Hepatobiliary Disposition of a Drug/Metabolite Pair: Comprehensive Pharmacokinetic Modeling in Sandwich-Cultured Rat Hepatocytes


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ABSTRACT

The hepatobiliary disposition of xenobiotics may involve passive and/or active uptake, metabolism by cytochromes P450, and excretion of the parent compound and/or metabolite(s) into bile. Although in vitro systems have been used to evaluate these individual processes discreetly, mechanistic in vitro studies of the sequential processes of uptake, metabolism, and biliary or basolateral excretion are limited. The current studies used sandwich-cultured (SC) rat hepatocytes combined with a comprehensive pharmacokinetic modeling approach to investigate the hepatobiliary disposition of terfenadine and fexofenadine, a model drug/metabolite pair. The metabolism of terfenadine and the biliary excretion of terfenadine and fexofenadine were determined in control and dexamethasone-treated SC rat hepatocytes. Dexamethasone (DEX) treatment increased the formation rates of the terfenadine metabolites azacyclonol and fexofenadine ∼20- and 2-fold, respectively. The biliary excretion index (BEI) of fexofenadine, when generated by terfenadine metabolism, was not significantly different from the BEI of preformed fexofenadine (15 ± 2% versus 19 ± 2%, respectively). Pharmacokinetic modeling revealed that the rate constant for hepatocyte uptake was faster for terfenadine compared with preformed fexofenadine (2.5 versus 0.08 h⁻¹, respectively), whereas the biliary excretion rate constant for preformed fexofenadine exceeded that of terfenadine (0.44 versus 0.039 h⁻¹, respectively). Interestingly, the rate constants for basolateral excretion of terfenadine and fexofenadine were comparable (3.2 versus 1.9 h⁻¹, respectively) and increased only slightly with DEX treatment. These studies demonstrate the utility of the SC hepatocyte model, coupled with pharmacokinetic modeling, to evaluate the hepatobiliary disposition of generated metabolites.

Biliary clearance is a predominant route of elimination for many compounds, including bile salts, organic anions, and organic cations. In recent years, the mechanisms involved in the hepatobiliary system have been elucidated. After transport into the hepatocyte, many compounds undergo phase I and/or phase II metabolism before excretion into bile (by canalicular transport proteins) or sinusoidal blood (by basolateral transport proteins). Pang and coworkers have clearly established that, in some cases, the hepatic disposition of an administered preformed metabolite may be very different from a metabolite generated in the intact organ (Pang et al., 1984; Tirona and Pang, 1996). However, differences in disposition of a generated versus preformed metabolite are by no means assured and must be demonstrated on a case-by-case basis.

Recent advances in molecular biology have allowed the mechanistic investigation of discrete transport or metabolic processes in isolation through administration of parent drug or metabolite to a variety of transfected, cell-based expression systems (Wrighton et al., 1995; Mizuno et al., 2003). Cummins et al. (2004) explored the interplay between transport and metabolism of midazolam and sirolimus in Caco-2 cells transfected with CYP3A4, whereas Sasaki et al. (2004) used double-transfected (OATP2/MRP2) Madin-Darby canine kidney cells to explore the potential rate-limiting step in the biliary disposition of a variety of substrates. The ability to quantify the impact of both hepatic transport and metabolism of drug candidates in a single in vitro system would offer significant advantages over existing methodologies.

ABBRiVATIONS: Oatp/OATP, organic anion transporting polypeptide; Mrp/MRP, multidrug resistance-associated protein; SC, sandwich-cultured; DEX, dexamethasone; DMEM, Dulbecco’s modified Eagle’s medium; HBSS, Hanks’ balanced salt solution; BEI, biliary excretion index; BC, bile-canaliculi; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; Cₘₜ in vitro, in vitro biliary clearance; AUC, area under the curve; TER, terfenadine; FEX, fexofenadine; AZA, azacyclonol; pre, preformed; gen, generated.
Primary rat hepatocytes have been used to explore both hepatic metabolism and transport processes (Li, 1997; Liu et al., 1999a; Kostrubsky et al., 2003). Rat hepatocytes cultured between two layers of gelled collagen in a sandwich configuration form extensive, functional bile canalicular networks (LeCluyse et al., 1994). Moreover, an in vitro/in vivo correlation of biliary clearance for nonmetabolically labile substrates such as methotrexate, [D-Pen2,5]enkephalin, and taurocholate (Li et al., 1999a) has been established in SC rat hepatocytes. When cultured over time, the expression of some phase I metabolizing enzymes in rat hepatocytes is diminished relative to the in vivo condition. However, dexamethasone (DEX) induces cytochrome P450s, including the role of CYP3A in the formation of the azacyclonol metabolite of terfenadine, has been well documented in microsomes and S9 fractions (Yun et al., 1993; Raeissi et al., 1999). The metabolism of terfenadine (Fig. 1) by cytochrome P450 CYP3A1/2 in primary rat hepatocytes and is useful in maintaining CYP3A1/2 activity in SC rat hepatocytes (LeCluyse et al., 1996; Li and Jurima-Romet, 1997). The function and expression of Oatp1, Oatp2, and Mdr1a/b in SC rat hepatocytes has been demonstrated (Annaert et al., 2001; Hoffmaster et al., 2004), and the effect of DEX treatment on the expression and function of these and other transport proteins (e.g., Bsep, Mrp2, and Ntcp) in SC rat hepatocytes also has been reported (Turncliff et al., 2004).

The metabolic and transport processes that mediate the hepatobiliary disposition of terfenadine and the terfenadine carboxylate metabolite fexofenadine have been described previously (Jurima-Romet et al., 1995; Cvetkovic et al., 1999). The metabolism of terfenadine (Fig. 1) by cytochrome P450s, including the role of CYP3A in the formation of the azacyclonol metabolite of terfenadine, has been well documented in microsomes and S9 fractions (Yun et al., 1993; Jurima-Romet et al., 1994; Raeissi et al., 1999). Hait et al. (1993) first suggested the involvement of P-glycoprotein in terfenadine transport. Oatp1a1 and Oatp1a4 probably mediate the hepatic uptake of fexofenadine in rat liver (Cvetkovic et al., 1999). Fexofenadine is a known Mdr1a/b substrate (Cvetkovic et al., 1999). However, recent work by Tahara et al. (2005) suggests that fexofenadine may be a substrate for multiple species-specific transport mechanisms. These observations further support the need for an in vitro model representative of in vivo hepatic transport and metabolism.

Pharmacokinetic modeling of in vivo or in vitro data can provide additional insight into the disposition of drugs and drug candidates. For example, modeling and simulation of verapamil disposition in Caco-2 cells suggested a nonlinear relationship between the extent of drug metabolism and the extent of drug transport (Johnson et al., 2003). Utilization of strategies to predict the extent of biliary clearance of parent compound and/or metabolite and to identify rate-limiting steps in hepatobiliary disposition for the purpose of predicting potential sites of drug interactions is becoming increasingly important in the drug development process.

In the current studies, terfenadine and fexofenadine were selected as a model drug/metabolite pair to examine differences in the hepatobiliary disposition of a metabolite when generated in vitro versus administered preformed in SC rat hepatocytes. Pharmacokinetic modeling of data were used to elucidate relative rates of transport and metabolic processes for terfenadine, fexofenadine, and azacyclonol. The hepatobiliary disposition of preformed fexofenadine was compared with fexofenadine generated by metabolism of terfenadine. This represents the first report using an in vitro model to examine the kinetics of hepatic uptake and biliary excretion of a drug/metabolite pair, including both preformed and generated metabolite.

**Materials and Methods**

**Chemicals.** Collagenase (type 1, class 1) was obtained from Worthington Biochemical Corporation (Freehold, NJ). Dulbecco's modified Eagle's medium (DMEM) and insulin were purchased from Invitrogen/GIBCO (Carlsbad, CA). ITS* culture supplement and rat tail collagen (type I) were purchased from BD Biosciences (Bedford, MA). DMEM (10%), penicillin-streptomycin solution, fetal bovine serum, taurocholic acid, DEX, Triton X-100, soybean trypsin inhibitor, terfenadine, fexofenadine, and loperamide were purchased from Sigma-Aldrich (St. Louis, MO). Azacyclonol (o-o-diphenyl-4-piperidine-methanol) was purchased from Arcos Organics (Fisher Chemical, Pittsburgh, PA). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

**Animals.** Male Wistar rats (270–325 g) obtained from Charles River Laboratories (Raleigh, NC) were used for hepatocyte isolation from whole liver. Animals had free access to water and food before surgery. All animal procedures were compliant with the guidelines of the University of North Carolina Institutional Animal Care and Use Committee.

**Isolation and In Vitro Culture of Primary Rat Hepatocytes.** Hepatocytes were isolated from male Wistar rats using a collagenase perfusion and were cultured as described previously (Liu et al., 1999b); hepatocyte viability was >85% as determined by trypan blue exclusion. In brief, hepatocytes were plated at a density of 3.0 × 10^6 cells/dish in 60-mm dishes previously coated with 0.2 ml of rat tail collagen type 1 solution (1.5 mg/ml, pH 7.4). After 24 h (day 1), medium was aspirated, and cells were overlaid with 200 µl of rat tail collagen type 1 solution (1.5 mg/ml, pH 7.4) to achieve a sandwich configuration. Thereafter, medium (DMEM supplemented with 1% ITS*, penicillin/streptomycin, l-glutamine, nonessential amino

![Fig. 1. Scheme of metabolic pathways of terfenadine.](image-url)
SC Rat Hepatocyte DEX Treatment. DEX (100 μM) or vehicle (dimethyl sulfoxide) was added to the culture medium beginning on day 2. The final concentration of dimethyl sulfoxide in culture medium was <0.1%. SC rat hepatocytes were treated with DEX or vehicle for 48 h.

Metabolism of Terfenadine and Fexofenadine in SC Rat Hepatocytes. On day 4, SC rat hepatocytes were rinsed with warm DMEM before addition of 3 ml of Hanks’ balanced salt solution (HBSS) (or early time points) or DMEM (for longer time points) containing terfenadine (5 μM) or fexofenadine (5 μM). HBSS samples (1 ml) were obtained at 0, 0.25, 0.5, and 1 h, whereas DMEM samples (1 ml) were obtained at 2, 4, 8, and 12 h. All samples were stored at −20°C until analysis. Three dishes of hepatocytes were sampled at each time point. After removal of the remaining incubation medium and rinsing with ice-cold HBSS (3 x 3 ml), methanol (1 ml) was added to each culture dish, and the cells were scraped off of the dishes and stored at −20°C until analysis.

Biliary Excretion of Terfenadine and Fexofenadine. The biliary excretion index (BEI) was calculated using B-CLEAR technology (Qualyst, Inc., Raleigh, NC) by dividing the difference in substrate accumulation between standard HBSS (cellular plus canalicular accumulation) and Ca²⁺-free HBSS (cellular accumulation) by the accumulation in standard HBSS (Liu et al., 1999b). In brief, day 4 SC rat hepatocytes were rinsed twice with 3 ml of warm standard HBSS (cells+bile canaliculi (BC)) or Ca²⁺-free HBSS [cells] and incubated with 3 ml of the same buffer for 10 min at 37°C. SC rat hepatocytes were incubated with 3 ml of terfenadine (5 μM) or preformed fexofenadine (5 μM) for 0.25, 0.5, and 1 h in standard HBSS. At each time point, an HBSS sample (1 ml) was removed for analysis; the remainder was aspirated. Thereafter, SC rat hepatocytes were rinsed vigorously three times with 3 ml of ice-cold HBSS. Hepatocytes were lysed with 1 ml of methanol. All dishes were scraped, and lysates were stored at −20°C until analysis by HPLC.

Non-specific Binding and Protein Determination. Accumulation data were corrected for non-specific binding of the relevant substrate to gelled collagen-coated hepatocyte-free culture dishes at each time point. All data were normalized to the average protein content in ~10 dishes/experiment (lysed with Triton X-100) as determined with a commercially available kit (BCA Protein Assay; Pierce Chemical Co., Rockford, IL), based on instructions provided by the manufacturer. Bovine serum albumin was used as the standard (supplied with the BCA kit).

HPLC Analysis. The amount of terfenadine and fexofenadine in both incubation medium and cell lysates from SC rat hepatocyte accumulation experiments was determined by HPLC with fluorescence detection using a method modified from Coutant et al. (1991). In brief, after centrifugation at 3000 g, 20°C until analysis. Three dishes of hepatocytes were sampled at each time point. All data were normalized to the average protein content in ~10 dishes/experiment (lysed with Triton X-100) as determined with a commercially available kit (BCA Protein Assay; Pierce Chemical Co., Rockford, IL), based on instructions provided by the manufacturer. Bovine serum albumin was used as the standard (supplied with the BCA kit).

LC/MS/MS Analysis. Due to the limited sensitivity of the HPLC method, concentrations of azacyclonol were determined by LC/MS/MS in a subset of samples. Concentrations of terfenadine and fexofenadine were also quantitated in these samples for comparison to concentrations determined by HPLC. Samples were prepared as described above. An aliquot of supernatant was transferred to an HPLC vial to which 250 μl of methanol containing internal standard (20 ng/ml loperamide) was added; samples were mixed by vortex for 30 s. Samples were injected (Agilent 1100 96-well plate autosampler) onto a 2.0 x 30-mm, 4 μM Synergi Max-RP column (Phenomenex, Torrance, CA) maintained at room temperature with a run time of 4 min. Analytes were eluted with a high-pressure linear gradient program consisting of 10 mM ammonium acetate, pH 6.8 (A) and methanol (B) delivered by an Agilent 1100 series binary pump (flow rate = 0.75 ml/min) as follows: 20% B to 95% B over 2 min, hold 95% B for 1 min, and returned rapidly to 20% B over 0.1 min. The system was allowed to re-equilibrate for 1 min. The entire column effluent was diverted from the Turbo Ionspray probe of an API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) for the first and last minute. Terfenadine, fexofenadine, azacyclonol, and loperamide (internal standard) were detected using selected reaction monitoring. The lower and upper limits of quantification of terfenadine, azacyclonol, and fexofenadine were 1 and 100 ng/ml, respectively. Terfenadine and fexofenadine concentrations determined by LC/MS/MS were in good agreement with results from the HPLC assay.

Data Analysis. The amount of terfenadine, fexofenadine, and azacyclonol in medium and cell lysates was determined in triplicate from individual livers. The BEI (%) of terfenadine, generated fexofenadine, and preformed fexofenadine was determined as:

\[
\text{BEI} = \left( \frac{\text{Accumulation}_{\text{cells} + \text{BC}} - \text{Accumulation}_{\text{cells}}} {\text{Accumulation}_{\text{cells} + \text{BC}}} \right) \times 100 \quad (1)
\]

BEI was calculated as a mean of BEI values from the individual livers and is expressed as mean ± S.E.M. The in vitro biliary clearance (CLB in vitro) of terfenadine and preformed fexofenadine was estimated by simultaneous modeling of accumulation data in cells + BC (shaded compartments represent BC and cells).
calculated as described previously (Liu et al., 1999a) with modifications as described in Appendix I. Area under the curve (AUC) values were calculated by either the linear or log-linear trapezoidal methods as noted in Appendix I.

Disposition of preformed Fexofenadine in SC Rat Hepatocytes. Estimates of the rate constants associated with the disposition of preformed fexofenadine in control (medium) and DEX-treated SC rat hepatocytes were fixed in model 2 to simulate the metabolism and biliary excretion of preformed fexofenadine. Estimates of the rate constants associated with the disposition of preformed fexofenadine in control (medium) and DEX-treated SC rat hepatocytes (Table 1). The BEI and ClB in vitro values for preformed fexofenadine were not significantly different between control and DEX-treated SC rat hepatocytes (Table 2); where appropriate, two-way analysis of variance was followed by a Tukey test. The criterion for statistical significance was $P < 0.05$.

Results

Nonspecific Binding of Terfenadine and Fexofenadine. The nonspecific binding of both terfenadine and fexofenadine to gelled collagen-coated polystyrene culture dishes was $<5\%$ of the initial mass and therefore was assumed to be insignificant.

Disposition of Preformed Fexofenadine in SC Rat Hepatocytes. Cumulative recovery of preformed fexofenadine in day 4 SC rat hepatocytes (medium+cell+BC) was $>90\%$ and was not significantly different between control and DEX-treated groups (data not shown). The accumulation of preformed fexofenadine in cells+BC versus cells of control and DEX-treated SC rat hepatocytes is shown in Fig. 3. The BEI and ClB in vitro values for preformed fexofenadine were not significantly different between control and DEX-treated SC rat hepatocytes (Table 1).

Metabolism of Terfenadine and Biliary Excretion of Terfenadine and Generated Fexofenadine in Day 4 SC Rat Hepatocytes. The disappearance of terfenadine from medium, cellular accumulation of terfenadine, generation of azacyclonol, and the amount of generated fexofenadine in cells and medium in day 4 control and DEX-treated SC rat hepatocytes is shown in Fig. 4. The accumulation of terfenadine and the fexofenadine metabolite in cells+BC versus cells of control and DEX-treated SC rat hepatocytes is shown in Fig. 5. The BEI of terfenadine in control day 4 SC rat hepatocytes was significantly higher compared with DEX-treated day 4 SC rat hepatocytes; however, ClB in vitro values were similar between the two groups (Table 1). At 1 h, the BEI of generated fexofenadine was similar in control and DEX-treated day 4 SC rat hepatocytes (Table 1).

Effect of DEX Treatment on the Metabolism of Terfenadine in Day 4 SC Rat Hepatocytes. Concentration-time profiles of terfenadine in control and DEX-treated day 4 SC rat hepatocytes generated in models 1 and 3 were fixed in model 2 to simulate the accumulation of terfenadine and formation of fexofenadine in cells+BC and cells in control and DEX-treated SC rat hepatocytes.

Statistical Analysis. Statistical differences in BEI or biliary clearance between control and DEX-treated day 4 SC rat hepatocytes were determined by Student’s $t$ test (Table 1). Two-way analysis of variance tables were constructed to compare the amount of substrate in medium and cells at selected time points in control versus DEX-treated SC rat hepatocytes (Table 2); where appropriate, two-way analysis of variance was followed by a Tukey test. The criterion for statistical significance was $P < 0.05$. 

### Table 1

Comparison of BEI and ClB in vitro values of terfenadine, generated fexofenadine, and preformed fexofenadine between control and DEX-treated (100 μM, 48 h) day 4 SC rat hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DEX-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BEI $^a$</td>
<td>ClB in vitro $^b$</td>
</tr>
<tr>
<td>TER</td>
<td>$15 \pm 4^a$</td>
<td>$9 \pm 5$</td>
</tr>
<tr>
<td>Generated FEX</td>
<td>$15 \pm 2$</td>
<td>$12 \pm 4$</td>
</tr>
<tr>
<td>Preformed FEX</td>
<td>$19 \pm 2$</td>
<td>$20 \pm 5$</td>
</tr>
</tbody>
</table>

$^a$ BEI values (1 h) were calculated as described under Materials and Methods.

$^b$ ClB in vitro values (1 h) were calculated according to equations in the Appendix I.

$^P < 0.05$ vs. treated.

### Table 2

Disposition of terfenadine in control and DEX-treated (100 μM; 48 h) day 4 SC rat hepatocytes.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>TER (nmol)</th>
<th>FEX (nmol)</th>
<th>AZA (nmol)</th>
<th>Total Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium Cells + BC</td>
<td>Medium Cells + BC</td>
<td>Medium Cells + BC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5 \pm 0.5</td>
<td>1.5 \pm 0.5</td>
<td>0.16 \pm 0.08</td>
<td>12 \pm 3</td>
</tr>
<tr>
<td></td>
<td>2.0 \pm 0.5</td>
<td>2.0 \pm 0.5</td>
<td>0.21 \pm 0.09</td>
<td>10 \pm 0.9</td>
</tr>
<tr>
<td></td>
<td>3.0 \pm 0.5</td>
<td>3.0 \pm 0.5</td>
<td>0.22 \pm 0.07</td>
<td>10 \pm 0.1</td>
</tr>
</tbody>
</table>

## $< $LOQ, below the lower limit of quantitation.

* $P < 0.05$ vs. 1 h (same treatment).

† $P < 0.05$ vs. control (same time point).
SC rat hepatocytes are shown in Fig. 6. A one-compartment model was used to estimate the elimination rate constant for terfenadine (0–4 h). The rate constant governing monoexponential terfenadine loss from day 4 SC rat hepatocytes (medium/cell) treated with DEX ($k = 1.04 \pm 0.2$ h$^{-1}$; $t_{1/2} = 0.7$ h) was increased compared with control ($k = 0.58 \pm 0.1$ h$^{-1}$, $t_{1/2} = 1.3$ h). The total amount (medium + cell + BC) of azacyclonol was significantly greater, whereas the amount (medium + cell + BC) of generated fexofenadine was similar at 1, 2, and 4 h after administration of terfenadine in DEX-treated day 4 SC rat hepatocytes compared with control (Table 2).

**Pharmacokinetic Modeling of Terfenadine and Fexofenadine Disposition in Day 4 SC Rat Hepatocytes.** The parameter estimates generated by compartmental modeling of terfenadine, generated fexofenadine, azacyclonol, and preformed fexofenadine data after incubation of terfenadine or preformed fexofenadine in control and DEX-treated day 4 SC rat hepatocytes are compiled in Table 3. The lines shown in Fig. 3 represent the fit of model 3 to preformed fexofenadine pooled data ($n = 3$). The lines shown in Fig. 4 represent the fit of model 2 to pooled terfenadine, generated fexofenadine, and azacyclonol data; rate constants determined in model 1 were fixed, and parameters describing the disposition of generated fexofenadine were estimated according to the scheme displayed in Fig. 2.

The rate constant for hepatocyte uptake was faster for terfenadine compared with preformed fexofenadine (2.5 versus 0.08 h$^{-1}$, respectively), whereas the biliary excretion rate constant for preformed fexofenadine exceeded that of terfenadine (0.44 versus 0.039 h$^{-1}$, respectively). The rate constants for basolateral excretion of terfenadine and fexofenadine were comparable (3.2 versus 1.9 h$^{-1}$, respectively). The rate constant for formation of azacyclonol was greater in DEX-treated day 4 SC rat hepatocytes (0.76 h$^{-1}$) compared with control cells (0.041 h$^{-1}$) (Fig. 4; Table 3). Although good correspondence of the model to the data was observed, the rate constants associated with the canalicular excretion and the basolateral reuptake of generated fexofenadine were poorly estimated by model 2 (coefficients of variation >60%; Table 3). However, the parameter estimates describing the transport processes of generated fexofenadine from model 2 were similar to parameter estimates describing hepatic transport of preformed fexofenadine estimated from model 3, and as such, parameter estimates from models 1 and 3 were used in subsequent simulations based on the assumption that the disposition of preformed fexofenadine and generated fexofenadine was identical.

**Simulation of Terfenadine and Generated Fexofenadine Disposition in Day 4 SC Rat Hepatocytes.** The disappearance of terfenadine from the incubation (medium + cell + BC) (Fig. 6; inset, data points) seemed to be
The time course of the disappearance of terfenadine (5 μM) from control (■) and DEX-treated (●) day 4 SC rat hepatocytes is shown in Fig. 6 (inset; solid and dashed lines, respectively). The influence of terfenadine accumulation in the bile canaliculi on the overall loss of terfenadine became more apparent after 4 h of incubation. Estimates of the rate constants associated with the disposition of terfenadine and fexofenadine in day 4 control and DEX-treated SC rat hepatocytes (Table 3, bold type) were fixed in model 2 (Fig. 2, Appendix II) to simulate the accumulation of terfenadine and formation of fexofenadine in control and DEX-treated SC rat hepatocytes in cells + BC and cells (Fig. 5). In general, the predicted values of terfenadine and generated fexofenadine were within the standard deviation of the observed data.

**Discussion**

These experiments represent the first report of an in vitro model that can be used to investigate the hepatic uptake, formation, and biliary excretion of a drug/metabolite pair compared with the hepatobiliary disposition of the preformed metabolite. Often, when therapeutic or toxic metabolites are identified in the drug discovery process, they are synthesized and administered to a relevant in vivo system to identify/confirm beneficial or deleterious effects. However, the disposition of preformed metabolites may not always mimic the disposition of those generated in vivo (Pang et al., 1984). In the case of fexofenadine, for example, a diffusional barrier for hepatic uptake exists for preformed fexofenadine. Although this barrier does not limit hepatic exposure when fexofenadine is generated from terfenadine in the hepatocyte, it could preclude comprehensive evaluation of preformed fexofenadine hepatobiliary disposition in the intact organ.

After intravenous administration of [14C]TER in rats, approximately 40% and 60% of the total radiolabeled dose was excreted in bile in 4 and 6 h, respectively, although unchanged terfenadine was not detected in the bile (Leeson et al., 1982). This result suggests that terfenadine metabolism, rather than biliary excretion, is the major route of terfenadine clearance in vivo in rats. The disposition of terfenadine and its carboxylate metabolite fexofenadine has been described previously in both in vivo and in vitro studies. The apparent K_{m} value for the formation of hydroxyterfenadine (Fig. 1) was reported to be ~32 μM in rat liver S9 (Jurima-Romet et al., 1994). Thus, a terfenadine concentration of 5 μM was used in these studies. The concentration of preformed fexofenadine used in these experiments (5 μM) was chosen based on both the described K_{m} values for Oatp1a1 (~32 μM) and Oatp1a4 (~6 μM) (Cvetkovic et al., 1999) and the sensitivity of the HPLC assay.

DEX has been shown to induce transport protein mRNA and protein expression levels in SC rat hepatocytes, including Oatp1a1 to a small extent; DEX did not induce the expression of Oatp1a1 in SC rat hepatocytes (Annaert et al., 2001; Luttringer et al., 2002; Turncliff et al., 2004). DEX treatment (100 μM; 48 h) of SC rat hepatocytes increased the expression of CYP3A1/2 (Turncliff et al., 2004). After incubation of terfenadine in day 4 SC rat hepatocytes, formation of fexofenadine and the N-demethylated metabolite azacyclonol was observed (Fig. 4, Table 2); the analysis of hydroxyterfenadine (Fig. 1) was not possible due to lack of available standard. Previously, Jurima-Romet et al. (1995) reported that the formation of azacyclonol was increased in DEX-

![Image](https://example.com/image1.png)

**Fig. 6.** Time course of the disappearance of terfenadine (5 μM) from control (■) and DEX-treated (●) day 4 SC rat hepatocytes (medium + cells + BC). Data represent mean ± S.E.M. (n = 3). Solid lines represent first-order elimination from a one-compartment model. Inset, time course of the disappearance of terfenadine (5 μM) from control day 4 SC rat hepatocytes (medium + cells + BC). Lines represent simulations of the compartmental model 2 (Fig. 2) in cells + BC (solid line) and cells (dashed line), respectively, using rate constants listed in Table 3 and equations described in Appendix II. The apparent biexponential decay of terfenadine is representative of accumulation in the bile canaliculi of day 4 SC rat hepatocytes; terfenadine loss from cells (in the absence of bile canaliculi) was monoeponential. The disposition of terfenadine in hepatocytes (model 2; Fig. 2) was simulated using the rate constants listed in Table 3; this simulation of the concentration-time profile of terfenadine in medium + cells + BC versus medium + cells is shown in Fig. 6 (inset; solid and dashed lines, respectively).
treated primary rat hepatocytes, whereas the formation of hydroxyterfenadine or fexofenadine was not induced. Likewise, in the present study the amount of fexofenadine formed was not affected by DEX treatment (Table 2), and only a modest increase was observed in the fexofenadine formation rate constant (Table 3). These results are in agreement with previous reports that the formation of fexofenadine in rats does not seem to be mediated specifically by the CYP3A1 isozyme (Jurima-Romet et al., 1995, 1996). However, DEX treatment did result in increased terfenadine clearance (Fig. 6) consistent with a greater than 10-fold increase in the formation rate constant of azacyclonol in DEX-treated SC rat hepatocytes (Table 3).

The in vitro biliary clearance of preformed fexofenadine determined in SC rat hepatocytes was ~6 ml/min/kg (Table 1). When this in vitro biliary clearance is scaled to the in vivo condition (Liu et al., 1999a), an in vivo intrinsic biliary clearance of ~16 ml/min/kg is predicted. The in vivo biliary clearance of fexofenadine in Sprague-Dawley rats recently was reported to be 11.4 ± 1.6 ml/min/kg (Tahara et al., 2005). Under the assumptions of the well-stirred model of hepatic disposition (Pang and Rowland, 1977), and assuming hepatic blood flow of 40 ml/min (Pollack et al., 1990), the intrinsic biliary clearance of preformed fexofenadine based on the work of Tahara et al. (2005) would be ~17 ml/min/kg. Likewise, the intrinsic biliary clearance of preformed fexofenadine in the isolated perfused rat liver would be ~20 ml/min/kg (Milne et al., 2000). Thus, our in vitro result (~16 ml/min/kg) is in good agreement with reported in vivo and in situ intrinsic biliary clearance values for fexofenadine.

A stepwise compartmental modeling approach was used to explore rate-limiting processes in the hepatobiliary disposition of terfenadine, fexofenadine, and azacyclonol in day SC rat hepatocytes. In control SC rat hepatocytes, the rate constant for terfenadine uptake (2.5 h⁻¹) was ~30-fold higher than that for fexofenadine (0.08 h⁻¹), consistent with a diffusional barrier for fexofenadine. Rate constants for terfenadine uptake and egress were ~2-fold greater in DEX-treated SC rat hepatocytes. However, the rate constants for terfenadine uptake (k₉TERM→TERC) and excretion (k₉TERC→M) across the basolateral membrane were approximately equal (Table 3), consistent with a predominant diffusional mechanism of TER flux across this membrane. A diffusional process for terfenadine accumulation is consistent with nearly 100% absorption of terfenadine after oral administration in humans (Okerholm et al., 1981).

Rate constants for terfenadine and fexofenadine basolateral excretion in control SC rat hepatocytes were of the same order of magnitude. If a diffusional barrier exists for fexofenadine, but not for terfenadine, the similarity in rate constants for basolateral egress suggests that fexofenadine excretion across the basolateral membrane is mediated by an active process. The role of Mrps in the basolateral excretion of fexofenadine is the subject of ongoing investigation. The appearance of fexofenadine in fresh HBSS after a 1-h incubation of terfenadine in day 4 SC rat hepatocytes provided evidence of basolateral excretion (Fig. 4). This observation is consistent with the fact that FEX is excreted in urine after terfenadine administration in vivo (Leeson et al., 1982); the sequential processes of terfenadine uptake in the liver, terfenadine metabolism to fexofenadine, and excretion of fexofenadine from the hepatocyte into sinusoidal blood would be prerequisite to the appearance of fexofenadine in urine.

In a simulation of the cellular accumulation of generated fexofenadine (Fig. 5), the parameter estimate for the biliary excretion of preformed fexofenadine adequately described the biliary elimination of generated fexofenadine, indicating that intracellular fexofenadine, at a given concentration, is excreted into bile at the same rate regardless of whether it is preformed or generated metabolite. Therefore, when diffusional barriers do not limit the access of a metabolite to a clearance mechanism (e.g., biliary excretion), the disposition of the generated metabolite may mimic that of the preformed administered metabolite.

The rate constant for the biliary excretion of fexofenadine (0.44 h⁻¹) was ~10-fold higher than that for terfenadine (0.039 h⁻¹), consistent with the extensive biliary excretion of fexofenadine compared with terfenadine observed in vivo (Leeson et al., 1982). The calculation of BEI (see Materials and Methods) is an algebraic approach to estimate the percentage of substrate taken up by hepatocytes that is excreted into bile. The BEI is analogous to the ratio of the biliary excretion rate constant to the sum of rate constants for ex-
cretion of substrate from SC hepatocytes \(e.g., k_{\text{FEXC-M}}/k_{\text{FEXC-B}}\cdot 100\), where the rate constants are determined based on the compartmental modeling approach. The BEI was similar to the related ratio of rate constants for preformed fexofenadine in control SC rat hepatocytes (19 versus 18%, respectively), but not for terfenadine (15 versus < 1%, respectively). This overprediction of the biliary excretion of terfenadine in SC rat hepatocytes using the BEI calculation occurs when the molar amount of terfenadine converted to metabolites residing in cells + medium + bile is not included in the denominator of the BEI equation. In vivo, the metabolism of terfenadine is rapid and parent terfenadine was not detected in the bile of rats administered an intravenous dose (Lee et al., 1982). Therefore, the BEI and \(\text{Cl}_{B\text{, in vitro}}\) values determined in these experiments might be more representative of the \textit{maximal} values expected in vivo for a metabolically labile compound. These observations are consistent with the pharmacokinetics modeling and simulation work of Liu and Pang (2005), who predicted that a decrease in the intrinsic metabolic clearance of enalapril would result in a significant increase in predicted biliary clearance.

The liver is a dynamic organ responsible for the uptake, metabolism, and excretion of many therapeutic agents. Although the utility of a double-transport protein-transfected cell line to examine the relative rates of transport across the apical and basolateral membranes is apparent (Sasaki et al., 2004), the availability of both transport and metabolism processes in a single experimental system, such as SC hepatocytes, can provide additional insight into the hepatobiliary disposition of novel therapeutic agents. Although a potential limitation of the current model is the decline in metabolic activity during the days in culture required for the transport proteins to become properly localized for biliary excretion experiments, relevant metabolic function can be maintained by use of media additives such as dexamethasone (LeCluyse et al., 1996; Turncliff et al., 2004). Another consideration is that the tight-junctional complexes that seal the bile canalicular spaces in SC rat hepatocytes can be disrupted continuously by \(\text{Ca}^{2+}\) modulation only for a limited time (Liu et al., 1999a); thus, accumulation studies to evaluate biliary excretion beyond 1 h may not be feasible. The SC model may be useful for assessing the overall magnitude of metabolism and/or biliary excretion of novel compounds, as well as to identify species-specific differences in these processes. The use of SC human hepatocytes for prediction of in vivo metabolism and biliary excretion is the focus of ongoing efforts.

Pharmacokinetic modeling of data from experiments in SC rat hepatocytes can yield the relative rates of hepatobiliary disposition of compounds. These studies represent a robust evaluation of the hepatobiliary disposition of a metabolite, when administered preformed versus when generated by metabolic conversion of a parent compound, and emphasize the utility of an in vitro model capable of assessing, in an integrated fashion, metabolism, biliary excretion, and basolateral transport processes.

### Appendix I: Calculation of In Vitro Biliary Clearance

The in vitro biliary clearance values of preformed fexofenadine (FEX\textit{pre}) and terfenadine (TER) in Table 2 were calculated according to eqs. 2 and 3 below. Rat liver weight and protein content in liver tissue were assumed to be 40 g/kg body weight and 0.2 g/g liver weight, respectively, in all calculations (Seglen, 1976). In these equations, abbreviations are as follows: M, medium; C, cells; B, bile canaliculi; and Accum, total accumulation of substrate under stated condition.

**Calculation of preformed FEX \(\text{Cl}_{B\text{, in vitro}}\)**

\[
\text{Cl}_{B\text{, in vitro}}^{\text{FEXC}} = \frac{\text{Accum FEX}_{\text{pre C, BC}} - \text{Accum FEX}_{\text{pre C, BC}}}{\text{AUC}_{0-60}^{\text{FEXC}}} \]

where:

\[
\text{AUC}_{0-60}^{\text{FEXC, M}} = \text{Time} \cdot \text{Concentration}_{\text{FEXC, M}} \]

**Calculation of TER \(\text{Cl}_{B\text{, in vitro}}\)**

\[
\text{Cl}_{B\text{, in vitro}}^{\text{TER}} = \frac{\text{Accum TER}_{\text{Cells, BC}} - \text{Accum TER}_{\text{Cells}}}{\text{AUC}_{0-60}^{\text{TER}}} \]

**Appendix II: Equations Describing the Metabolic and Biliary Disposition of TER, Generated FEX, Preformed FEX, and AZA in Day 4 SC Rat Hepatocytes**

The following equations, based on model schemes shown in Fig. 2, were used in the stepwise regression analysis of observed data. Parameter estimates of the transport and metabolic processes responsible for the hepatobiliary disposition of TER and FEX and AZA are described in Table 3. The following abbreviations and subscripts are used: M, incubation medium; C, cellular; B, canalicular network; and T, total (M + C + B). All data were modeled as amounts (pmol/mg protein).

**Model 1**

Accumulation in cells + BC

\[
\frac{d\text{TER}}{dt} = -k_{\text{TER} \rightarrow \text{TERC}} \cdot \text{TER} + k_{\text{TERC} \rightarrow \text{TERB}} \cdot \text{TERC} \]

Accumulation in cells

\[
\frac{d\text{TER}}{dt} = k_{\text{TERC}} \cdot \text{TERC} - \frac{k_{\text{TER} \rightarrow \text{Other}}} {\text{TER} + k_{\text{TERC} \rightarrow \text{AZA}}} \cdot \text{TERC} \]

Accumulation in bile

\[
\frac{d\text{AZA}}{dt} = k_{\text{TERC} \rightarrow \text{AZA}} \cdot \text{TERC} \]

Accumulation in gavage

\[
\frac{d\text{FEX}}{dt} = k_{\text{TERC} \rightarrow \text{FEX}} \cdot \text{TERC} \]
Model 2
Accumulation in cells + BC: eqs. 4 to 7. Additionally,

\[
\frac{dFEX_{\text{genM}}}{dt} = \frac{k_{\text{FEXc}} + k_{\text{FEXm}}}{k_{\text{FEXc}} + k_{\text{FEXm}}} \cdot FEX_{\text{genM}} \\
- (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{genC}}
\]

(10)

\[
\frac{dFEX_{\text{genM}}}{dt} = -k_{\text{FEXcM}} \cdot FEX_{\text{genM}} + (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{genC}}
\]

(11)

\[
\frac{dFEX_{\text{genB}}}{dt} = k_{\text{FEXc}} \cdot FEX_{\text{genB}}
\]

(12)

Accumulation in cells: eqs. 5, 7, 9, and 10. Additionally,

\[
\frac{dFEX_{\text{genM}}}{dt} = -k_{\text{FEXcM}} \cdot FEX_{\text{genM}} + (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{genC}}
\]

(13)

Model 3
Accumulation in cells + BC

\[
\frac{dFEX_{\text{preM}}}{dt} = -k_{\text{FEXcM}} \cdot FEX_{\text{preM}} + (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{preC}}
\]

(14)

\[
\frac{dFEX_{\text{preC}}}{dt} = k_{\text{FEXcM}} \cdot FEX_{\text{preC}} - (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{preC}}
\]

(15)

\[
\frac{dFEX_{\text{preB}}}{dt} = k_{\text{FEXc}} \cdot FEX_{\text{preB}}
\]

(16)

Accumulation in cells: eq. 15. Additionally,

\[
\frac{dFEX_{\text{preM}}}{dt} = -k_{\text{FEXcM}} \cdot FEX_{\text{preM}} + (k_{\text{FEXc}} + k_{\text{FEXm}} + k_{\text{FEXd}}) \cdot FEX_{\text{preC}}
\]

(17)

References


Tahara H, Kusahara H, Fuse S, and Sugiyama Y (2005) P-glycoprotein plays a major role in the efflux of fexofenadine in the small intestine and blood-brain barrier, but only a limited role in its biliary excretion. Drug Metab Dispos 33:963–968.


