Multiple Transport Systems Mediate the Hepatic Uptake and Biliary Excretion of the Metabolically Stable Opioid Peptide [D-penicillamine$^{2,5}$]enkephalin

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Non-standard Abbreviations: Oatp1a1, rat organic anion transporting polypeptide 1a1 (formerly Oatp1); Oatp1a4, rat organic anion transporting polypeptide 1a4 (formerly Oatp2); Oatp1b2, rat organic anion transporting polypeptide 1b2 (formerly Oatp4); OATP1B1, human organic anion transporting polypeptide 1B1 (formerly OATPC);
OATP1B3, human organic anion transporting polypeptide 1B3 (formerly OATP8); Bcrp, breast cancer resistance protein; DPDPE, [D-penicillamine\textsuperscript{2,5}]enkephalin; Mrp2, multidrug resistance-associated protein 2; TR\textsuperscript{-} rats, Mrp2-deficient rats; P-gp, P-glycoprotein; Mdr1a/b, multidrug resistance protein 1a/b; Ntcp, sodium taurocholate co-transporting polypeptide; SC, sandwich-cultured; BEI, biliary excretion index; DIG, digoxin; BSP, bromosulfophthalein; DELT II, deltorphin II; TEA, tetraethylammonium; PAH, \textit{p}-aminohippurate; AIC, Akaike’s Information Criterion; BC, bile canaliculi; HRP, horseradish peroxidase; TBS-T, tris-buffered saline containing 0.3\% Tween 20
ABSTRACT

Rapid and extensive biliary excretion of [D-penicillamine\textsuperscript{2,5}]enkephalin (DPDPE) in rats as the unchanged peptide suggests that multiple transport proteins may be involved in the hepatobiliary disposition of this zwitterionic peptide. Although DPDPE is a P-gp substrate, the role of other transport proteins in the hepatic clearance of DPDPE has not been established. Furthermore, the ability of various experimental approaches to quantify the contribution of a specific hepatic uptake or excretion process when multiple transport systems are involved has not been addressed. \textsuperscript{3}H-DPDPE uptake in suspended Wistar rat hepatocytes was primarily (>95\%) due to temperature-dependent transport mechanisms; similar results were obtained in suspended hepatocytes from Mrp2-deficient (TR\textsuperscript{−}) rats. Pharmacokinetic modeling revealed that saturable and linear processes were involved in \textsuperscript{3}H-DPDPE uptake in hepatocytes. The use of transport modulators suggested that hepatic uptake of \textsuperscript{3}H-DPDPE was mediated by Oatp1a1, Oatp1a4, and likely Oatp1b2. Accumulation of \textsuperscript{3}H-DPDPE in sandwich-cultured (SC) hepatocytes was rapid; uptake of \textsuperscript{3}H-DPDPE in SC rat hepatocytes from control and TR\textsuperscript{−} rats was similar. However, the biliary excretion index and biliary clearance decreased by 83 and 85\%, respectively, in TR\textsuperscript{−} SC rat hepatocytes indicating that DPDPE is an Mrp2 substrate. Rate constants for uptake and excretion of \textsuperscript{3}H-DPDPE in SC rat hepatocytes were determined by pharmacokinetic modeling; data were consistent with basolateral excretion of \textsuperscript{3}H-DPDPE from the hepatocyte. These results demonstrate the complexities of hepatobiliary disposition when multiple transport mechanisms are involved for a given...
substrate, and emphasize the necessity of multi-experimental approaches for the comprehensive resolution of these processes.
INTRODUCTION

Determination of the contribution of multiple transport systems to the hepatic disposition of xenobiotics is critical to understanding and predicting the kinetic behavior of a compound in the liver. The focus on in vitro systems to study drug transport has led to identification of important transport proteins that govern the pharmacokinetics of many xenobiotics. Although some in vitro observations have been translated successfully to clinically relevant predictions (Fromm et al., 1999; Nishizato et al., 2003; Shitara et al., 2003), determination of the relative impact of drug/transporter interactions in vivo remains difficult. A particular obstacle to the effective prediction of drug transport in vivo is that many experimental systems examine the in vitro behavior of individual transporters in isolation, while substrates in vivo have the opportunity to interact with multiple transporters in parallel or in sequence. For example, biliary excretion of the HMG-CoA reductase inhibitor pravastatin and the conjugated steroid estradiol-17β-glucuronide is extensive (Takikawa et al., 1996; Yamazaki et al., 1997). Subsequent studies evaluating the vectorial transport of these compounds in MDCK cells expressing only OATP1B1 or only MRP2 indicated that transport by these proteins was negligible (Sasaki et al., 2002). However, when both OATP1B1 and MRP2 were expressed together, a significant transcellular flux of both compounds was demonstrated. Similar results were obtained for rifampin and several other xenobiotics in MDCK cells transfected with both OATP1B3 and MRP2 compared to the single transfectants (Cui et al., 2001). Therefore, in vitro systems that express all relevant transporters involved in the disposition of a given drug are desirable when making predictions regarding transport in vivo.
Isolated hepatocytes should express the full complement of hepatic transport proteins. Hepatocytes can be obtained from multiple species (including genetic or naturally occurring mutants) and can be used to identify both substrates and inhibitors of hepatic transport systems. Furthermore, when cultured in a sandwich configuration between two layers of extracellular matrix (e.g., collagen), hepatocytes repolarize (Dunn et al., 1991; Talamini et al., 1997), express relevant drug transport proteins at both the basolateral and the apical domain, and allow for investigation of vectorial drug transport, including canalicular excretion (Liu et al., 1999b; Annaert et al., 2001; Hoffmaster et al., 2004a).

D-Penicillamine<sup>2-5</sup>enkephalin (DPDPE; Fig. 1), a cyclic opioid peptide, is resistant to degradation by endogenous peptidases (Weber et al., 1991), and undergoes negligible biotransformation in rodents (Weber et al., 1991; Chen and Pollack, 1997). Similar to other small therapeutic peptides such as somatostatin analogs (Cathapermal et al., 1991) and renin inhibitors (Greenfield et al., 1989), DPDPE is cleared rapidly and extensively by the liver via biliary excretion (Chen and Pollack, 1997). Recent studies indicated that active transport processes at both the basolateral and canalicular membranes of the hepatocyte are responsible for the efficient excretion of several of these peptides (Yamada et al., 1998; Niinuma et al., 1999). DPDPE is transported by several members of the organic anion transporting polypeptide (Oatp) family (Gao et al., 2000; Cattori et al., 2001) based on studies conducted in cRNA microinjected Xenopus laevis oocytes expressing a single transporter. The relative impact of these transporters on the overall hepatobiliary disposition of DPDPE has not been determined.

P-glycoprotein (P-gp; Mdr1a/b) plays a major role in limiting the brain
penetration of DPDPE (Chen and Pollack, 1998; Chen and Pollack, 1999). Although P-gp is expressed in the hepatic canalicular membrane, in vivo studies in Mdr1a-deficient mice revealed no change in DPDPE systemic pharmacokinetics compared to Mdr1a-competent mice (Chen and Pollack, 1998). A subsequent study with the Mdr1a/b inhibitor GF120918 in Mdr1a/b-competent mice resulted in modulation of DPDPE blood-brain disposition with no alteration in DPDPE systemic pharmacokinetics (Chen and Pollack, 1999).

In order to understand the role of P-gp in the hepatic clearance of DPDPE, the disposition of DPDPE was studied in suspended and sandwich-cultured (SC) rat hepatocytes from control and Mrp2-deficient (TR-) rats. The transport proteins responsible for DPDPE hepatic uptake and biliary excretion were determined, and pharmacokinetic modeling was utilized to examine the relative rates of potential transport pathways to the overall hepatobiliary disposition of the peptide.

METHODS

Chemicals and Reagents. Unlabeled DPDPE was obtained from American Peptide Company (Sunnyvale, CA); ^3^H-DPDPE (44 Ci/mmol, >95% purity) and ^14^C-inulin (1.4 Ci/mmol, >98% purity) were purchased from Perkin Elmer Life Sciences (Boston, MA). Collagenase (type I, class I) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), insulin, penicillin-streptomycin, non-essential amino acids, and L-glutamine were obtained from Gibco/Invitrogen (Carlsbad, CA). Rat tail collagen (type I) and ITS^{+™}
were purchased from BD Biosciences (Bedford, MA). Polyclonal anti-Mdr1a/b (Ab-1) and monoclonal anti-Bcrp (BXP-21) antibodies were purchased from Oncogene/EMD Biosciences (Darmstadt, Germany). Monoclonal anti-rat Mrp2 (M2III-6) antibody was obtained from Alexis Biochemical (San Diego, CA). Monoclonal anti-rat Oatp1a4 [previously Oatp2; for new nomenclature see (Hagenbuch and Meier, 2004)] and monoclonal anti-rat actin antibodies were purchased from Chemicon (Temecula, CA). Polyclonal antibody against Oatp1a1 (previously Oatp1), raised in rabbits using a C-terminal fusion protein (aa 631-670) as the antigen (Jacquemin et al., 1994), was a generous gift of Dr. P. Meier (Zurich, Switzerland). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham (Piscataway, NJ). Complete™ protease inhibitor was acquired from Roche Diagnostics (Indianapolis, IN) and SuperSignal West Dura™ was acquired from Pierce Biotechnology (Rockford, IL). Dexamethasone, Hank’s Balanced Salt Solution (HBSS; with or without calcium), mineral oil, silicone oil, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade and were available from commercial sources.

Animals. Male Wistar (control) rats (220-300 g) from Charles River Laboratories Inc. (Raleigh, NC) or male Mrp2-deficient (TR-) rats bred at the University of North Carolina (250-300 g; breeding stock obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY) were used as liver donors for hepatocyte studies. Rats were housed in an alternating 12-hr light and dark cycle with rat chow and water provided ad libitum. All animals were allowed to acclimate for at least one week prior to experimentation. Rats were anesthetized with ketamine/xylazine (60/12 mg/kg i.p.). The Institutional Animal
Care and Use Committee at the University of North Carolina approved all animal procedures.

**Suspended Hepatocyte Isolation/Uptake Studies.** Rat hepatocytes (control or TR⁻) were isolated by collagenase perfusion as described previously (Brock and Vore, 1984). Viability, as determined by Trypan Blue exclusion, was 91-97% (mean =93%). Cells were suspended in modified HBSS containing 10 mM Tris/5 mM glucose (pH = 7.4) and stored on ice prior to conducting uptake studies (Brouwer et al., 1987). Isolated hepatocytes (n=3-5 livers, in duplicate or triplicate) were pre-incubated under 95%/5% O₂/CO₂ for 5 min at 37°C or 4°C before addition of ³H-DPDPE (250 nCi) and inhibitor or vehicle (0.1% DMSO). The following concentrations of inhibitors were chosen based upon reported affinities for the given active hepatic transport processes: 20 µM bromosulfophthalein [BSP; Oatp1a1, Oatp1b2 (previously Oatp4)], 10 µM digoxin (DIG; Oatp1a4), 1 mM deltorphin II (DELT II; Oatp1a1), 200 µM tetraethylammonium (TEA; Oct1), 1 mM p-aminohippurate (PAH; Oat2). Inhibitor concentrations were 4-10 fold above the reported Kₘ (assuming competitive inhibition) or Kᵢ for each transport process except for DIG (~40 fold) (Urakami et al., 1998; Cattori et al., 2001; Sugiyama et al., 2001). Aliquots of the suspension were removed at timed intervals (up to 5 min) and centrifuged immediately through silicone oil (ρ=1.03, adjusted with mineral oil). ³H-DPDPE in the cell pellet and supernatant was determined by liquid scintillation spectrometry. Adherent fluid volume was estimated with ¹⁴C-inulin (500 nCi) as previously described (Baur et al., 1975). Protein concentrations for individual hepatocyte suspensions were determined by the bicinchoninic acid (BCA) method (Smith et al., 1985).
Sandwich-Cultured Hepatocyte Isolation, Uptake, and Biliary Excretion Studies.

Rat hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Liu et al., 1998). Hepatocyte suspensions (viability 88-95%; mean 91%) were plated in 60-mm polystyrene dishes coated with gelled collagen at a density of ~1.25 x 10^5 cells/cm^2 in supplemented DMEM containing FBS and 0.1 µM dexamethasone. One to three hr post plating, hepatocyte cultures were shaken lightly and non-adherent cells were aspirated. Following 24 hr of incubation at 37°C, cells were overlaid with gelled collagen and cultures were maintained in FBS-free DMEM containing ITS™ with 0.1 µM dexamethasone for 72 hr (Day 4). Medium was replaced every 24 hr. As reported previously, depletion of calcium from the culture medium for 10 min opened the tight junctions that sealed the hepatocyte bile canalicular spaces, permitting quantification of substrate that had been excreted into the canalicular space (Liu et al., 1999b). On Day 4, cells were rinsed twice with 2 ml HBSS (37°C) and pre-incubated (10 min, 37°C) in 3 ml standard [cells+bile canaliculi (BC)] or Ca^{2+}-free HBSS [cells] to maintain or disrupt canalicular networks, respectively. SC hepatocytes (n=3 livers, in triplicate) were incubated with ^3H-DPDPE for 1-10 min in 3 ml standard HBSS, rinsed vigorously 3 times with HBSS (4°C), and lysed with 0.5% Triton-X 100. Non-specific binding was evaluated by including a blank collagen-coated dish in each group. Lysates were analyzed by liquid scintillation spectrometry. Total protein in each dish was determined by the BCA method (Smith et al., 1985). Accumulation data were normalized for total protein in each dish. The biliary excretion index (BEI; the percentage of accumulated ^3H-DPDPE in canalicular networks) and the in vitro biliary clearance (C_{lb, in vitro}) were calculated using B-CLEAR™ technology (Qualyst, Inc.,
Research Triangle Park, NC) based on the following equations (Liu et al., 1999c):

\[
BEI = \frac{(Accumulation_{\text{cells}} + BC - Accumulation_{\text{cells}})}{Accumulation_{\text{cells}} + BC} \times 100
\]

(Eqn. 1)

\[
Cl_{b, \text{in vitro}} = \frac{(Accumulation_{\text{cells}} + BC - Accumulation_{\text{cells}})}{(AUC_{0-T})}
\]

(Eqn. 2)

where \(AUC_{0-T}\) represents the product of the incubation time (T) and the initial \(^3\)H-DPDPE concentration in the medium. \(Cl_{b, \text{in vitro}}\) values were scaled per kg body weight using 0.2 g protein/g liver; 40 g liver/kg body weight (Seglen, 1976).

**Western Blotting.** Hepatocyte cultures were lysed with Complete™ protease inhibitor in phosphate buffered saline (PBS) with 1% SDS/1 mM EDTA and stored at -80°C until analysis. Protein (~30 µg) was loaded onto a NuPage 4-12% Bis-Tris gel (Invitrogen; Carlsbad, CA) without heat denaturation. Proteins were separated by electrophoresis and transferred onto a PVDF membrane. Membranes were blocked overnight at 4°C with 5% non-fat milk in Tris-buffered saline with 0.3% Tween-20 (TBS-T; pH = 7.4) and then incubated with primary antibody for 1 hr [anti-Oatp1a4 (1:1000), anti-Bcrp (1:750), anti-Mrp2 (1:2000), anti-Actin (1:10,000)] or 2 hr [anti-Oatp1a1 (1:2000), anti-Mdr1a/b (1:2000)]. Membranes were washed twice with TBS-T for 15 min, twice with TBS-T with 0.5% non-fat milk, probed with HRP-conjugated secondary antibodies (1:10000), and washed again as above before detection by chemiluminescence with SuperSignal West Dura™. Immunoreactive protein was visualized using a Versa-Doc 1000 molecular imager and Quantity One v.4.1 software (Bio-Rad Laboratories; Hercules, CA).

**Pharmacokinetic Modeling.** An integrated pharmacokinetic model was utilized to evaluate the mechanisms of \(^3\)H-DPDPE uptake in suspended rat hepatocytes. Models incorporating various combinations of linear and/or nonlinear parameters governing
DPDPE flux were fit to the average $^3$H-DPDPE (0.5-250 µM) uptake rate vs. concentration data in suspended hepatocytes. A compartmental modeling approach was utilized to describe the uptake and biliary excretion of $^3$H-DPDPE in SC rat hepatocytes. Data in SC rat hepatocytes in the presence of intact and disrupted bile canaliculi (0.5-250 µM) were fit simultaneously with the model described in Figure 2 by nonlinear least-squares regression (WinNonlin v.4.1, Mountain View, CA). The equations based on the model presented in Figure 2 are as follows:

$$V_{\text{medium}} \cdot \frac{dC_{\text{medium}}}{dt} = \left( K_{\text{uptake}} \cdot V_{\text{medium}} + \frac{V_{\text{m, uptake}}}{K_{\text{m, uptake}} + C_{\text{medium}}} \right) \cdot C_{\text{medium}} + \left( \frac{V_{\text{m, billefflux}}}{K_{\text{m, billefflux}} + C_{\text{hepatocyte}}} \right) \cdot C_{\text{hepatocyte}} + K_{\text{leakage}} \cdot X_{\text{bile}},$$

$$C_{\text{medium}}^0 = \frac{X_0}{V_{\text{medium}}} \quad \text{(Eqn. 3)}$$

$$V_{\text{hepatocyte}} \cdot \frac{dC_{\text{hepatocyte}}}{dt} = \left( K_{\text{uptake}} \cdot V_{\text{medium}} + \frac{V_{\text{m, uptake}}}{K_{\text{m, uptake}} + C_{\text{medium}}} \right) \cdot C_{\text{medium}} - \left( \frac{V_{\text{m, billefflux}}}{K_{\text{m, billefflux}} + C_{\text{hepatocyte}}} + K_{\text{bile}} \cdot V_{\text{hepatocyte}} \right) \cdot C_{\text{hepatocyte}},$$

$$C_{\text{hepatocyte}}^0 = 0 \quad \text{(Eqn. 4)}$$

$$\frac{dX_{\text{bile}}}{dt} = K_{\text{bile}} \cdot V_{\text{hepatocyte}} \cdot C_{\text{hepatocyte}} - K_{\text{leakage}} \cdot X_{\text{bile}}, \quad X_{\text{bile}}^0 = 0 \quad \text{(Eqn. 5)}$$

where variable and parameters are defined as in Figure 2. The protein content of each dish of hepatocytes should be proportional to the intracellular cytosolic volume of the hepatocytes; the average protein content (1.85 ± 0.1 mg/dish) was utilized as a surrogate for hepatocyte volume. Model selection was based upon Akaike’s Information Criterion (AIC), the coefficients of variation of the recovered parameters, the rank and condition number of the matrix of partial derivatives, and distribution of the residual error. A weighting scheme of $1/Y$ and the Gauss-Newton (Levenberg and Hartley) minimization method were utilized for all modeling routines.
Data Analysis. Accumulation data in suspended and SC rat hepatocytes were normalized to the total amount of protein in each tube or dish, respectively. Uptake clearance in suspended hepatocytes and SC hepatocytes was determined as the combination of two saturable processes, one operating in the linear range (Cl_{linear}):

\[
Cl_{uptake, \text{suspended}} = \left( \frac{V_{\text{max}}}{K_m + C} + Cl_{\text{linear}} \right)
\]

(Eqn. 6)

\[
Cl_{uptake, \text{SC}} = \left( \frac{V_{\text{m, uptake}}}{K_{m, \text{uptake}} + C} + k_{\text{uptake}} \cdot V_{\text{medium}} \right)
\]

(Eqn. 7)

Statistical significance was determined by Student’s t-test with Bonferroni’s correction for multiple comparisons, or two-way ANOVA with Tukey’s post hoc test, as appropriate. The criterion for significance in all cases was \( p < 0.05 \).

RESULTS

Uptake of \(^3\)H-DPDPE in Suspended Rat Hepatocytes. Preliminary experiments in suspended hepatocytes over 5 min indicated that uptake of \(^3\)H-DPDPE was linear over the initial 90 sec (data not shown). Therefore, all subsequent experiments were conducted through 1 min (15, 30, 45, and 60 sec) to evaluate the initial uptake rate of \(^3\)H-DPDPE into hepatocytes. The initial uptake velocity of 1 µM \(^3\)H-DPDPE in suspended hepatocytes from control and TR’ rats was 15.9 ± 2.1 and 15.5 ± 1.2 pmol/min/mg protein, respectively (Fig. 3). \(^3\)H-DPDPE accumulation in suspended hepatocytes at 4°C was less than 5% of the total observed at 37°C (data not shown). The initial uptake of 0.5-250 µM \(^3\)H-DPDPE in suspended hepatocytes involved parallel saturable and linear kinetic processes. Pharmacokinetic modeling yielded an apparent V_{max} = 385 ± 10
pmol/min/mg protein, $K_m = 28.9 \pm 2 \mu M$, and a linear clearance, $Cl = 1.93 \pm 0.04 \mu L/min/mg protein$ (Fig. 4A). The saturable component of $^3$H-DPDPE uptake at 0.5 µM was > 85% and decreased to ~44% at 250 µM (Fig. 4B). TEA, PAH, DMSO (as vehicle, 0.1%), or the absence of sodium (substitution with choline-based buffer) had no effect on the uptake of $^3$H-DPDPE in suspended hepatocytes (Fig. 5). The Oatp1a1 substrate DELT II, the Oatp1a4 substrate DIG, and the Oatp1a1/Oatp1b2 substrate BSP all significantly decreased the initial uptake of $^3$H-DPDPE (Fig. 5) over 60 sec; the combination of BSP and DIG further decreased $^3$H-DPDPE uptake to <12% of control. In suspended hepatocytes from TR− rats, transport modulators inhibited $^3$H-DPDPE uptake to the same extent as in control hepatocytes (data not shown).

**Expression of Hepatic Transport Proteins in Control and TR− SC Rat Hepatocytes.**

Immunoblot analysis revealed that Oatp1a1 expression was similar between control and TR− SC rat hepatocytes on Day 4 in culture (Fig. 6). Oatp1a4 expression was slightly lower in TR− SC rat hepatocytes. Expression of the canalicular proteins Mdr1a/b and Bcrp was similar between control and TR− SC hepatocytes. As expected, Mrp2 protein was detected in control SC rat hepatocytes and was absent in SC hepatocytes from TR− rats (Fig. 6).

**Accumulation of $^3$H-DPDPE in SC Rat Hepatocytes.** $^3$H-DPDPE accumulation in SC rat hepatocytes was determined over 10 min. Uptake and excretion were rapid, mimicking the *in vivo* situation (Fig. 7); at 10 min, a 0.5-µM $^3$H-DPDPE dose resulted in a BEI of 32 ± 6% and a $Cl_{b,\text{in vitro}}$ of 1.37 ± 0.41 µL/min/mg protein. Accumulation of 15 µM $^3$H-DPDPE at 10 min in cells + BC of SC hepatocytes from TR− rats was similar to control SC hepatocytes (Fig. 8). As expected, accumulation of $^3$H-DPDPE was
significantly lower in control SC hepatocytes with disrupted canalicular spaces; these data yielded a BEI of 37 ± 10% and a Clb,\textit{in vitro} of 1.48 ± 0.36 µL/min/mg protein. In contrast, ³H-DPDPE accumulation in TR− SC hepatocytes was not significantly affected by the disruption of bile canaliculi (BEI = 6 ± 4%; Clb,\textit{in vitro} = 0.23 ± 0.17 µL/min/mg protein).

**Pharmacokinetic Modeling of ³H-DPDPE Accumulation in SC Rat Hepatocytes.**

Accumulation of ³H-DPDPE (0.5-250 µM) was investigated over 10 min in Day 4 SC rat hepatocytes with intact (Fig. 9A) and disrupted (Fig. 9B) canalicular spaces. Based upon the pharmacokinetic parameters for ³H-DPDPE uptake in suspended hepatocytes (Fig. 4), the model structure in SC hepatocytes consisted of parallel saturable and linear uptake processes. The Kₘ for the saturable component of ³H-DPDPE uptake was obtained from suspended hepatocytes and fixed for modeling of the SC hepatocyte data. Based upon kinetic modeling of ³H-DPDPE in isolated perfused livers (Hoffmaster et al., 2004b), models consisting of basolateral efflux from the hepatocyte were considered. The remaining model structure was determined by evaluating various combinations of first-order and nonlinear processes describing canalicular excretion and leakage from the bile space (Fig. 2); appropriate equations were fit to the mean ³H-DPDPE (0.5-250 µM) accumulation vs. time data plotted in Fig. 2. All models were of full rank, and the condition number for each model was less than 10⁶, suggesting sufficient data to estimate all parameters and a low degree of co-linearity between parameters. Based on visual inspection of the residuals, coefficients of variation of the recovered parameters, and AIC values, the model structure presented in Fig. 2 provided the best description of the entire data set. This model scheme incorporated parallel linear and saturable processes for
basolateral uptake of $^3$H-DPDPE ($k_{uptake}$, $K_{m,uptake}$, $V_{m,uptake}$), a saturable process for $^3$H-DPDPE basolateral excretion ($K_{m,blefflux}$, $V_{m,blefflux}$), a first-order rate constant ($k_{bile}$) associated with $^3$H-DPDPE flux into the bile canalicular spaces, and a first-order rate constant ($k_{leakage}$) for $^3$H-DPDPE leakage from the bile canalicular spaces. Estimates of kinetic parameters obtained from the model are shown in Table 1; the fit of the model to the data is depicted in Fig. 9. The model recovered good estimates of all parameters with the exception of $K_{m,blefflux}$. Several attempts to recover this parameter resulted in an estimate (with a very high coefficient of variation) well below cellular concentrations achieved under the experimental conditions examined in this study. Therefore, the model treated basolateral efflux of $^3$H-DPDPE as a zero-order process operating at the $V_{max}$ for transport over the dose range studied; no estimate for $K_{m,blefflux}$ is reported.

DISCUSSION

$^3$H-DPDPE was taken up rapidly in suspended hepatocytes, consistent with rapid hepatic uptake and biliary excretion of $^3$H-DPDPE in vivo (within 5 minutes) following an intravenous dose (Chen and Pollack, 1997). Pharmacokinetic modeling revealed that saturable and linear processes were involved in $^3$H-DPDPE uptake in suspended hepatocytes over the dose range studied. The apparent $K_m$ for this process (28.9 µM) was similar to the individual $K_m$ values reported in cRNA microinjected oocytes [Oatp1a1, 48 µM; Oatp1a4, 19 µM; Oatp1b2, 22 µM] (Cattori et al., 2001). However, an apparently linear component of $^3$H-DPDPE uptake was maintained at concentrations well above the reported $K_m$ values for individual Oatp isoforms. To assess sodium-dependent $^3$H-
DPDPE transport in the liver, choline-based buffer was substituted for sodium-based buffer in suspended hepatocytes. As expected, $^{3}$H-DPDPE uptake was sodium-independent, indicating that the sodium-dependent taurocholate co-transporting polypeptide (Ntcp) was not involved in $^{3}$H-DPDPE uptake into hepatocytes. The lack of inhibition by PAH and TEA indicated that $^{3}$H-DPDPE is not transported by Oat or Oct in the liver (Urakami et al., 1998; Sugiyama et al., 2001). However, inhibition by DIG, an Oatp1a4 substrate [$K_m = 0.24 \mu M$; (Sugiyama et al., 2001)] and DELT II, an Oatp1a1 substrate [$K_m = 137 \mu M$; (Gao et al., 2000; Cattori et al., 2001)] suggested that these individual Oatp-mediated transport processes are important in the uptake of $^{3}$H-DPDPE in suspended hepatocytes. Inhibition of $^{3}$H-DPDPE uptake by BSP, an Oatp1a1 and Oatp1b2 substrate [$K_m = 1.5$ and $1.1 \mu M$, respectively; (Cattori et al., 2001)], coupled with further inhibition by DIG resulting in ~90% decrease in $^{3}$H-DPDPE uptake, suggests that the hepatic uptake of $^{3}$H-DPDPE is mediated by multiple Oatp isoforms.

Interestingly, at 250 $\mu M$ $^{3}$H-DPDPE (5 times higher concentration than the reported $K_m$ values for $^{3}$H-DPDPE in cRNA microinjected oocytes) an apparently linear process accounted for greater than 50% of the opioid uptake in suspended hepatocytes. Since passive uptake of $^{3}$H-DPDPE is negligible, the linear portion of uptake may be due to a saturable process with a $K_m$ that exceeds the concentrations of $^{3}$H-DPDPE utilized in these studies (i.e. the active process operating in the linear range).

As demonstrated in previous in vivo studies, $^{3}$H-DPDPE appeared to be sensitive to P-gp modulation by the P-gp inhibitor GF120918 at the blood-brain barrier but not in the liver (Chen and Pollack, 1998; Chen and Pollack, 1999). One explanation for these observations is that in the liver, $^{3}$H-DPDPE and the inhibitor must cross the basolateral
membrane of the hepatocyte in order to access the substrate/inhibitor recognition site for P-gp. Therefore, it is possible that local concentrations of the inhibitor in the liver were inadequate to modulate P-gp-mediated biliary excretion of $^3$H-DPDPE. An alternative explanation for the lack of an apparent influence of P-gp on the systemic disposition of $^3$H-DPDPE is that P-gp may not be the primary transporter responsible for biliary excretion of the opioid peptide \textit{in vivo}.

To investigate the dynamic process of $^3$H-DPDPE biliary excretion, SC rat hepatocytes were used to study mechanisms of hepatic uptake and biliary excretion. SC hepatocytes repolarize, establish canalicular networks, and express functional transport proteins at the appropriate domain of the cell (Liu et al., 1999b; Hoffmaster et al., 2004a). Similar to \textit{in vivo} studies, uptake and biliary excretion of $^3$H-DPDPE in Day 4 SC rat hepatocytes was rapid and extensive, consistent with efficient transport processes at both the basolateral and apical domains of the hepatocyte. $\text{Cl}_{\text{b,in vitro}}$ of 15 $\mu$M $^3$H-DPDPE scaled per kg body weight determined in this study (11.8 ± 2.9 ml/min/kg) was similar to the value previously reported in SC rat hepatocytes [12.6 ± 2.2 ml/min/kg (Liu et al., 1999a)] and correlated well with \textit{in vivo} observations (Chen and Pollack, 1996).

However, when evaluated in SC rat hepatocytes from TR$^-$ rats, both BEI and $\text{Cl}_{\text{b,in vitro}}$ were significantly decreased compared to control, suggesting a major role for Mrp2 in $^3$H-DPDPE biliary excretion. Uptake of $^3$H-DPDPE in suspended hepatocytes from TR$^-$ rats was identical to that in control hepatocytes, and initial uptake in suspended TR$^-$ rat hepatocytes was inhibited by transport modulators to the same extent as control hepatocytes. Accumulation of $^3$H-DPDPE in Day 4 TR$^-$ SC hepatocytes with intact canalicular networks (cells + BC) was identical to control hepatocytes, and the expression
of the other established xenobiotic canalicular transport proteins, P-gp and Bcrp, was identical in Day 4 SC hepatocytes from both control and TR- rats. Therefore, Mrp2 plays a significant role in the biliary excretion of $^{3}$H-DPDPE.

In order to better understand the overall hepatic disposition of $^{3}$H-DPDPE, a comprehensive pharmacokinetic analysis was performed on the time course of accumulation in SC rat hepatocytes with intact and disrupted canalicular networks. Based on the rapid accumulation of $^{3}$H-DPDPE in the bile canaliculi of SC rat hepatocytes, a three-compartment model was utilized to describe the accumulation of 0.5-250 µM $^{3}$H-DPDPE in SC rat hepatocytes. Model structure was based in part on kinetic modeling results from suspended hepatocytes in which $^{3}$H-DPDPE uptake was mediated by a combination of saturable and linear processes. $^{3}$H-DPDPE disposition in isolated perfused livers indicated the presence of a high affinity, low capacity basolateral efflux mechanism (Hoffmaster et al., 2004b). Therefore, for the final model structure, only those models that combined nonlinear and linear kinetic processes for uptake in SC hepatocytes and a nonlinear basolateral efflux process were considered; different combinations of the remaining processes governing $^{3}$H-DPDPE flux into and out of each compartment [including possible leakage from the bile space, based upon previous observations (Liu et al., 1999c)] were explored. Modeling of $^{3}$H-DPDPE uptake in suspended hepatocytes recovered an apparent $K_m$ (28.9 µM) for the saturable uptake process. Because the affinity of the saturable uptake process should not differ between suspended and SC hepatocytes, the $K_m$ for uptake was fixed for modeling of the SC data. Efficient uptake and biliary excretion were observed over the entire dose range, indicating sequential high-capacity processes for vectorial transport of $^{3}$H-DPDPE.
When normalized per mg protein, uptake clearance (Eqn. 7) in SC hepatocytes correlated well with uptake clearance (Eqn. 6) in suspended hepatocytes (11.4 µL/min/mg protein vs. 15.3 µL/min/mg protein, respectively). Pharmacokinetic modeling was consistent with a high affinity basolateral efflux process for $^3$H-DPDPE in SC hepatocytes. Although modeling could not recover an accurate estimate of the $K_m$ for this process, the recovered estimates were consistently below hepatocyte concentrations observed in these studies, indicating that efflux occurred as a zero-order process. Mrp2 substrates often are substrates for anionic transporters on the basolateral membrane [e.g. Mrp3 (Kool et al., 1999; Xiong et al., 2002) or Mrp4 (Rius et al., 2003)]. Mrp3 expression is increased in TR$^-$ rats and may prevent the accumulation of organic anions in the liver in the absence of functional Mrp2 (Xiong et al., 2002). It is possible that Mrp3, potentially coupled with Mrp4 or some other basolateral transport protein, is responsible for the basolateral flux of $^3$H-DPDPE from the liver back across the basolateral membrane. However, the specific transport protein(s) responsible for the basolateral excretion of $^3$H-DPDPE, and the impact of this process on $^3$H-DPDPE hepatobiliary disposition in both control and TR$^-$ rats, are the subject of continuing investigations. Similar to previous observations in SC rat hepatocytes, kinetic modeling supported a first-order leakage from the bile space (Liu et al., 1999c). In vivo and in vitro, bile motility is the result of multiple cycles of contraction and relaxation of the canalicular lumen which may cause the pseudo first-order release of $^3$H-DPDPE back into the medium (Phillips et al., 1982; Watanabe et al., 1991).

In transfected systems used to study drug transport, protein expression often is maximized to obtain the highest transport activity possible. Although protein levels and
function may correspond to data obtained in intact hepatocytes (Kouzuki et al., 1998), often this arbitrary expression level results in transport data that are difficult to correlate to other *in vitro* or *in vivo* experimental systems (Kouzuki et al., 1999). Based upon the analysis of the fraction of transport due to nonlinear processes, the saturable portion of uptake clearance in the linear range of $^3$H-DPDPE uptake $\left( \frac{V_{\text{m, uptake}}}{K_{\text{m, uptake}}} \right)$ accounts for ~87% of the total uptake in suspended hepatocytes and only ~56% in Day 4 SC hepatocytes. In SC rat hepatocytes, Oatp1a1 expression remained constant over days in culture whereas Oatp1a4 decreased slightly (Hoffmaster et al., 2004a). The slight decrease in Oatp1a4 expression could explain, in part, the fractional decrease in the uptake clearance of $^3$H-DPDPE in Day 4 SC rat hepatocytes. Due to lack of availability of specific antibody, detection of Oatp1b2 was not possible. Therefore, the contribution of Oatp1b2 to the decrease in $^3$H-DPDPE uptake in SC hepatocytes cannot be ruled out.

In conclusion, the hepatic uptake of $^3$H-DPDPE is mediated by multiple isoforms of Oatp. Mrp2 plays a substantial role in the biliary excretion of $^3$H-DPDPE. Pharmacokinetic modeling was consistent with excretion of this opioid peptide across the basolateral membrane of the hepatocyte. These data highlight the complexities of hepatobiliary drug transport, and underscore the need to utilize a variety of experimental strategies in elucidating the processes involved in hepatobiliary disposition.

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Footnotes

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Figure Legends.

Figure 1. Structure of the metabolically stable opioid peptide DPDPE

Figure 2. Model scheme describing the accumulation of $^3$H-DPDPE in SC rat hepatocytes. The optimal model structure was determined by evaluating models with saturable and linear uptake process and saturable basolateral efflux, with different combinations of first-order and nonlinear pharmacokinetic parameters describing canalicular excretion and leakage from the bile space. $X_0$, initial bolus dose of $^3$H-DPDPE into the medium compartment; $C_{\text{medium}}$, concentration of $^3$H-DPDPE in medium; $V_{\text{medium}}$, volume of the medium (3 mL); $C_{\text{hepatocyte}}$, concentration of $^3$H-DPDPE in hepatocyte; $V_{\text{hepatocyte}}$, volume of the cell (1.85 mg protein/dish); $X_{\text{bile}}$, amount of $^3$H-DPDPE in the bile canaliculi; $K_{\text{uptake}}$, first-order rate constant for basolateral uptake; $K_{m,\text{uptake}}$, Michaelis-Menten constant for basolateral uptake; $V_{m,\text{uptake}}$, maximal velocity for basolateral uptake; $K_{\text{bile}}$, first-order rate constant for canalicular excretion; $K_{m,\text{blefflux}}$, Michaelis-Menten constant for basolateral efflux from cell to media; $V_{m,\text{blefflux}}$, maximal velocity for basolateral efflux from cell to medium; $K_{\text{leakage}}$, first-order rate constant for leakage from bile canalicul space to medium.

Figure 3. Uptake of 1 µM $^3$H-DPDPE into suspended hepatocytes from control (◆, solid line) and TR− (■, dashed line) rats over 60 sec. Lines represent linear regression of the data ($r^2 > 0.997$). Data are presented as mean ± S.E.M. (n=5 livers, 2-3 determinations/liver).
Figure 4. Initial uptake rate of $^3$H-DPDPE (0.5-250 µM; 15-60 sec) in suspended rat hepatocytes (A). A pharmacokinetic model with one linear (---; $C_{\text{linear}} = 1.93$ µL/min/mg protein) and one saturable process (——; $V_{\text{max}} = 385$ pmol/min/mg protein, $K_m = 28.9$ µM) was fit to the data. Data represent mean ± S.E.M. (n=3 livers, 2-4 measurements/liver). Fractional contribution of the saturable uptake component (φ) to $^3$H-DPDPE uptake in suspended rat hepatocytes (B).

Figure 5. Initial uptake of 1 µM $^3$H-DPDPE (15-60 sec) in suspended rat hepatocytes in the presence of transport modulators. Data represent mean % ± S.E.M. (n=3-4 livers, 2-3 measurements/liver); *$p < 0.05$ vs. control.

Figure 6. Representative Western blots showing expression of Mdr1a/b, Mrp2, Bcrp, Oatp1a1, Oatp1a4, and actin in SC hepatocytes from control (C) and TR$^-$ rats on Day 4 in culture.

Figure 7. (A) Accumulation of 15 µM $^3$H-DPDPE over time in Day 4 control SC hepatocytes with intact (◆, cell + BC accumulation) and disrupted (◇, cellular accumulation) bile canaliculi. The difference between the closed and open symbols represents the amount of $^3$H-DPDPE excreted into the bile canaliculi. Data represent mean ± S.E.M. (n=3 livers in triplicate); $p < 0.05$ at 5 and 10 minutes. (B) Accumulation of 15 µM $^3$H-DPDPE over 10 min in SC rat hepatocytes from control and TR$^-$ rats with intact (solid, cell + BC accumulation) and disrupted (hatched, cellular accumulation) bile
canaliculi. The difference between the closed and open bars represents the amount of $^3$H-DPDPE excreted into the bile canaliculi. Data are presented as mean ± S.E.M. (n=5 livers, in triplicate); *p < 0.05 compared to hepatocytes with intact bile canaliculi.

Figure 8. Accumulation of $^3$H-DPDPE at 250 µM (●), 100 µM (■), 50 µM (▲), 15 µM (●), 5 µM (■, inset), 1 µM (●, inset), and 0.5 µM (▼, inset) over 10 min in Day 4 SC rat hepatocytes with intact (A; closed symbols, cells + BC accumulation) and disrupted (B; open symbols, cellular accumulation) bile canaliculi. Data are presented as mean ± S.E.M. (n=3 livers, in triplicate). Regression lines represent the best fit of the pharmacokinetic model to the data (Figure 9, Table 1).
Table 1. Kinetic parameters obtained from nonlinear least-squares regression analysis of the average $^3$H-DPDPE accumulation vs. time data (Fig. 8) using the model equations (3-5) based on the scheme depicted in Fig. 9. Average $^3$H-DPDPE determinations from 3 livers in triplicate were utilized for estimation of kinetic parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimate</th>
<th>CV%</th>
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<tr>
<td>$k_{\text{uptake}}$ (min$^{-1}$)</td>
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<tr>
<td>$K_{\text{m,uptake}}$ (µM)</td>
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<td>n/a$^a$</td>
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<td>$V_{\text{m,uptake}}$ (pmol • min$^{-1}$)</td>
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<td>$V_{\text{m,blefflux}}$ (pmol • min$^{-1}$)</td>
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<td>$k_{\text{bile}}$ (min$^{-1}$)</td>
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<tr>
<td>$k_{\text{leakage}}$ (min$^{-1}$)</td>
<td>1.6</td>
<td>34</td>
</tr>
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</table>

$^a$parameter estimate fixed from suspended hepatocyte results
Figure 1
Figure 2

$X_o$

$C_{medium}$

$V_{medium}$

$V_{m,uptake}$

$K_{m,uptake}$

$k_{uptake}$

$V_{m,blefflux}$

$K_{m,blefflux}$

$C_{hepatocyte}$

$V_{hepatocyte}$

$k_{bile}$

$k_{leakage}$

$X_{bile}$

$C_{medium}$

$V_{medium}$
Figure 3
Figure 4

A

Uptake Rate (pmol/min/mg protein)

0 50 100 150 200 250

3H-DPDPE Concentration (µM)

B

ϕ

0 50 100 150 200 250

3H-DPDPE Concentration (µM)
Figure 5

% Control Uptake

Control | Choline Buffer | BSP (20 µM) | DIG (10 µM) | DIG (10 µM) + BSP (20 µM) | DELT II (1 mM) | TEA (200 µM) | PAH (1 mM)

* indicates significant difference from control.
Figure 6

**Basolateral Membrane**

- Oatp1a1 (~80 kDa) →
- Oatp1a4 (~80 kDa) →
- Actin (~43 kDa) →

**Canalicular Membrane**

- Mrp2 (~190 kDa) →
- Mdr1a/b (~170 kDa) →
- Bcrp (~70 kDa) →
- Actin (~43 kDa) →

C  TR⁻  C  TR⁻
Figure 7

A

Accumulation (pmol/mg protein)

Time (min)

B

Accumulation (pmol/mg protein)

Control   TR⁻
Figure 8

A

B