highest for BQ-485 and lowest for compound A. The \( CL_{\text{uptake, vitro}} \) was comparable for compound A than for BQ-123, but this difference was not very marked and less than 2-fold (Table 1), suggesting that the large difference in net biliary excretion (\( CL_{\text{bile, p}} \)) between these two compounds (Table 1) cannot be explained simply by a difference in
efficiency of the uptake process at the sinusoidal membrane. To compare the efficiency of excretion across the bile canalicular membrane for each compound, we determined \( CL_{\text{bile}, h} \) in the infusion study (Table 1). This \( CL_{\text{bile}, h} \) is defined in terms of the total substrate concentration in the liver and therefore should be dependent on tissue binding in liver. If only unbound molecules can penetrate the bile canalicular membrane, the intrinsic transport activity on this membrane should be represented as \( CL_{\text{bile}, h}/f_T \), which was defined in terms of the hepatic unbound concentration. The \( CL_{\text{bile}, h}/f_T \) determined in the present study for compound A was lowest and only half that of BQ-123 (Table 1). Thus, the efficiency of the biliary secretion process also is one of the factors that determines differences in the degree of net biliary excretion.

To demonstrate that these in vivo kinetic parameters (\( CL_{\text{uptake, vivo}} \) and \( CL_{\text{bile}, h}/f_T \)) directly reflect transport activity on sinusoidal and bile canalicular membranes, we compared these in vivo parameters with those extrapolated from data obtained in vitro in isolated rat hepatocytes and isolated rat bile canalicular membrane vesicles (CMV), respectively. In the hepatic uptake process, both \( Na^+ \)-dependent and -independent active transport mechanisms operate in the case of all four peptides (S. Akhteruzzaman, Y. Kato, H. Kouzuki, H. Suzuki, A. Hiasaka, B. Stieger, P. J. Meier and Y. Sugiyama, submitted for publication). Based on eqs. 8 and 9, hepatic uptake clearance can be predicted based on in vitro data, and the \( CL_{\text{uptake, vivo}} \) thus obtained was almost the same as that observed in vivo (\( CL_{\text{uptake, vivo}} \)) for each compound (Fig. 4). This demonstrates that the \( CL_{\text{uptake, vivo}} \) observed in vivo in the present study reflects membrane transport across the sinusoidal membrane. On the other hand, cMOAT primarily mediates the transport of BQ-123, BQ-485, and BQ-518 in the excretion process on the bile canalicular membrane, whereas a primary active transporter other than cMOAT is responsible for the biliary excretion of compound A (Akhteruzzaman et al., 1999). The clearance for the ATP-dependent transport of BQ-123, BQ-485, BQ-518, and compound A in CMV was 11.3, 7.53, 2.41, and 0.6 \( \mu l/min/mg \) protein at a substrate concentration of 10 \( \mu M \) (Akhteruzzaman et al., 1999). Also, both the \( CL_{\text{bile}, h}/f_T \) (Table 1) and the clearance for the ATP-dependent transport observed in CMV (Akhteruzzaman et al., 1999) were highest for BQ-485 and lowest for compound A. Thus, the biliary excretion of peptides observed in vivo reflects membrane transport activity across the bile canalicular membrane.

The difference in \( CL_{\text{uptake, vivo}} \) and \( CL_{\text{bile}, h}/f_T \) between BQ-123 and compound A was not very marked (less than 2-fold) compared with that in \( CL_{\text{bile}, h} \) between each compound (an 8-fold difference). This may suggest that the difference in the efflux process from hepatocytes back into the circulation is also involved in determining such a large difference in \( CL_{\text{bile}, h} \) between these compounds. In the present study, we actually determined the clearances for the net biliary excretion (\( CL_{\text{bile}, h} \)), uptake process (\( CL_{\text{uptake, vivo}} \)), and excretion process (\( CL_{\text{bile}, h}/f_T \)). Because the degree of metabolism of these compounds is minor, it is possible to calculate the efflux clearance for each compound based on these three parameters. Assuming the venous equilibrium model (Pang et al., 1977), the apparent intrinsic clearance (\( CL_{\text{int, app}} \)) and the influx clearance across basolateral membrane (\( PS_2 \)) can be defined as:

\[
CL_{\text{bile}, h} = \frac{Q_p \cdot f_u \cdot CL_{\text{int, app}} \cdot (1 - H_{et})}{R_b + (Q_p \cdot CL_{\text{int, app}})}
\]

\[
CL_{\text{uptake, vivo}} = \frac{Q_p \cdot f_u \cdot PS_1 \cdot (1 - H_{et})}{R_b + (Q_p \cdot CL_{\text{int, app}})}
\]

where \( CL_{\text{int, app}} \) can be written as:

\[
CL_{\text{int, app}} = PS_1 \times \frac{CL_{\text{bile}, h}/f_T}{PS_2 + CL_{\text{bile}, h}/f_T}
\]

\( PS_2 \) is the efflux clearance across the basolateral membrane. Based on the pharmacokinetic parameters obtained in the present study (Table 1), both \( CL_{\text{int, app}} \) and \( PS_1 \) can be estimated for each peptide using eqs. 10 and 11, respectively. The \( PS_2 \) can then be calculated based on eq. 12. For both BQ-123 and BQ-485, the \( CL_{\text{int, app}} \) was almost equal to \( PS_1 \) because the \( CL_{\text{bile}, h} \) was almost equal to the \( CL_{\text{uptake, vivo}} \) for both compounds (Table 1). Therefore, based on eq. 12, the \( PS_2 \) for these two compounds should be much less than their own \( CL_{\text{bile}, h}/f_T \) (\( PS_2 < 15.8 \) ml/min/kg for BQ-123 and \( PS_2 < 363 \) ml/min/kg for BQ-485). From our calculation, the \( PS_2 \) for BQ-518 and compound A was 33.8 and 58.4 ml/min/kg, respectively. Thus, one of the reasons for the lower \( CL_{\text{bile}, h} \) of compound A compared with BQ-123 may be its higher efflux across the basolateral membrane as well as its lower transport across the bile canalicular membrane. Further studies are needed to determine the \( PS_2 \) for each peptide more directly and to identify the reason for this discrepancy in \( PS_2 \) between the two compounds.

The present study (Fig. 4) shows that the hepatic uptake clearance, assessed by in vivo integration plot analysis, can be reasonably predicted for all four endothelin antagonists from the initial uptake rate obtained in vitro using freshly isolated hepatocytes. We have also confirmed this prediction for other therapeutic agents such as pravastatin (Yamazaki et al., 1993) and octreotide (Yamada et al., 1997), as well as the good agreement in influx clearance into hepatocytes between a liver perfusion system and isolated hepatocytes for 15 drugs with different membrane permeability (Miyauchi et al., 1993). These results indicate that in humans, too, the efficiency of the hepatic uptake of therapeutic agents can be predicted if their initial uptake rate can be determined in freshly isolated human hepatocytes. It might be difficult to predict the absolute values for hepatic uptake clearance in vivo in humans because freshly isolated human hepatocytes are not always available, and so the viability of human hepatocytes is critical. Nevertheless, the relative degree of uptake activity may be assessed for the different compounds. Therefore, such human hepatocyte systems are suitable for screening drugs during their developmental stage.

The present study has allowed us to conclude that hepatobiliary transport plays a major role in determining the overall elimination of endothelin antagonists from the circulation. The efficiency in net biliary excretion greatly differs between each compound and can be affected by transport.
activity in hepatic uptake across the basolateral membrane and/or biliary excretion across the bile canicular membrane.

References


