PBPK modeling to unravel nonlinear pharmacokinetics of verapamil
to estimate the fractional clearance for verapamil N-demethylation
in the recirculating rat liver preparation

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Abstract

We applied physiologically-based pharmacokinetic (PBPK) modeling to study the dose-dependent metabolism and excretion of verapamil and of its preformed metabolite, norverapamil, to unravel the kinetics of norverapamil formation via N-demethylation. Various initial verapamil (1, 50 and 100 μM) and preformed norverapamil (1.5 and 5 μM) concentrations, perfused at 12 mL·min⁻¹, were investigated in the perfused rat liver preparation. Perfusate and bile were collected over 90 min, and livers were harvested at the end of perfusion for HPLC analysis. After correction for the adsorption of 10-25% dose verapamil and norverapamil onto Tygon® tubing and binding to albumin and red blood cell, fitting of verapamil and formed and preformed norverapamil data with ADAPT5® revealed nonlinearity for protein binding, N-demethylation ($V_{\text{VER} \rightarrow \text{NOR}}_{\text{max},\text{met1}} = 96.6\pm33.4$ nmol·min⁻¹; $K_{m,\text{met1}}^{\text{VER} \rightarrow \text{NOR}} = 10.4\pm4.1$ μM), formation of other metabolites ($V_{\text{max,met2}}^{\text{VER \rightarrow others}} = 288\pm51$ nmol·min⁻¹; $K_{m,\text{met2}}^{\text{VER \rightarrow others}} = 14.1\pm4.9$ μM), as well as biliary excretion ($V_{\text{max,sec}}^{\text{VER}} = 0.911\pm0.505$ nmol·min⁻¹; $K_{m,\text{sec}}^{\text{VER}} = 4.75\pm2.29$ μM). The hepatic clearance of verapamil ($CL_{\text{VER}}^L$) decreased with dose (8.16-10.2 mL·min⁻¹), with values remaining high relative to perfusate blood flow rate among the doses. The hepatic clearance of preformed norverapamil (11 mL·min⁻¹) remained unchanged for the concentrations studied and approximated perfusate blood flow rate, suggesting a high norverapamil extraction ratio. The fractional formation of norverapamil and biliary excretion of verapamil based on fitted constants were 31.1% and 0.64% of $CL_{\text{VER}}^L$, respectively. Enantiomeric disposition and auto-inhibition of verapamil failed to perturb these estimations according to PBPK modeling, due to the low values of $K_m$ and $k_i$. 
Introduction

Verapamil, a calcium channel blocker (Fleckenstein, 1977) used for the treatment of cardiac arrhythmias and hypertension (McTavish and Sorkin, 1989), is given as a racemic mixture. Much is known about the stereoselective disposition of the more potent S- versus R-verapamil in human and rats (Busse et al., 2006; Bhatti et al., 1997). Binding of verapamil to bovine serum albumin (BSA) revealed that the unbound plasma fraction of R-verapamil (~0.65) exceeds that of S-verapamil (~0.55) (Mehvar et al., 1994). The reverse is true for binding to human serum albumin and α1-acid glycoprotein (Mehvar and Reynolds, 1996; Hanada et al., 1998), whereas the plasma bound fraction of verapamil in human is concentration-independent within the therapeutic range and unaltered by the presence of norverapamil (Keefe et al., 1981). The distribution of verapamil and norverapamil into red blood cells is also stereoselective [human (S>R) versus rat (R>S)] (Robinson and Mehvar, 1996).

Much species difference exists for the metabolism of verapamil. First-pass removal of verapamil is attributed to both the intestine and liver in rats (Hanada et al., 2008) and man (Fromm et al., 1998), though intestinal elimination is lacking in dogs (Lee et al., 2001). At least 25 phase I and 14 phase II metabolites have been identified in the rat (Walles et al., 2003). First-pass metabolism in rats is saturable and stereoselective: hepatic bioavailability for R-verapamil is higher than that for S-verapamil, but the opposite was observed for intestinal bioavailability, resulting in a higher systemic bioavailability for R-verapamil following oral dosing (Hanada et al., 2008). Extensive first-pass removal and liver clearance have been reported, with verapamil being mostly metabolized via N- and O-dealkylation (98%) in both rats and humans (Eichelbaum et al., 1979; Woodcock et al., 1981; Choi and Burm, 2008), with norverapamil and D617 as major metabolites formed via N-dealkylation, predominantly by CYP3A4, CYP3A5, CYP2C8 and CYP2E1 (Eichelbaum et al. 1979; Kroemer et al., 1992; Tracy et al., 1999; Sun et al., 2004). Nonlinear metabolism was observed in man following long-term, repeated oral dosing (1-1.6 mg·kg⁻¹ over one month), leading to prolongation of half-life and decreased apparent clearance.
High doses of verapamil (6-19 mg·kg\(^{-1}\)) infused intravenously in man resulted in saturable kinetics and decreased systemic clearance with dose escalation (Toffoli et al., 1997). In addition, verapamil and norverapamil were reported to be mechanism-based inhibitors of cytochrome P450 and P-glycoprotein (P-gp) that resulted in auto-inhibition upon long term use clinically (Lemma et al., 2006; Wang et al., 2013). Verapamil and norverapamil were present at significantly higher concentrations in human intestine lumen and bile compared to plasma, suggesting excretion mediated by transporters, probably P-gp (von Richter et al., 2001). Norverapamil is transported by P-gp in Caco-2 cells and P-gp-overexpressing L-MDR1 cells, but to a lesser extent than other verapamil metabolites, D-617 and D-620 (Pauli-Magnus et al., 2000).

The need for understanding metabolite formation kinetics is paramount, especially when the metabolite in question is active or toxic (Baillie et al., 2002; Pang, 2009). The purpose of this investigation is to develop a strategy to study dose-dependent verapamil metabolism and biliary excretion and describe metabolite formation kinetics on the importance of a given metabolic pathway (N-demethylation) relative to other elimination pathways (metabolism or biliary excretion) with use of verapamil and its active metabolite, norverapamil. To this end, Mehvar and colleagues estimated the extent of formation of norverapamil from verapamil in the perfused rat liver preparation and examined removal of verapamil from plasma (Mehvar et al., 1994), but had not addressed the occurrence of sequential metabolism of formed norverapamil (Pang and Gillette, 1979) nor accounted for verapamil partitioning into red blood cells. Since the \(K_m\) for metabolism ranges from 60 to 140 μM (Hanada et al., 2008), we employed initial concentrations of 1 to 100 μM verapamil to revisit the problem of metabolite kinetics in the recirculating perfused rat liver preparation. Mechanism-based auto-inhibition for verapamil that exists in man (Wang et al., 2004; Wang et al., 2013) was not observed in rat liver microsomes, hepatocytes and precision-cut liver slices (Obach, 1999; Shibata et al., 2002; Axelsson et al., 2003; Guo et al., 2007), except with gel entrainment after prolonged exposure with verapamil (Yin et
al., 2011). Hence, auto-inhibition is not expected to occur within the short time-frame for liver perfusion studies. We applied physiological-based pharmacokinetic (PBPK) modeling, to account for tubing adsorption of verapamil and norverapamil and stereoselective vascular binding and saturable metabolism and excretion.
Materials and methods

Materials

Verapamil (racemic, R- and S-; C_{27}H_{38}N_{2}O_{4}) and norverapamil (C_{26}H_{36}N_{2}O_{4}) were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich; dextrose (50%) was purchased from Abbott laboratories (Montreal, QC, Canada). HPLC grade acetonitrile, methanol, and ethyl acetate were obtained from Sigma Aldrich. Male Sprague-Dawley rats (324±31.1 g) were supplied by Charles River Laboratories (St. Constant, QC, Canada). [3H]Verapamil, produced via general tritium exchange (specific activity 1 mCi·mL⁻¹; >97% radiochemical purity by HPLC), was obtained from American Radiolabeled Chemicals Inc., St. Louis, MO.

Albumin binding and RBC partitioning of verapamil

A mixture of labeled and unlabeled verapamil was used to provide for sensitivity for the binding studies. The total verapamil concentration was given as the sum of the labeled (from specific activity) and unlabeled verapamil, and the dpm·mL⁻¹ of plasma was the equivalent of assayed HPLC concentration in plasma. Binding of [³H]verapamil to BSA (2%) was measured in equilibrium dialysis half-cells that were separated by a semi-permeable membrane (molecular cutoff 12-14,000; Spectrum Laboratories Inc., Rancho Dominguez, CA). 1 mL 2% BSA in KHB (Krebs Henseleit buffer, pH 7.4) containing various concentrations of verapamil (0.4-140 μM) was added to the protein-side half-cell, and 1 mL of KHB was introduced to the other (buffer side) half-cell. The cell was incubated at 37°C using a rotating water bath. After 5 h of equilibration, the time predetermined for equilibration, samples (100 μL) from both sides were removed for protein determination and liquid scintillation counting (LSC, with LS 5801 Counter; Beckman Coulter Canada, Mississauga, ON). Preservation of protein and water volumes was checked at the end of the experiment. The dpms in the protein and buffer sides were
The plasma unbound fraction of verapamil (f_P) was calculated as the ratio of verapamil concentrations in buffer side to that in the protein side, at the plasma concentration determined at the end of the binding study.

The distribution of verapamil into red blood cell (RBC) was studied by mixing [3H]verapamil and unlabeled (1-440 μM) verapamil in 2% BSA plasma perfusate. Upon mixing this protein solution with an equal volume of blood perfusate containing 40% washed bovine erythrocytes, RBC (or 2x the normal 20% RBC, a kind gift from Ryding-Regency Meat Packers Ltd., Toronto, ON), a blood perfusate of composition identical to that used for perfusion was obtained. Aliquots of the resultant labeled and unlabeled verapamil concentration in blood perfusate (C_B) (1/2 the concentration of initial plasma perfusate), were removed for assay by HPLC, and for incubation at 37 °C for 5 min. Then 200 μL blood perfusate was removed at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 min after incubation, and hematocrit (Hct) was measured at 0.25 and 5 min. The total verapamil concentration in plasma (C_P) was estimated using liquid scintillation counting; the concentration in red blood cells (C_RBC) was calculated: 

\[ C_{RBC} = \frac{C_B - C_P (1-Hct)}{Hct} \]

Data of verapamil binding to BSA and distribution to RBC were then fitted simultaneously according to the binding model with one class of binding site (see Appendix A for mass balance equations) to furnish constants such as the binding association constant (K_A) and modified rate constants for partitioning (k'_p) into and out of RBC (k'_pr) based on total drug concentration using ADAPT5® (BMSR version 5, USC). The fraction unbound in plasma (f_P) was predicted according to best fitted binding parameters.

**Recirculating rat liver perfusion and drug adsorption**

The TWO/TEN Perfuser (perfusion apparatus, MX International, Aurora, CO) was used for recirculation of verapamil and norverapamil at the flow rate of 12 mL·min\(^{-1}\) and 37 °C. Perfusate consisted of 1% BSA, 0.6% Dextrose and 20% washed bovine erythrocyte in oxygenated (95% O\(_2\) and 5% CO\(_2\) at 1 L·min\(^{-1}\)) Krebs-Henseleit bicarbonate solution (KHB), pH 7.4. The surgical and liver perfusion...
procedures were identical to those described previously (Tan and Pang, 2001). Male Sprague-Dawley rats (300-365 g) were anesthetized with a mixture of ketamine (90 mg·kg⁻¹) and xylazine (10 mg·kg⁻¹), and recirculating liver perfusion was conducted after cannulation of the bile duct and portal and hepatic veins for the inflow and outflow, respectively; the hepatic artery was ligated. Drug-free blank perfusate was first used to recirculate the liver for 20 min for equilibration, followed by perfusion with drug-containing perfusate from a second reservoir (200 mL) consisting of the designated concentration of verapamil (1, 50 or 100 μM, n=4) or preformed norverapamil (1.5 μM, n=4 or 5 μM, n=3) for the next 90 min. Reservoir perfusate (1 mL) was removed at 2.5, 7.5, 12.5, 17.5, 22.5, 27.5, 35, 45, 55 and 70, and 2 mL was removed at 0 and 90 min. Bile was sampled at 5 min intervals between 0 to 30 min, or at 10- or 20- min intervals thereafter. At the end of perfusion (90 min), the liver was flushed with 50 mL cold blank KHB to remove any residual blood perfusate, and the liver was weighed, minced, blast frozen with liquid nitrogen and stored at –80 °C until analysis by HPLC.

Since adsorption of verapamil and norverapamil to tubing was found, Tygon® tubing of constant length, obtained from Saint-Gobain Performance Plastics (Valley Forge, PA, USA) that exhibited less binding to the compounds, was used for perfusion. For characterization of binding, liver perfusion with drug-free (20 min) then verapamil- or norverapamil-containing (90 min) blood perfusate was conducted in absence of the rat liver to characterize for the loss of drug due to binding to tubing.

**HPLC**

**For measuring C_B and distribution of verapamil into RBC.** A HPLC method was used for the separation of verapamil and its internal standard (50 μL of 100 μg·mL⁻¹ diltiazem) at the wavelength of 230 nm (Garcia et al., 1997). The HPLC system consisted of a Shimadzu 6A UV spectrophotometric detector, LC-6AD liquid chromatograph, SIL-9A auto injector, CR-4A chromatopac, and a 15-20 μm μBondapak C₁₈ reverse column (3.9 x 300 mm, Waters, UK). The mobile phase [40% 0.04 M ammonium acetate: triethylamine (2:0.04 v/v) + 60% methanol] was delivered at a flow rate of 0.9
mL·min$^{-1}$. The typical retention times for verapamil and diltiazem were 18 and 23 min, respectively. The extraction procedure of verapamil for blood perfusate samples was as follows: to 1 mL sample, 12 mL polypropylene, 50 μL 100 μg·mL$^{-1}$ diltiazem and 75 μL 1 M NaOH were added and mixed thoroughly, and then 4 mL of a hexane and 2-propanol mixture (2:1 v/v) was added. The content was mixed for 2 min, followed by centrifuging samples at 3000 rpm. After removal and drying of the extract under N$_2$, the residue was reconstituted in 200 μL ACN and water (1:1 v/v) for HPLC injection.

**Verapamil and norverapamil determination in tubing adsorption and liver perfusion studies.** In these studies, the Shimadzu 10A HPLC system was used to separate verapamil and norverapamil at the detection wavelength of 278 nm. The system consisted of a SCL-10A system controller, a SPD-10A UV-visible detector, a SIL-10XL automatic injector, a FCV-10AL solvent delivery unit and a LD-10AT liquid chromatograph. A 10-μm C$_{18}$ reverse column (4.6 x 250 mm, Altech Associates, Deerfield, IL) was used to separate verapamil, norverapamil and the internal standard (diazepam). The mobile phase consisted of 55% of 0.02 M ammonium acetate buffer (0.4% triethylamine, pH adjusted to 6 with acetic acid) and 45% of acetonitrile, and was maintained for 30 min for each injection at flow rate of 1 mL·min$^{-1}$. The calibration curves for verapamil and norverapamil were linear over the range of 0.25 to 60, and 0.125 to 30 nmole, respectively, in 2 ml perfusate, with correlation coefficients of ~0.999 (n = 5). The LOQs were 0.25 nmole for verapamil and 0.125 nmole for norverapamil. The typical retention times for verapamil, norverapamil and diazepam were 15, 12 and 23 min, respectively.

Blood perfusate samples (0.25–1.8 mL) were made up to a final volume of 2 mL with blank perfusate, mixed with 50 μL internal standard (50 μg·mL$^{-1}$ diazepam) and 50 μL 1 M NaOH, and extracted against 6 mL ethyl acetate. After repeated, 3-min vigorous mixing, the mixture was centrifuged at 3000 rpm for 10 min and the supernatant was transferred and dried under nitrogen gas. The dried residue that was reconstituted in 100 μL mobile phase was centrifuged, and 50 μL was injected. Liver tissue was obtained from homogenization in 1.5-time volume of ice-cold saline for 30 s
thrice. Then 0.2 mL was removed, added 1 mL ethyl acetate and mixed for 3 min, followed by centrifugation at 3000 rpm for 10 min. The organic layer was removed and dried under nitrogen gas and the residue was reconstituted with 200 μL mobile phase, and 150 μL was injected into HPLC. Calibration curves were prepared based on a set of standards containing varying known concentrations of verapamil and norverapamil in perfusate blood or tissue, prepared under identical conditions as the samples.

Bile samples were made up to 60 μL with H₂O, added 10 μL acetonitrile and 10 μL internal standard (50 μg·mL⁻¹ diazepam), then 1 mL ethyl acetate for extraction. The nitrogen-dried supernatant was reconstituted in 200 μL mobile phase and 90 μL was injected into the HPLC. For the norverapamil perfusion studies, bile collected during the entire 90-min of perfusion was pooled, mixed with 50 μL acetonitrile, 50 μL of 50 μg·mL⁻¹ diazepam, 50 μL NaOH (1 M), and extracted with 3 mL ethyl acetate. After the supernatant was nitrogen-dried, the residue was reconstituted in 200 μL mobile phase, and 50 μL was injected into the HPLC. The concentrations of verapamil and norverapamil were determined with use of calibration curves, prepared under identical conditions.

**PBPK modeling**

We applied a PBPK model to fit perfusate, liver and bile data of verapamil and preformed and formed norverapamil simultaneously (Figure 1). The model was constructed under a number of assumptions, with mass balance equations shown in the Appendices. The tubing adsorption rate constants, estimated for reservoir for verapamil (k₁⁻VT or k₉⁻VT) and norverapamil (k₁⁻NT or k₉⁻NT) via fitting of perfusion data in absence of liver (sham experiments), were viewed to be identical to other perfusion experiments with liver. These tubing adsorption rate constants, along with other physiological parameters such as liver volume (Vₗ) and hepatic blood perfusate flow (Qₗ), were assigned for modeling of the liver perfusion data. The recirculating liver preparation was constructed as two compartments,
liver blood (LB) and liver tissue (L), which were connected to the reservoir (R) by hepatic perfusate blood flow (QL). Flow-limited distribution of verapamil and preformed and formed norverapamil was assumed because these drugs are lipophilic compounds (logP = 3.79 and 3.3, respectively) and therefore can diffuse freely across the membrane. The influx clearance (CL_{in}^{VER} or CL_{in}^{NOR}) and efflux clearance (CL_{ef}^{VER} or CL_{ef}^{NOR}) were assumed to be the same, and were assigned as 5x the value of perfusate blood flow (QL) as initial estimates for the influx/efflux clearances in the PBPK model. Since only unbound verapamil and norverapamil were able to be transported or eliminated, the unbound fractions of drug in blood perfusate (f_B; measured experimentally and incorporated in the model, see Appendix A-C) and liver (f_L; fitted in the model) were considered. Verapamil was metabolized to norverapamil or other metabolites, with the metabolic intrinsic clearances, CL_{int,met1}^{VER→NOR} and CL_{int,met2}^{VER→others} [\frac{V_{\text{max}}}{K_m} and \frac{V_{\text{max}}}{c_{L,u} + K_m}] for linear and nonlinear conditions, respectively, where \(c_{L,u}\) denotes the unbound liver concentration. The assumption that stereoselective metabolism of verapamil under our high concentrations being unimportant was made. Norverapamil was further metabolized with the intrinsic metabolic clearance or CL_{int,met}^{NOR} (or \(\frac{V_{\text{max}}}{K_m}\)). Verapamil and norverapamil were secreted into bile with secretory intrinsic clearances, CL_{int,sec}^{VER} (or \(\frac{V_{\text{max,sec}}}{K_m^{\text{VER,sec}}}\) ) or CL_{int,sec}^{NOR} (or \(\frac{V_{\text{max,sec}}}{K_m^{\text{NOR,sec}}}\) ), respectively. Transit compartments (\(A_t\)) containing time delays (represented as \(\tau\) function) were incorporated to describe the delayed biliary secretion of verapamil and norverapamil (see Appendix C for mass balance equations).

Our strategy was to first consider linearity in the removal processes for verapamil metabolism and excretion, then consideration of each pathway as nonlinear. Michaelis-Menten equations, with the appropriate \(V_{\text{max}}\) (maximum velocity of the enzyme or transporter) and \(K_m\) (Michaelis-Menten constant) terms were used in lieu of CL_{int} for metabolism and biliary secretion: \(\text{CL}_{\text{int,met}}\) or \(\text{CL}_{\text{int,sec}} = \frac{V_{\text{max}}}{c_{L,u} + K_m}\), namely, the intrinsic clearance terms under nonlinear conditions were given as \(V_{\text{max}}\) divided by the sum of the unbound substrate concentration in liver (\(c_{L,u}\)) and the \(K_m\) term. In parallel, data for preformed
norverapamil metabolism and excretion were fitted, with and without nonlinearity. The final fit consisted of simultaneously fitting of both verapamil (1, 50 and 100 μM) and formed and preformed norverapamil (1.5 and 5 μM) data.

Model fitting criterion was evaluated based on the Akaike information criteria (AIC) score, or $2k -2\ln(\text{Likelihood})$, where $k$ is the number of parameters in the model and Likelihood is the maximum value of the likelihood function for the model. AIC evaluates the goodness-of-fit with consideration of the penalty for adding additional parameters to fit the data set; the lower the AIC score, the better the goodness-of-fit. Also, the correlation between predicted and observed concentrations and weighted residuals, calculated as $\frac{\text{Observations-Predictions}}{\text{Observations}}$ versus time plots were used to examine systematic trends and extreme values. The sum of squared weighted residuals was calculated for each of the candidate model to define which model was most appropriate to describe the pharmacokinetics of verapamil and formed and preformed norverapamil.

**Statistical Analysis**

The estimated parameters were shown as mean±SD (CV%). The %coefficient of variation, denoted by CV%, was calculated as \( \left( \frac{\text{SD}}{\text{mean}} \right) \times 100\% \). The two-tailed student t-test with unequal variance was used to compare the means between two groups, and a \( P \) value less than .05 was considered as significant. One-way ANOVA and post-hoc Tukey HSD test were used to evaluate the means in three dosing groups (1, 50 and 100 μM) using SPSS (IBM Corp, version 22, NY). The F test was used to assess model improvement (Boxenbaum et al., 1974). The error proportional method was used to calculate uncertainties: $\Delta Z = \sqrt{(\Delta X)^2 + (\Delta Y)^2 + \cdots}$ was used when adding or subtracting two or more values with standard deviations; $\frac{\Delta Z}{Z} = \sqrt{\left(\frac{\Delta X}{X}\right)^2 + \left(\frac{\Delta Y}{Y}\right)^2 + \cdots}$ was used when multiplying or dividing two or more values with standard deviations.
Results

Protein binding and RBC partitioning

Binding of verapamil (0.4-140 μM) to BSA (2%) in plasma was found to be concentration-dependent, with the unbound fraction in plasma \( f_p \) varying from 0.4 to 0.6 within the concentration range studied (Figure 2A). The ratio of verapamil concentration in perfusate blood to plasma \( C_B/C_P \) was found to be concentration-dependent, with value increasing from 1.11±0.07 to 1.29±0.05 \( (P < .001) \) within the concentration range of 1 to 441 μM examined. An average unbound fraction of verapamil in blood perfusate (0.45±0.09) may be estimated, based on the average value of \( f_p \) \( (f_B = \frac{C_P}{C_B} f_p) \) (Pang and Rowland, 1977). The ratio of verapamil concentrations in red blood cells and plasma \( C_{RBC}/C_P \) was around 2-3 within the concentration range studied when Hct, the hematocrit, was 0.134±0.014, showing that verapamil can distribute into red blood cells. Upon fitting the data to the model (Appendix A), nonlinear protein binding and distribution into red blood cell were adequately described by the model with one class of binding site (Figure 2B), yielding fitted values of modified rate constants \( k_{pr}' \) (13.2±4.4 min\(^{-1}\)) and \( k_{rp}' \) (19.5±8.7 min\(^{-1}\)), the number of protein binding sites \( (n=0.301±0.160) \), and binding association constant \( (K_A =0.0091±0.0057 \mu M^{-1}) \). Since the turnover time \((1/k_{pr}')\) of verapamil in red cells was 3.1 sec, a value much lower than the mean transit time of red blood cells or 14-15 sec (Pang et al., 1995), red blood cell binding of verapamil can rapidly achieve equilibrium and will not affect verapamil removal. Nonlinear protein binding due to one single class of binding site and concentration-dependent \( C_B/C_P \) were incorporated in PBPK modeling (Appendix B and C). Protein binding due to two classes of binding sites was also considered during fitting, though the fit was suboptimal (Figure 2A). Binding would not materially affect the clearance of verapamil since the drug is highly cleared, and the trend was verified, namely, small changes in \( f_B \) (with values fluctuating by +0.1 or -0.1), would not materially alter subsequent metabolism or excretion (simulations not shown).

Tubing adsorption
The adsorption profiles of verapamil and norverapamil to Tygon® tubing are shown in Figure 3. The rate constants for binding (k_{R → t}) to tubing and debinding (k_{t → R}), obtained from fitting the adsorption data without rat liver, are summarized in Table 1. Although the absolute amounts of drug adsorbed to Tygon® tubing increased with dose, the %dose of verapamil and preformed norverapamil adsorbed decreased with increasing concentrations perfused. The lowest adsorbed amount (50 n mole or 25% dose) occurred for the lowest concentration (for initial concentration of 1 μM), whereas about 10% dose (1 or 2 μmole) was adsorbed for higher doses (for initial concentrations of 50 and 100 μM). Similarly, the amount of norverapamil adsorbed onto the Tygon® tubing increased from 0.06 to 0.1 μmole after perfusion with initial concentrations of 1.5 and 5 μM norverapamil. The rate constants for binding to Tygon® tubing (k_{R → t}^{VER} and k_{R → t}^{NOR}) decreased as the dose escalated, suggesting that tubing binding sites might be saturated at higher drug concentrations. There was no change in the return rate constants (k_{t → R}^{VER} and k_{t → R}^{NOR}). The tubing rate constants (Table 1) were assigned as constants into the PBPK model (See Appendix C for mass balance equations).

**Nonlinear kinetics of verapamil and norverapamil**

The physiological and experimentally-derived estimates for the verapamil (Table 2 and Supplemental Table 1) and preformed norverapamil (Table 3 and Supplemental Table 2) perfusion studies are summarized. Preformed norverapamil was investigated to provide information on the sequential handling of norverapamil formed from verapamil. Temporal profiles of verapamil and its preformed and formed metabolite norverapamil in perfusate, liver and biliary secretion are shown in Figure 4. Volume recovery was ~85%, showing small experimental error. In reservoir perfusate, verapamil concentrations decreased rapidly following an apparent log-linear decay, and the terminal elimination rate constant (calculated from the slope of elimination phase, β) was significantly decreased from 0.0536±0.0008 to 0.0386±0.0075 min⁻¹ for the high dose groups (50 and 100 μM) compared with the low dose group (1 μM; P = 0.0007), suggesting that the apparent half-life was prolonged with dose,
exhibiting concentration-dependent or nonlinear kinetics. Similarly, the hepatic clearance ($\text{CL}_L^{\text{VER}}$) for verapamil decreased from 10.2 to 8.75 mL·min$^{-1}$ ($P = 0.04$) though the values remained high and close to perfusate blood flow rate; $E_L^{\text{VER}}$ or extraction ratio of verapamil $= \frac{\text{CL}_L^{\text{VER}}}{Q_L}$ was high (0.8). Concentrations of formed norverapamil in perfusate were very low (< 4 μM), and no apparent trend was observed over time. The dose-normalized $\text{AUC}_{\infty}\text{VER}$ and $\text{AUC}_{\infty}\text{VER} \rightarrow \text{NOR}$ increased with increasing input concentrations, suggesting accumulation of drug and metabolite in perfusate with dose (Supplemental Table 1).

Preformed norverapamil decayed rapidly and monoexponentially in reservoir perfusate (Figure 5), yielding similar terminal elimination rate constants (0.0484±0.0022 vs. 0.0612±0.008 min$^{-1}$; $P = 0.15$) for the 1.5 and 5 μM input concentration groups. Biliary excretion of norverapamil was not remarkable.

The estimated extraction ratio of preformed norverapamil ($E_L^{\text{NOR}} = \frac{\text{CL}_L^{\text{NOR}}}{Q_L}$) was high (0.9). The fraction of hepatic clearance of verapamil forming norverapamil ($h_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}}$), based on the assumption of linearity for the lowest verapamil concentration (1 μM) and the equation comparing the AUC of formed vs. preformed norverapamil, normalized to the availability of norverapamil $\left[ \frac{\text{AUC}_{\text{VER} \rightarrow \text{NOR}}}{\text{dose}_{\text{IV}}^{\text{VER}}} \right] / F_L^{\text{NORP}}$, was 23.2% (Pang and Kwan, 1983). Biliary excretion clearance of verapamil was 1% of $\text{CL}_L^{\text{VER}}$ for the lowest input concentration of 1 μM (Table 2); the biliary secretion of preformed norverapamil was only 0.3 - 0.4% of $\text{CL}_L^{\text{NOR}}$.

**PBPK modeling**

Data of verapamil, formed and preformed norverapamil were fitted simultaneously using ADAPT5® (BMSR version 5, USC). The strategy was to first consider all metabolism and excretion as first-order processes; nonlinearity in metabolism or excretion was then included stepwise into the fitting routine. Physiological parameters such as volume of tissue (V) and perfusate blood flow rates ($Q_L$) were
assigned (see Tables 5). Temporal profiles of fitted verapamil, formed and preformed norverapamil in perfusate, liver and bile (Figures 4 and 5) for the best model included nonlinearity in N-demethylation, formation of other verapamil metabolites, and biliary excretion. Evidence on improvement of fit was summarized in Table 4. According to F-test, incorporation of nonlinearity in $\text{CL}_{\text{int,met1}}^{\text{VER} \rightarrow \text{NOR}}$, $\text{CL}_{\text{int,met2}}^{\text{VER} \rightarrow \text{others}}$, $\text{CL}_{\text{int,sec}}^{\text{VER}}$ and $\text{CL}_{\text{int,sec}}^{\text{NOR}}$ to the base model significantly improved the goodness-of-fit, evidenced by the lowest sum of squared weighted residues (WSS) and AIC score (Table 4). With the nonlinear pathways, the predicted concentrations matched the measured concentrations well and fell on the line of identity; the predicted vs. observed and weighted residuals revealed a random distribution around 0 (Supplemental Figures 1 and 2).

In the final model (Model H) that best fit the data, the coefficient of variation (CV%) was between 5 to 48% of all parameter estimates (Table 5). The unbound fraction in perfusate blood estimated for norverapamil was 0.44, a value similar to that for verapamil, whereas liver tissue binding to verapamil and norverapamil were high. Reasonable transfer clearances ($\text{CL}_{\text{in}}^{\text{VER} = \text{CL}_{\text{ef}}^{\text{VER}}}$) and ($\text{CL}_{\text{in}}^{\text{NOR} = \text{CL}_{\text{ef}}^{\text{NOR}}}$) as well as $V_{\text{max}}$ and $K_m$ parameters with low CV% were obtained. The $K_m$ values for the secretion of verapamil and norverapamil were around 5 μM, which suggests that the P-gp pump could be readily saturated. $h_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}}$ or the fraction of hepatic clearance of verapamil that forms norverapamil via N-demethylation according to the fitted constants ($h_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}} = \frac{V_{\text{max}}^{\text{VER} \rightarrow \text{NOR}}}{K_{m,\text{int,met1}}^{\text{NOR}}} + \frac{V_{\text{max}}^{\text{VER} \rightarrow \text{others}}}{K_{m,\text{met2}}^{\text{others}}} + \frac{V_{\text{max}}^{\text{VER}}}{K_{m,\text{sec}}} + \frac{V_{\text{max}}^{\text{VER} \rightarrow \text{NOR}}}{K_{m,\text{int,sec}}^{\text{NOR}}}$) was 0.311±0.189, and 68.3% of $\text{CL}_{L}^{\text{VER}}$ was conducive to formation of other verapamil metabolites; biliary secretion of verapamil was only 0.64% $\text{CL}_{L}^{\text{VER}}$. Based on the ratio of the fitted $V_{\text{max}}$ and $K_m$, the recalculated $h_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}}$ (31.1%) was around 10% higher than that based the lowest dose under linear condition (or 23.3%), and was significantly higher than that of Mehvar and coworkers (12%) (1994).

**PBPK modeling on stereoselective disposition and auto-inhibition of verapamil metabolism**
One limitation of the present study, however, was that stereoselectivity in verapamil and norverapamil disposition has not been fully addressed. In terms of stereoselective binding, it has been reported that R-VER has a higher (~1.6x) unbound fraction in plasma \( (f_p) \) over S-VER at both low and high concentrations (Mehvar et al., 1994; Mehvar and Reynolds, 1995; Robinson and Mehvar, 1996), but the \( C_B/C_P \) ratio for R-VER is ~1.5x times that of S-VER (Hanada et al. 2008). Since the unbound fraction in blood \( f_B = f_P*C_P/C_B \), we conclude that the unbound fractions in blood for S-VER and R-VER are similar, as are blood profiles of (R-) and (S)-verapamil. Therefore it is reasonable to assume that the \( f_B \) value for the racemic mixture was the same as those for S-VER and R-VER. We further found that small changes in \( f_B \) would not materially affect the clearances of S-VER, R-VER or racemic VER (simulations not shown). This is reasonable since the compounds are all highly cleared.

We had assumed that stereoselectivity in metabolism was not an important factor under our dosing input concentrations, which were high in relation to the \( K_m \)s (initial concentrations of 50 and 100 \( \mu \)M), and stereoselectivity effects in verapamil metabolism could be negligible (Mehvar and Reynolds, 1995; Hanada et al., 2008). As shown in the simulations based on literature data on \( K_m \)s for R- and S-VER (see Supplemental Table 3), the S-VER/R-VER \( K_m \) ratios were 1.9 and 1.34, respectively, pathways for the other metabolite formation and N-demethylation. The \( K_m \) estimates obtained for racemic or (R,S)-VER for the “other metabolites” or “N-demethylation” pathways (Table 5) were 10.4 and 14.1 \( \mu \)M, respectively. These averaged \( K_m \)s for (R,S)-verapamil corresponded to \( (1.9x +1x)K_m,R-VER/2 \) and \( (1.3x+1x)K_m,R-VER/2 \), respectively, for the “other metabolites” and “N-demethylation” pathways. From our calculations, the \( K_m \)s for R- and S-VER for “N-demethylation” are 9.04 and 11.8 \( \mu \)M, whereas those for the “other metabolites pathways” are 9.72 and 18.5 \( \mu \)M, respectively (Supplemental Table 3). Simulations with these \( K_m \)s constants obtained for R-VER and S-VER showed that the difference in stereoisomeric kinetics was minor (Figure 6), and existed only for the early-in-time
liver profiles at the lowest concentration. Also, the summed simulated R- and S-VER taken to represent racemic (R,S) profiles were similar to those observed (see Figure 7).

In addition, inhibitory effects of verapamil on its own P-gp-mediated biliary secretion and metabolite formation, events observed in humans, should be negligible during the short 90-min perfusion, inasmuch as lack of precedence in rat liver for auto-inhibition (Obach, 1999; Shibata et al., 2002; Axelsson et al., 2003; Guo et al., 2007; Yin et al., 2011). Moreover, incorporation of time-dependent inhibition components ($e^{-k_I t}$, where $k_I$ is the \textit{in vivo} inhibition rate constant, which equals the \textit{in vitro} inactivation rate constant, $k_{\text{obs}}$) (Wang et al., 2004) to the various verapamil eliminatory processes (or $CL_{\text{int}}$) failed to significantly improve the goodness-of-fit of the models (see Supplemental Table 4). The fitted $k_I$ values for metabolic formation ($CL_{\text{int,met1}}^{\text{VER->NOR}}$ and $CL_{\text{int,met2}}^{\text{VER->others}}$) and biliary secretions ($CL_{\text{int,sec}}^{\text{VER}}$) were low, ranging from 0.002 to 0.14 min$^{-1}$ (data not shown). Fitting results, with and without auto-inhibition, support the notion that time-dependent inactivation of various verapamil elimination pathways would not perturb the pharmacokinetics of verapamil in the perfused rat liver.
Discussion

Verapamil has been considered to be a good candidate to study transporter-enzyme interplay, due to the fact that it is a substrate of both CYP450 and P-gp. Under linear kinetic conditions, inhibition or induction of metabolism vs. excretion has been studied, since induction of the competing pathway reduces the intracellular substrate concentration for the given pathway (Pang et al., 2009). Data in the perfused rat liver preparation not only revealed nonlinear metabolism and secretion of verapamil (1, 50 and 100 μM) but a poor extent of biliary excretion (Table 5), suggesting that verapamil is a poor candidate for the examination of transporter-enzyme interplay between CYP450 and P-gp in liver. Additionally, the fact that fitted $K_m$ values for P-gp mediated excretion (4.75 and 5.36 μM) are lower than those for the metabolic pathways, including N-demethylation or formation of other metabolites (10.4 and 14.1 μM), suggests that transporter-enzyme interplay kinetics would be extremely complex under these nonlinear conditions. Induction of liver metabolism would not greatly perturb the extent of secretion since biliary excretion of verapamil was low, despite that verapamil would serve as a better candidate for the study of transporter-enzyme interplay in the intestine since there is more secreted (Johnson et al. 2001). However complexity in data interpretation still applies when nonlinearity metabolism and P-gp-mediated secretion exists, and when the secreted substrate can undergo reabsorption (Tam et al., 2003). Under nonlinear conditions, induction of intestinal P-gp that increases secretion could result in an increased extent of metabolism upon reabsorption of the secreted drug to furnish a more efficient metabolic system, as suggested by Pang and colleagues (Tam et al., 2003; Pang et al., 2009; Fan et al., 2010).

By contrast, verapamil is ideal for the consideration of metabolite (norverapamil) kinetics since there are competing metabolic pathways. Thus, we have utilized this precursor-product pair to develop strategies on the estimation of formation clearances of metabolites under linear and nonlinear conditions, for a drug that is bound to both red blood cell and plasma. After consideration of the substantial loss
due to tubing adsorption, we found that the extraction ratios of verapamil and preformed norverapamil were both high in the rat liver preparation, and the measured verapamil (~7-8 % dose) and formed norverapamil (~ 3-5 % dose) recovered in the liver tissue at the end of perfusion were similar to those in previous perfusion studies involving a high dose of verapamil administered (Mehvar et al., 1994). However, our reported average unbound fraction of verapamil in the blood perfusate ($f_{UB}^{VER}$) was higher (0.45 versus 0.19), and total intrinsic hepatic clearance of verapamil ($Cl_{int, tot}^{VER}$) was lower (16 vs. 50-130 mL·min$^{-1}$) compared to those reported by Mehvar and coworkers (Mehvar et al., 1994). The likely explanation is due to the consideration of verapamil binding to red blood cells and tubing as well as nonlinear metabolism in our study. That verapamil elimination is dose-dependent, with $\text{AUC}_{\infty}^{VER}$ increasing and $\text{Cl}_{L}^{VER}$ decreasing at higher dosing levels confirmed nonlinearity in verapamil disposition, an observation that is in agreement with that inferred for the clinical pharmacokinetics of verapamil (Freedman et al., 1981; Shand et al., 1981; Anderson et al., 1982; Tartaglione et al., 1983; Toffoli et al., 1997; Maeda et al., 2011).

Our strategy to study the kinetics of verapamil in the determination of whether formation of norverapamil is the major clearance pathway rests on capacity-limiting PBPK models, since these models are excellent for including sequential metabolism and drug- or metabolite-specific transporters and enzymes, distinguishing between kinetics of formed and preformed metabolites (Pang and Durk, 2010). In this study, we considered the nonlinear binding to plasma proteins and distribution into red cells and accounted for binding to tubing to fit the liver perfusion data of verapamil (1, 50 and 100 μM) and preformed (1.5 and 5 μM) and formed norverapamil simultaneously. For this reason, metabolite modeling of formed norverapamil was achieved with greater assurance. Additionally, the amount of verapamil and norverapamil binding to Tygon$^\text{®}$ tubing was not negligible (~10-25% of total dose), suggesting the necessity to account for drug loss due to tubing adsorption to avoid overestimation of hepatic clearance (calculated from $\text{Cl}_{L}^{VER} = \text{Cl}_{tot}^{VER} - \text{Cl}_{tubing}^{VER}$). We further emphasized that plasma
protein binding estimated in a test tube must be extended to include the total plasma space (plasma + Disse space) within the liver vasculature to consider equilibrium of drug between plasma and red blood cells (see Appendix B). Different PBPK models with increasing degrees of nonlinearity were then built to account for linear vs. nonlinear metabolism and biliary excretion by defining the intrinsic clearance \( \text{CL}_{\text{int}} \) in terms of \( V_{\text{max}} \) and \( K_m \), and the best-fitted model was determined according to AIC score, sum of squared weighted residuals (F-test) and diagnostic plots (prediction vs. observation and weighted residuals vs. time plots).

Our final PBPK model that showed the most significant improvement, with minimum AIC and sum of squared weighted residuals, incorporated multiple nonlinear factors describing metabolic and biliary secretion pathways of verapamil and norverapamil. PBPK modeling showed that stereoselective binding, metabolism and auto-inhibition were unimportant during our dosing conditions (see Supplemental Tables 3-4, Figures 6 and 7). Although PBPK modeling of verapamil and norverapamil that included nonlinear metabolic clearance and biliary excretion has been previously published (Neuhoff et al., 2013; Wang et al., 2013), to our knowledge, we are the first one to describe sequential metabolism and nonlinear kinetics of verapamil by fitting verapamil and its preformed and formed metabolite levels in blood perfusate and bile simultaneously. The fitted results have shown that rate of verapamil and formed and preformed norverapamil diffuse into the hepatocytes (\( \text{CL}_{\text{in}}^\text{VER} \) and \( \text{CL}_{\text{in}}^\text{NOR} \)) at rates that are much greater than the perfusate blood flow (\( Q_L \)), implying that these compounds did equilibrate rapidly between perfusate and liver. This finding is reasonable because verapamil and norverapamil are both lipophilic compounds. Since the efflux process outcompeted the biliary secretion (\( \text{CL}_{\text{ef}}^\text{VER} \gg \text{CL}_{\text{int,sec}}^\text{VER}, \text{CL}_{\text{ef}}^\text{NOR} \gg \text{CL}_{\text{int,sec}}^\text{NOR} \)), verapamil and norverapamil were expected to accumulate in the reservoir, with limited amounts appearing in bile, events that are consistent with our observations.

Our fitted unbound fraction of verapamil in the liver tissue (\( f_L^\text{VER} \)) matched literature values in rats (0.02) (Yamano et al., 2000), but was lower than that in healthy subjects (0.09) (Giacomini et al., 1984).
The $K_m$ values determined in the final model (H, Table 4) are quite low relative to perfusate concentration, ranging from 5-14 μM, suggesting at the high concentrations (50 and 100 μM), most of the enzymes and P-gp are saturated, and nonlinear kinetics will likely occur. The fitted $K_m$ values for metabolizing verapamil to norverapamil and other metabolites ($K_{m,met1}^{VER\rightarrow NOR}$ and $K_{m,met2}^{VER\rightarrow others}$, respectively) are in agreement with literature values (Tracy et al., 1999; Hanada et al., 2008) after adjusting for $f_L^{VER}$ or unbound fraction in liver. The reported $K_m$ for P-gp mediated biliary secretion of verapamil ($K_{m,sec}^{VER}$) are similar to that found in a previous study ($K_m = 4.1 \mu M$), where the biliary excretion of verapamil mediated by P-gp (~ 8 μM) was measured using human MDR1 reconstituted in liposomes (Kimura et al., 2007).

Proper estimation of norverapamil formation was adequately addressed in the study. With the assumption of linear pharmacokinetics, the fraction ($h_{mi}^{VER\rightarrow NOR}$) of hepatic clearance ($CL_L^{VER}$) that forms norverapamil can be calculated using the formula $h_{mi}^{VER\rightarrow NOR} = \frac{f_{mi}^{VER\rightarrow NOR}}{F_L^{NOR}}$ (Table 2) where $f_{mi}^{VER\rightarrow NOR}$ represents the fraction of $CL_L^{VER}$ that furnishes the formed norverapamil to the circulation, and $F_L^{NOR}$ denotes the hepatic availability of norverapamil, since $h_{mi}^{VER\rightarrow NOR}$ needs to account for the sequential metabolism of locally formed norverapamil, which never reaches the systemic circulation (Pang and Gillette, 1979; Pang and Kwan, 1983). The $h_{mi}^{VER\rightarrow NOR}$ value (0.12) from Mehvar and coworkers (Mehvar et al., 1994) was much underestimated due to their failure to account for sequential metabolism ($F_L^{NOR}$), the attendant nonlinearity in metabolism and binding to red blood cells. The $h_{mi}^{VER\rightarrow NOR}$ value (0.23) based on lowest dose data under assumption of linear kinetics (Table 2) was closer to the $h_{mi}^{VER\rightarrow NOR}$ value (0.31) based on the ratio of the formation intrinsic clearance, normalized to the summed intrinsic clearances using fitted $V_{max}$ and $K_m$ values for metabolism and biliary excretion (Table 5).

In conclusion, our comprehensive analysis with PBPK modeling revealed that the metabolic pathways converting verapamil to norverapamil and to other metabolites as well as the biliary excretion
of verapamil and norverapamil are concentration-dependent in verapamil and norverapamil liver perfusion data using PBPK modeling. Stereoselective binding and metabolism (Figures 6 and 7) and auto-inhibition (Supplemental Table 4) were unimportant. With the careful consideration of nonlinearity stepwise in PBPK modeling, we are able to unravel which elimination pathways are nonlinear and demonstrate a strategy to assess the fractional pathway of hepatic clearance responsible for either excretion of drug into bile or in formation of metabolites, after consideration of sequential metabolism of the metabolite and nonlinear protein binding of drug.
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**Authorship Contributions**

Participated in research design: Si, Sveigaard, and Pang

Conducted experiments: Si, Yang, Tang, Chow, Sveigaard, and Pang

Performed data analysis: Yang, Si, Tang, and Pang

Wrote or contributed to the writing of the manuscript: Yang, Si, and Pang
Reference


Footnotes

Qi Joy Yang and Luqin Si are co-first authors

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Figure Legends

Figure 1. Physiological-based pharmacokinetic (PBPK) model of verapamil (racemic) and norverapamil in the recirculating perfused rat liver. Perfusate blood flow rate \( Q_L \) interconnects the reservoir with liver blood compartment. The compartments are denoted as the reservoir (R), Tygon\textsuperscript{®} tubing (t), liver blood (LB), liver tissue (L), and bile. Enzymes (Cyps) and transporters (P-gp) are considered in the model. Both verapamil and norverapamil are adsorbed onto the Tygon\textsuperscript{®} tubing (top), and concentrations of unbound verapamil and norverapamil in reservoir blood (denoted by \( f_\text{VER}^{LB} \) and \( f_\text{NOR}^{LB} \), respectively) reach liver blood and rapidly diffuse into and out of the liver (via \( CL_{\text{in}} \) and \( CL_{\text{ef}} \), respectively, transport clearances that equal each other). Unbound verapamil in liver (of concentration \( f_\text{L}^{\text{VER}} \)) is metabolized to norverapamil via N-demethylation, with the intrinsic clearance, \( CL_{\text{int,met1}}^{\text{VER}} \). VER is also eliminated in liver to form other metabolites or is excreted into bile, with intrinsic clearances of \( CL_{\text{int,met2}}^{\text{VER\rightarrow others}} \) and \( CL_{\text{int,sec}}^{\text{VER}} \), respectively. Similarly, unbound norverapamil (of concentration \( f_\text{L}^{\text{NOR}} \)), the formed metabolite, is further metabolized (\( CL_{\text{int,met}}^{\text{NOR}} \)) and secreted (\( CL_{\text{int,sec}}^{\text{NOR}} \)). Since all these metabolic and secretory pathways are saturable, the intrinsic clearance parameters may be expressed in terms of \( V_{\text{max}} \) and \( K_m \) and well as unbound liver concentration (see Appendix C for mass balance equations). Two transit compartments (of amounts, \( A_{\text{tr1}}^{\text{VER}} \) and \( A_{\text{tr2}}^{\text{VER}} \); \( A_{\text{tr1}}^{\text{NOR}} \) and \( A_{\text{tr2}}^{\text{NOR}} \)) were defined in the model to account for the time delay (\( \tau \)) in biliary secretion.

Figure 2. Nonlinear plasma protein binding of verapamil (racemic) to BSA (2%) (A), and distribution of verapamil into red blood cells (B). (A) The unbound fraction of verapamil in plasma (\( f_P \)) was plotted against \( \log(C_P) \) to reveal concentration-dependent binding within the range of 0.4 - 140 \( \mu \text{M} \). The sold line represents \( f_P \) values predicted according to one class of binding site (fitted constants, \( n = 0.301\pm0.160 \) and \( K_A= 0.0099\pm0.0057 \mu\text{M}^{-1} \), Appendix A). (B) Observed (circle) and fitted (line)
are \( C_{\text{RBC}} \) and \( C_p \) over time \((n=6)\) in red blood cell distribution study (see Method for details). After the model was fitted to the data of verapamil in RBC and plasma, the transfer rate constants, \( k'_{rp} \) and \( k'_{pr} \), were found to be \(19.5 \pm 8.7\) and \(13.2 \pm 4.4\) min\(^{-1}\), respectively (Appendix A). The numbers next to the fitted curves are the total initial \( C_p \) (1 - 441 \( \mu \)M in 2% BSA) used for admixture with 40%-RBC containing blood perfusate (in 2% BSA).

**Figure 3.** The amounts (%dose) of verapamil (A) and preformed norverapamil (B) adsorbed onto Tygon® tubing of the perfusion apparatus. The solid symbols denote measured amounts (%dose), and the solid lines denote the fitted amounts over time (see Appendix C for mass balance equations). Different colored symbols and lines denote the different initial concentrations of verapamil (racemic) and norverapamil (racemic) used for perfusion.

**Figure 4.** Temporal profiles of fitted and observed verapamil and formed norverapamil in the perfusate (red), liver (green) and the cumulative secreted amounts in bile (blue) at initial concentrations of 1, 50 and 100 \( \mu \)M of racemic verapamil \((n=4)\) in recirculating rat liver preparations. The lines denote predictions according as the final fit with the PBPK model (Model H) describing various nonlinear pathways \( \frac{\text{CL}_{\text{int,met}}}{\text{VER}}\), \( \frac{\text{CL}_{\text{int,met}}}{\text{NOR}}\), \( \frac{\text{CL}_{\text{int,met}}}{\text{other}}\), \( \frac{\text{CL}_{\text{int,sec}}}{\text{VER}}\) and \( \frac{\text{CL}_{\text{int,sec}}}{\text{NOR}}\); see Appendix C for mass balance equations). The solid and open symbols are the observed concentrations or amounts of verapamil and formed norverapamil, respectively, in liver and bile.

**Figure 5.** Temporal profiles of observed (open symbols) and fitted preformed norverapamil in the reservoir perfusate (red), liver (green) and cumulative excreted amounts in bile (blue) at initial norverapamil (racemic) concentrations of 1.5 (A, \(n=4\)) and 5 (B, \(n=3\)) \( \mu \)M in recirculating rat liver preparations. The solid lines denote the fitted values according to the final PBPK model (model H) with nonlinear \( \text{CL}_{\text{sec}}\) (see Appendix C for mass balance equations).
Figure 6. Simulated concentrations of verapamil enantiomers (S- and R-verapamil) and their corresponding formed metabolites, S- and R-norverapamil, in reservoir perfusate, and amounts in liver and bile at initial concentrations of 0.5 (A), 25 (B) and 50 μM (C), concentrations (½ of the concentrations of racemic verapamil used experimentally). Stereoselective elimination, with K_m's shown in Supplemental Table 3 was considered for simulations (with equations shown in Appendix C). The black arrow in (A) showed that concentrations of R-VER in liver were slightly lower than those of S-VER at comparable perfusion time, due to the lower K_m for faster metabolism of R-VER at the lowest dose.

Figure 7. Simulated, concentrations or amounts of racemic verapamil (S-VER + R-VER) and formed norverapamil (S-NOR + R-NOR) in reservoir perfusate and liver (A, C, and E, left panel) and excreted amounts in bile (B, D, and F, right panel) (data of Figure 6, with consideration of stereoselective elimination, black solid or dotted lines) were presented against experimental (colored symbols) and fitted results on racemic verapamil and formed norverapamil from PBPK modeling (colored lines, also shown in Figure 4), without consideration of stereoselective elimination. The observed, fitted (lack of consideration of stereoselective elimination) and simulated data (with consideration of stereoselective elimination) of summed enantiomeric verapamil and norverapamil concentrations/amounts were virtually identical, showing that stereoselective elimination was unimportant under our conditions examined.
Table 1. Binding ($k_{R \rightarrow t}$) and debinding ($k_{t \rightarrow R}$) constants for verapamil and norverapamil to tubing of the perfusion apparatus, obtained by fitting of perfusate data in absence of a rat liver in sham-liver perfusion studies (Figure 1)

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<thead>
<tr>
<th>Verapamil (µM)</th>
<th>$k_{t \rightarrow R}^{VER}$ (min$^{-1}$)</th>
<th>$k_{R \rightarrow t}^{VER}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0206±0.0161</td>
<td>0.0083±0.0012</td>
</tr>
<tr>
<td>50</td>
<td>0.0400±0.0382</td>
<td>0.0052±0.0041</td>
</tr>
<tr>
<td>100</td>
<td>0.0197±0.0171</td>
<td>0.0032±0.0023</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Norverapamil (µM)</th>
<th>$k_{t \rightarrow R}^{NOR}$ (min$^{-1}$)</th>
<th>$k_{R \rightarrow t}^{NOR}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.0156±0.0013</td>
<td>0.0044±0.0005</td>
</tr>
<tr>
<td>5</td>
<td>0.0047±0.0063</td>
<td>0.0024±0.0007</td>
</tr>
</tbody>
</table>
Table 2. Experimentally-derived parameters with different verapamil initial concentrations delivered to the recirculating, red blood cell-perfused rat liver preparation at 12 mL·min⁻¹

| Intended and measured Verapamil concentration at t=0 (μM) |
|---|---|---|---|---|
| | 1 | 50 | 100 | P value |
| | 0.947 ± 0.064 | 47.5 ± 1.8 | 103 ± 0.7 | |

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formula</th>
<th>1 (n=4)</th>
<th>50 (n=4)</th>
<th>100 (n=4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CL}_{\text{L}}$ (mL·min⁻¹)</td>
<td>$\frac{\text{Dose}<em>{\text{VER}}}{\text{AUC}</em>{\infty}}$</td>
<td>10.5 ± 1.3</td>
<td>8.34 ± 0.60</td>
<td>8.91 ± 2.27</td>
<td>.244</td>
</tr>
<tr>
<td>$\text{CL}_{\text{tubing}}$ (mL·min⁻¹)</td>
<td></td>
<td>0.288 ± 0.043</td>
<td>0.180 ± 0.02</td>
<td>0.163 ± 0.043</td>
<td>.012*</td>
</tr>
<tr>
<td>$\text{CL}<em>{\text{VER}} = \text{CL}</em>{\text{L}} - \text{CL}_{\text{tubing}}$ (mL·min⁻¹)</td>
<td></td>
<td>10.2 ± 1.3</td>
<td>8.16 ± 0.63</td>
<td>8.75 ± 2.27</td>
<td>.274</td>
</tr>
<tr>
<td>$\text{CL}<em>{\text{bile}} = \frac{\text{AUC}</em>{\text{VER}}}{\text{AUC}_{\text{VER}}}$ (mL·min⁻¹)</td>
<td></td>
<td>0.118 ± 0.029</td>
<td>0.012 ± 0.004</td>
<td>0.022 ± 0.009</td>
<td>&lt;.0001*</td>
</tr>
<tr>
<td>$\text{CL}<em>{\text{met}} = \text{CL}</em>{\text{VER}} - \text{CL}_{\text{bile}}$ (mL·min⁻¹)</td>
<td></td>
<td>10.1 ± 1.3</td>
<td>8.15 ± 0.63</td>
<td>8.73 ± 2.27</td>
<td>.285</td>
</tr>
<tr>
<td>$\text{E}<em>{\text{VER}} = \frac{\text{CL}</em>{\text{L}}}{Q_L}$</td>
<td></td>
<td>0.850 ± 0.107</td>
<td>0.680 ± 0.053</td>
<td>0.729 ± 0.189</td>
<td>.282</td>
</tr>
<tr>
<td>$\text{F}<em>{\text{VER}} = 1 - \text{E}</em>{\text{VER}}$</td>
<td></td>
<td>0.150 ± 0.107</td>
<td>0.320 ± 0.053</td>
<td>0.271 ± 0.189</td>
<td>.365</td>
</tr>
<tr>
<td>$\text{F}<em>{\text{mi}}^{\text{VER} \rightarrow \text{NOR}} = \frac{\text{AUC}</em>{\text{VER}}}{\text{Dose}<em>{\text{VER}}} / \frac{\text{AUC}</em>{\infty}}{\text{Dose}_{\text{NOR}}}$</td>
<td></td>
<td>0.027 ± 0.008</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>$\text{f}_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}}$</td>
<td></td>
<td>0.012 ± 0.003</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>$h_{\text{mi}}^{\text{VER} \rightarrow \text{NOR}} = \frac{\text{f}<em>{\text{mi}}^{\text{VER} \rightarrow \text{NOR}}}{\text{NOR}</em>{\text{mi}}}$</td>
<td></td>
<td>0.232 ± 0.189</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

*significant difference between 1, 50 and 100 μM verapamil dosing groups P < .05.

*b fraction of hepatic clearance that furnishes norverapamil to circulation (Pang and Kwan, 1983); preformed norverapamil data was from Table 3.

c fraction of hepatic clearance that forms norverapamil from verapamil where $F_{\text{L}}^{\text{NOR}}$ is the hepatic availability of preformed norverapamil at the lowest dose from Table 3.
Table 3. Experimentally-derived parameters with dosing of preformed norverapamil at initial concentrations of approximately 1.5 and 5 μM

<table>
<thead>
<tr>
<th>Intended and measured preformed norverapamil concentration at t=0 (μM)</th>
<th>1.5 (n=4)</th>
<th>5 (n=3)</th>
<th>P Value</th>
</tr>
</thead>
</table>
| \[ CL_{\text{tot}}^{\text{NORp}} (\text{mL·min}^{-1}) = \]
| \[ CL^{\text{NORp}}_L + CL^{\text{NORp}}_{\text{tubing}} = \frac{\text{Dose}^{\text{NORp}}}{\text{AUC}_{90\text{min}}} \] | 11.2 ± 1.1 | 11.0 ± 1.0 | .857 |
| \[ CL^{\text{NORp}}_{\text{tubing}} (\text{mL·min}^{-1}) \] | 0.554 ± 0.079 | 0.253 ± 0.013 | .001<sup>a</sup> |
| \[ CL^{\text{NORp}}_L (\text{mL·min}^{-1}) = CL^{\text{NORp}}_{\text{tot}} - CL^{\text{NORp}}_{\text{tubing}} \] | 10.6 ± 1.1 | 10.8 ± 1.0 | .826 |
| \[ CL^{\text{NORp}}_{\text{bile}} (\text{mL·min}^{-1}) = \frac{\text{AUC}_{90\text{min}}^{\text{NORp}}}{\text{AUC}_{90\text{min}}} \] | 0.032 ± 0.004 | 0.044 ± 0.006 | .001<sup>a</sup> |
| \[ CL^{\text{NORp}}_{\text{met}} (\text{mL·min}^{-1}) = CL^{\text{NORp}}_L - CL^{\text{NORp}}_{\text{bile}} \] | 10.6 ± 1.1 | 10.8 ± 1.0 | .826 |
| \[ E_{\text{L}}^{\text{NORp}} = \frac{CL^{\text{NORp}}_L}{Q_L} \] | 0.883 ± 0.090 | 0.900 ± 0.081 | .796 |
| \[ F_{\text{L}}^{\text{NORp}} = 1 - E_{\text{L}}^{\text{NORp}} \] | 0.117 ± 0.090 | 0.100 ± 0.081 | .823 |
| \[ f_{\text{NOR}}^{\text{L}} = \frac{CL_{\text{bile}}^{\text{NORp}}}{CL_L} \] | 0.0031 ± 0.0005 | 0.0041 ± 0.0007 | .107 |

<sup>a</sup> Significant difference in norverapamil dosing groups (1.5 and 5 μM) P < .05.
Table 4. F-tests comparing various PBPK models (A to H) with different combinations of linear and nonlinear elimination pathways

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
<th>df&lt;sup&gt;a&lt;/sup&gt;</th>
<th>WSS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>F score&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Critical F value&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Significance&lt;sup&gt;e&lt;/sup&gt;</th>
<th>AIC values&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>No nonlinear pathway</td>
<td>189</td>
<td>653</td>
<td>N.A.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>3297</td>
</tr>
<tr>
<td>B</td>
<td>Nonlinear Cl&lt;sub&gt;VER→NOR&lt;/sub&gt;&lt;sub&gt;int,met1&lt;/sub&gt; only</td>
<td>188</td>
<td>837</td>
<td>-41.3</td>
<td>F(.05,1,188) = 3.83</td>
<td>No</td>
<td>3370</td>
</tr>
<tr>
<td>C</td>
<td>Nonlinear Cl&lt;sub&gt;VER→others&lt;/sub&gt;&lt;sub&gt;int,met2&lt;/sub&gt; only</td>
<td>188</td>
<td>808</td>
<td>-36.0</td>
<td>No</td>
<td>3322</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Nonlinear Cl&lt;sub&gt;int,sec&lt;/sub&gt; only</td>
<td>188</td>
<td>695</td>
<td>-11.4</td>
<td>No</td>
<td>3293</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Nonlinear Cl&lt;sub&gt;int,sec&lt;/sub&gt; only</td>
<td>187</td>
<td>287</td>
<td>119</td>
<td>F(.05,2,187) = 3.00</td>
<td>Yes</td>
<td>3244</td>
</tr>
<tr>
<td>F</td>
<td>Nonlinear Cl&lt;sub&gt;VER→NOR&lt;/sub&gt;&lt;sub&gt;int,met1&lt;/sub&gt;, Cl&lt;sub&gt;VER→sec&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt; &amp; Cl&lt;sub&gt;NOR&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt;</td>
<td>187</td>
<td>250</td>
<td>100</td>
<td>F(.05,3,186) = 2.60</td>
<td>Yes</td>
<td>3235</td>
</tr>
<tr>
<td>G</td>
<td>Nonlinear Cl&lt;sub&gt;VER→others&lt;/sub&gt;&lt;sub&gt;int,met1&lt;/sub&gt;, Cl&lt;sub&gt;VER→sec&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt; &amp; Cl&lt;sub&gt;NOR&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt;</td>
<td>186</td>
<td>234</td>
<td>111</td>
<td>Yes</td>
<td>3225</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Nonlinear Cl&lt;sub&gt;VER→NOR&lt;/sub&gt;&lt;sub&gt;int,met1&lt;/sub&gt;, Cl&lt;sub&gt;VER→others&lt;/sub&gt;&lt;sub&gt;int,met2&lt;/sub&gt;, Cl&lt;sub&gt;VER→sec&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt; &amp; Cl&lt;sub&gt;NOR&lt;/sub&gt;&lt;sub&gt;int,sec&lt;/sub&gt;</td>
<td>185</td>
<td>71.4</td>
<td>377</td>
<td>F(.05,4,185) = 2.37</td>
<td>Yes</td>
<td>3214</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degree of freedom is the number of data points (n = 198) used in the model minus the number of parameters being fitted.

<sup>b</sup>WSS is the sum of squared weighted residuals, which was calculated using \( \sum_{i=1}^{n} \frac{(\text{observed value}_i - \text{fitted value}_i)^2}{\text{observed value}_i} \). The total WSS for verapamil, preformed and formed norverapamil data in perfusate, bile and liver compartments was calculated.

<sup>c</sup>F score was calculated using \( \frac{\text{WSS}_0 - \text{WSS}_i}{\text{WSS}_0} \times \frac{\text{df}_i}{\text{df}_0 - \text{df}_i} \), where df denotes the degree of freedom and df<sub>i</sub> > df<sub>f</sub>.

<sup>d</sup>Critical F value was obtained from F-table with numerator of df<sub>f</sub> – df<sub>i</sub> and denominator of df<sub>i</sub>.

<sup>e</sup>Significance level was \( \alpha = .05 \); Model E-H showed significant improvement in goodness-of-fitting compared to the model A.

<sup>f</sup>Akaike information criteria (AIC), a measurement of goodness-of-fit, is calculated as 2k – 2ln(Likelihood), where k is the number of parameters fitted in the model, and Likelihood is the maximized value of the likelihood function.
Table 5. Fitted estimates obtained with fitting with the final model (Model H) to liver perfusion data (Table 4), with incorporation of nonlinear protein binding and nonlinear metabolism and biliary secretion of verapamil and norverapamil (see Appendix C for mass balance equations)

<table>
<thead>
<tr>
<th></th>
<th>Assigned Parameters</th>
<th>Fitted Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD (CV%)</td>
<td></td>
</tr>
<tr>
<td>Verapamil (VER, racemic) (μM) (n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of sinusoid, V&lt;sub&gt;LB&lt;/sub&gt; (mL)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.79 ± 0.23</td>
<td>2.70 ± 0.16</td>
</tr>
<tr>
<td>Volume of liver tissue, V&lt;sub&gt;L&lt;/sub&gt; (mL)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.16 ± 0.68</td>
<td>10.6 ± 0.6</td>
</tr>
<tr>
<td>Norverapamil (NOR, racemic) (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of sinusoid, V&lt;sub&gt;LB&lt;/sub&gt; (mL)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.93 ± 0.08 (2.73)</td>
<td>2.35 ± 0.15 (6.38)</td>
</tr>
<tr>
<td>Volume of liver tissue, V&lt;sub&gt;L&lt;/sub&gt; (mL)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.57 ± 0.22 (2.57)</td>
<td>9.23 ± 0.59 (6.39)</td>
</tr>
<tr>
<td>f&lt;sub&gt;L&lt;/sub&gt;VER: unbound fraction of VER in liver tissue</td>
<td>0.0878± 0.0050 (5.69)</td>
<td></td>
</tr>
<tr>
<td>f&lt;sub&gt;B&lt;/sub&gt;NOR: unbound fraction of NOR in liver blood perfusate</td>
<td>0.44 ± 0.05 (11.3)</td>
<td></td>
</tr>
<tr>
<td>f&lt;sub&gt;L&lt;/sub&gt;NOR: unbound fraction of NOR in liver tissue</td>
<td>0.00874 ± 0.00046 (5.26)</td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;in&lt;/sub&gt;VER = CL&lt;sub&gt;ef&lt;/sub&gt;VER: influx and efflux clearances of VER (mL·min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>526 ± 96 (18.3)</td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;in&lt;/sub&gt;NOR and CL&lt;sub&gt;ef&lt;/sub&gt;NOR: influx and efflux clearances for NOR (mL·min&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>482 ± 181 (37.6)</td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;int,met1&lt;/sub&gt;VER→NOR = V&lt;sub&gt;max,met1&lt;/sub&gt;&lt;sub&gt;VER→NOR&lt;/sub&gt; / K&lt;sub&gt;m,met1&lt;/sub&gt;&lt;sub&gt;VER→NOR&lt;/sub&gt;: intrinsic formation clearance of NOR from VER (mL·min&lt;sup&gt;-1&lt;/sup&gt;) under linear conditions</td>
<td>V&lt;sub&gt;VER→NOR&lt;/sub&gt;&lt;sub&gt;max,met1&lt;/sub&gt; = 96.6 ± 33.4 (34.6) nmole·min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;m,met1&lt;/sub&gt;&lt;sub&gt;VER→NOR&lt;/sub&gt; = 10.4 ± 4.1 (38.9) μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;int,met2&lt;/sub&gt;VER→others = V&lt;sub&gt;max,met2&lt;/sub&gt;&lt;sub&gt;VER→others&lt;/sub&gt; / K&lt;sub&gt;m,met2&lt;/sub&gt;&lt;sub&gt;VER→others&lt;/sub&gt;: metabolic intrinsic clearance of VER to form other metabolites (mL·min&lt;sup&gt;-1&lt;/sup&gt;) under linear conditions</td>
<td>V&lt;sub&gt;VER→others&lt;/sub&gt;&lt;sub&gt;max,met2&lt;/sub&gt; = 288 ± 51 (17.7) nmole·min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;m,met2&lt;/sub&gt;&lt;sub&gt;VER→others&lt;/sub&gt; = 14.1 ± 4.9 (34.7) μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;int,met&lt;/sub&gt;NOR: metabolic intrinsic clearance of NOR (mL·min&lt;sup&gt;-1&lt;/sup&gt;) under linear conditions</td>
<td>36.5± 18.4 (50.4)</td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;int,sec&lt;/sub&gt;VER = V&lt;sub&gt;max,sec&lt;/sub&gt;&lt;sub&gt;VER&lt;/sub&gt; / K&lt;sub&gt;m,sec&lt;/sub&gt;&lt;sub&gt;VER&lt;/sub&gt;: biliary intrinsic clearance of VER (mL·min&lt;sup&gt;-1&lt;/sup&gt;) under linear conditions</td>
<td>V&lt;sub&gt;max,sec&lt;/sub&gt;&lt;sub&gt;VER&lt;/sub&gt; = 0.911 ± 0.505 (55.4) nmole·min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;m,sec&lt;/sub&gt;&lt;sub&gt;VER&lt;/sub&gt; = 4.75 ± 2.29 (48.2) μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;int,sec&lt;/sub&gt;NOR = V&lt;sub&gt;max,sec&lt;/sub&gt;&lt;sub&gt;NOR&lt;/sub&gt; / K&lt;sub&gt;m,sec&lt;/sub&gt;&lt;sub&gt;NOR&lt;/sub&gt;: biliary intrinsic clearance of NOR (mL·min&lt;sup&gt;-1&lt;/sup&gt;) under linear conditions</td>
<td>V&lt;sub&gt;max,sec&lt;/sub&gt;&lt;sub&gt;NOR&lt;/sub&gt; = 0.776 ± 0.220 (28.4) nmole·min&lt;sup&gt;-1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;m,sec&lt;/sub&gt;&lt;sub&gt;NOR&lt;/sub&gt; = 5.36 ± 1.66 (31.0) μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>τ, time delay for biliary secretion (min)</td>
<td>4.49 ± 1.41 (31.4)</td>
<td></td>
</tr>
</tbody>
</table>
\[
\begin{align*}
\text{h}^\text{VER→NOR}_{\text{mi}} &= \frac{\frac{V_{\text{VER→NOR}}}{K_m,\text{mi}}}{\frac{V_{\text{VER→NOR}}}{K_m,\text{met1}} + \frac{V_{\text{VER→NOR}}}{K_m,\text{met2}} + \frac{V_{\text{VER→other}}}{K_m,\text{sec}}} \\
&= 0.311 \pm 0.189 \\
\text{h}^\text{VER→other}_{\text{mi}} &= \frac{\frac{V_{\text{VER→other}}}{K_m,\text{met1}}}{\frac{V_{\text{VER→other}}}{K_m,\text{met1}} + \frac{V_{\text{VER→other}}}{K_m,\text{met2}} + \frac{V_{\text{VER→other}}}{K_m,\text{sec}}} \\
&= 0.683 \pm 0.341 \\
\text{h}^\text{VER}_{e} &= \frac{\frac{V_{\text{VER}}}{K_{m,\text{sec}}}}{\frac{V_{\text{VER}}}{K_{m,\text{met1}}} + \frac{V_{\text{VER}}}{K_{m,\text{met2}}} + \frac{V_{\text{VER}}}{K_{m,\text{sec}}}} \\
&= 0.0064 \pm 0.0051
\end{align*}
\]

-the assigned parameters in PBPK model for fitting of verapamil, preformed and formed norverapamil simultaneously.

-from multiple indicator dilution studies in rat (Pang et al., 1988).

-ratio of intrinsic clearances is the same as ratio of hepatic clearance.
Appendix A: *In vitro* distribution of verapamil into RBC and plasma protein binding

The above schematics show the exchange of verapamil between RBC and plasma and for binding of verapamil to BSA. Here, $f_{RBC}$ is the unbound fraction of drug in RBC and $f_{RBC}C_{RBC}$ is the unbound drug concentration in RBC; correspondingly, $f_P$ is the unbound fraction of drug in plasma and $f_P C_P$ is the unbound drug concentration in plasma. The rates of change of verapamil in RBC and plasma in *in vitro* red cell distribution studies, where verapamil in plasma (2% BSA) was incubated with blank blood perfusate (40% RBC and 2% BSA) to result in a perfusate composition of 20% RBC and 2% BSA, appear in the equations shown below with rate constants, $k'_{rp} (= f_{RBC}*k'_{rp})$ and $k'_{pr} (= f_P*k'_{pr})$, based on total concentrations, since $f_{RBC}$ is unknown.

\[
\frac{dc_{RBC}}{dt} = k'_{pr} C_P \frac{(1-Hct)}{Hct} - k'_{rp} C_{RBC}
\]

\[
\frac{dc_P}{dt} = k'_{rp} C_{RBC} \frac{Hct}{(1-Hct)} - k'_{pr} C_P
\]

The binding of verapamil to one class of BSA binding site is given by:

\[
(1 - f_P) C_P = C_{P,\text{bound}} = \frac{nK_A C_{P,u} [P]}{1 + K_A C_{P,u}} \quad (A3)
\]

where $K_A$ is the binding association constant, and equals $k_1/k_2$ or ratio of the on- and off-rate constants for protein binding, $n$ is the number of binding site, $[P]$ is the total BSA concentration, $f_P$ is the unbound fraction in plasma and $C_{P,u}$ is the unbound concentration of verapamil in plasma. Equations A1-A3 were used simultaneously to fit data obtained in the red blood cell distribution and protein binding studies, and the fitted results are shown in Figure 2.
Appendix B: Incorporation of verapamil distribution into RBC and plasma protein binding for modeling liver perfusion data

In perfused liver experiments, drug in sinusoidal RBC equilibrates with drug in the total sucrose space or $V_{\text{plasma, tot}}$ (sinusoidal plasma and sucrose Disse space). The mass balance equation at equilibrium is summarized as the following:

$$V_B \text{ Hct } k'_{rp} C_{\text{RBC}} = V_{\text{plasma, tot}} k'_{pr} C_P$$  \hspace{1cm} (B1)

Upon substitution that $V_{\text{plasma, tot}}$ is $(1 + Y_{\text{Suc}}) V_p$, where $Y_{\text{Suc}}$ is the interstitial or sucrose Disse space/plasma space $V_p$, and knowing that $V_p = V_B(1 - \text{Hct})$, we obtain (Pang et al., 1988; Liu et al., 2005)

$$\frac{C_{\text{RBC}}}{C_P} = \frac{k'_{pr}(1 + Y_{\text{Suc}})(1 - \text{Hct})}{\text{Hct } k'_{rp}}$$  \hspace{1cm} (B2)

From mass balance, the sinusoidal blood ($C_B$), plasma ($C_P$) and red blood cell ($C_{\text{RBC}}$) concentrations are expressed as shown below.

$$C_B V_B = (1 - \text{Hct}) C_p V_B + \text{Hct } C_{\text{RBC}} V_B$$  \hspace{1cm} (B3)

Upon substitution of Eq. B2 into Eq. B3, one obtains,

$$\frac{C_B}{C_P} = R = \text{Hct } \left( \frac{C_{\text{RBC}}}{C_P} \right) + (1 - \text{Hct}) = \frac{k'_{pr}(1 + Y_{\text{Suc}})(1 - \text{Hct})}{k'_{rp}} + (1 - \text{Hct})$$  \hspace{1cm} (B4)

Nonlinearity in protein binding will result in a changing $C_B/C_P$ ratio, and this could be accommodated by Eq. B4, where $Y_{\text{Suc}}$ equals 0.52 (Pang et al., 1988).

The fitted $k'_{rp}$ and $k'_{pr}$ were used to calculate the ratio of drug concentrations in red blood cells and plasma ($\frac{C_{\text{RBC}}}{C_P}$) (Eq. B2) and $C_B$ from ($\frac{C_{\text{RBC}}}{C_P}$) (Eq. B4), ratio of drug concentrations in perfusate blood to plasma. However, for the sake of simplicity, binding of verapamil to liver tissue and norverapamil to liver and plasma proteins was assumed to be linear.
Appendix C: PBPK modeling of verapamil and formed and preformed norverapamil, with consideration of tubing adsorption in perfusion experiments

In following equations, superscripts VER, NOR and NORₚ denote the parent drug (verapamil) and its formed and preformed metabolite (norverapamil), respectively. A and C denote amount and concentration, respectively; Qₜ, V and f represent the perfusate blood flow rate, volume, and the unbound fraction, respectively; subscripts t → R, R → t, in and ef denote transfer from tubing to reservoir, from reservoir to tubing, influx from liver blood (LB) into liver and efflux from liver back to liver blood, respectively. Other pharmacokinetic parameters such as metabolic clearance and biliary secretion have been described previously under the Method Section.

Tubing adsorption was first considered in the model by incorporating dose-specific adsorption rate constants for VER and NORₚ. Then the influx clearance (CLᵢᵥₑᵣ or CLᵢⁿᵢᵣ) and efflux clearance (CLₑᵣᵥₑᵣ or CLₑᵣᵣᵣ) were assumed to be the same because verapamil and norverapamil are lipophilic drugs (logP = 3.79 and 3.3, respectively) and can diffuse freely across the membrane. Transit compartments were used to account for the time delay (τ) of verapamil and norverapamil biliary secretion, and two transit compartments (Transit 1 and Transit 2, denoted with amounts, Aₜ₁ and Aₜ₂, respectively) best described the bile data (data not shown). Additionally, CLᵢⁿᵢᵣ, CLₑᵣᵥₑᵣ, CLₑᵣᵣᵣ, CLᵢᵣᵣᵣ and kᵣᵣᵣ and kᵣᵣᵣ were assigned as the same constants for preformed and formed norverapamil. Data of three input concentrations of verapamil, two input concentrations of preformed norverapamil and the corresponding formed norverapamil from verapamil in perfusate, liver and bile were fitted simultaneously using PBPK model (Figure 1).

For rates of change of verapamil in perfusate (B) adsorbed on to Tygon® tubing (t) during verapamil perfusion without liver:

\[
\frac{dA_{B}^{VER}}{dt} = k_{t \rightarrow R}^{VER} A_{tubing}^{VER} - k_{R \rightarrow t}^{VER} A_{B}^{VER} \quad (C1)
\]

\[
\frac{dA_{tubing}^{VER}}{dt} = -k_{t \rightarrow R}^{VER} A_{tubing}^{VER} + k_{R \rightarrow t}^{VER} A_{B}^{VER} \quad (C2)
\]

For rates of change of norverapamil (both preformed and formed) in perfusate (B) and adsorbed onto Tygon® tubing (t) during norverapamil perfusion without liver:

\[
\frac{dA_{B}^{NOR}}{dt} = k_{t \rightarrow R}^{NOR} A_{tubing}^{NOR} - k_{R \rightarrow t}^{NOR} A_{B}^{NOR} \quad (C3)
\]

\[
\frac{dA_{tubing}^{NOR}}{dt} = -k_{t \rightarrow R}^{NOR} A_{tubing}^{NOR} + k_{R \rightarrow t}^{NOR} A_{B}^{NOR} \quad (C4)
\]

Fitted values of \( k_{t \rightarrow R}^{VER}, k_{R \rightarrow t}^{VER}, k_{t \rightarrow R}^{NOR} \) and \( k_{R \rightarrow t}^{NOR} \) are summarized in Table 1, and these values were fixed for later modeling with verapamil and preformed and formed norverapamil data in perfused liver preparations.
For rates of change of verapamil in reservoir perfusate (B), tubing, liver blood (LB, whole liver blood; L_{RBC}, liver red blood cells; L_{P}, liver plasma), and liver (L) and bile compartments:

\[
\frac{dA_{\text{VER}}}{dt} = k_{t \to R} A_{\text{VER}}^{\text{tubing}} - k_{R \to t} A_{\text{VER}}^{\text{LB}} + Q_L (C_{B,\text{LB}} - C_{B,\text{VER}}) \tag{C5}
\]

\[
\frac{dA_{\text{VER}}}{dt}^{\text{tubing}} = -k_{t \to R} A_{\text{VER}}^{\text{tubing}} + k_{R \to t} A_{\text{VER}}^{\text{LB}} \tag{C6}
\]

\[
\frac{dA_{\text{VER}}}{dt}^{\text{LB}} = -Q_L (C_{B,\text{LB}} - C_{B,\text{VER}}) - f_{\text{VER}}^{\text{LB}} C_{\text{LB}}^{\text{CL}_{\text{L}}^{\text{in}}} + f_{\text{L}}^{\text{VER}} C_{\text{L}}^{\text{CL}_{\text{e}}_{\text{f}}^{\text{VER}}} \tag{C7}
\]

\[
\frac{dA_{\text{VER}}}{dt}^{\text{L}} = f_{\text{B}}^{\text{VER}} C_{\text{L}}^{\text{CL}_{\text{L}}^{\text{in}}} - f_{\text{L}}^{\text{VER}} C_{\text{L}}^{\text{CL}_{\text{e}}_{\text{f}}^{\text{VER}}} + f_{\text{CL}_{\text{int},\text{met1}}}^{\text{VER}} + f_{\text{CL}_{\text{int},\text{met2}}}^{\text{VER}} + f_{\text{CL}_{\text{int},\text{sec}}}^{\text{VER}} \tag{C8}
\]

For modeling of the perfusion data, the unbound concentration of verapamil in blood perfusate (C_{B,u}) was assumed to equal C_{P,u} based on one class of binding sites.

\[
C_{B,u} = C_{P,u} = \frac{\left((-1+n[P_t]K_A-K_A C_P)+\sqrt{\left([-1+n[P_t]K_A-K_A C_P]\right)^2+4K_A C_P}\right)}{2K_A} \tag{C9}
\]

We then converted the C_P to C_B based on the C_B/C_P ratio (R) in Eq. B4 according to the fitted k\text{'}_{P,B} and k\text{'}_{P,R} and Y_{Suc} values, and express the unbound concentration in blood perfusate as

\[
f_{\text{B}}^{\text{VER}} C_{\text{B}}^{\text{VER}} = \frac{\left((-1+n[P_t]K_A-K_A (C_{LB}/R))+\sqrt{\left([-1+n[P_t]K_A-K_A (C_{LB}/R)]\right)^2+4K_A (C_{LB}/R)}\right)}{2K_A} \tag{C10}
\]

Fitted constants obtained from the in vitro binding and distribution studies on n=0.301, the binding association constant K_A (0.0091 μM^{-1}) and [P_t], total BSA concentration in plasma (151.5 μM) were used (appendix A).

For biliary excretion,

\[
\frac{dA_{\text{VER}}}{dt}^{\text{bile}} = f_{\text{L}}^{\text{VER}} C_{\text{L}}^{\text{CL}_{\text{int},\text{sec}}} \tag{C11}
\]

For the time delay of verapamil biliary secretion:

\[
\frac{dA_{\text{VER}}}{dt}^{\text{Tr1}} = \frac{A_{\text{VER}}^{\text{bile}} - A_{\text{VER}}^{\text{Tr1}}}{\tau} \tag{C12}
\]

\[
\frac{dA_{\text{VER}}}{dt}^{\text{Tr2}} = \frac{A_{\text{VER}}^{\text{Tr2}} - A_{\text{VER}}^{\text{Tr1}}}{\tau} \tag{C13}
\]
For rates of **formed** norverapamil in the reservoir, tubing, liver blood perfusate, liver and blood:

\[
\frac{dA_{\text{bile}}^{\text{NOR}}}{dt} = k_{t \rightarrow R}^{\text{NOR}} A_{\text{tubing}}^{\text{NOR}} - k_{R \rightarrow t}^{\text{NOR}} A_{B}^{\text{NOR}} + Q_L (C_{\text{LB}}^{\text{NOR}} - C_{B}^{\text{NOR}})
\]  

(C14)

\[
\frac{dA_{\text{tubing}}^{\text{NOR}}}{dt} = -k_{t \rightarrow R}^{\text{NOR}} A_{\text{tubing}}^{\text{NOR}} + k_{R \rightarrow t}^{\text{NOR}} A_{B}^{\text{NOR}}
\]  

(C15)

\[
\frac{dA_{B}^{\text{NOR}}}{dt} = -Q_L (C_{\text{LB}}^{\text{NOR}} - C_{B}^{\text{NOR}}) - f_{B}^{\text{NOR}} C_{LB}^{\text{NOR}} C_{\text{LB}}^{\text{NOR}} + f_{L}^{\text{NOR}} C_{L}^{\text{NOR}} C_{\text{LB}}^{\text{NOR}} + f_{L}^{\text{NOR}} C_{L}^{\text{NOR}} C_{\text{LB}}^{\text{NOR}}
\]  

(C16)

\[
\frac{dA_{\text{bile}}^{\text{NOR}}}{dt} = f_{L}^{\text{NOR}} C_{L}^{\text{NOR}} C_{\text{LB}}^{\text{NOR}} + f_{L}^{\text{VER}} C_{L}^{\text{VER}} C_{\text{int,met1}}^{\text{VER}} - f_{L}^{\text{NOR}} C_{L}^{\text{NOR}} (C_{\text{eff}}^{\text{NOR}} + C_{\text{int,met}}^{\text{NOR}} + C_{\text{int,sec}}^{\text{NOR}})
\]  

(C17)

For the time delay of norverapamil biliary secretion:

\[
\frac{dA_{\text{tubing}}^{\text{NOR}}}{dt} = \frac{A_{\text{bile}}^{\text{NOR}} - A_{\text{tubing}}^{\text{NOR}}}{\tau}
\]  

(C19)

\[
\frac{dA_{\text{tubing}}^{\text{NOR}}}{dt} = \frac{A_{\text{tubing}}^{\text{NOR}} - A_{\text{tubing}}^{\text{NOR}}}{\tau}
\]  

(C20)

For saturable N-demethylation, \(C_{\text{int,met1}}^{\text{VER}}\) is:

\[
C_{\text{int,met1}}^{\text{VER}} = \frac{V_{\text{VER}}^{\text{NOR}}}{V_{\text{VER}}^{\text{max,met1}}} + f_{L}^{\text{VER}}
\]  

(C21)

For saturable metabolic pathway: verapamil to other metabolites, \(C_{\text{int,met2}}^{\text{VER}}\) is:

\[
C_{\text{int,met2}}^{\text{VER}} = \frac{V_{\text{VER}}^{\text{others}}}{V_{\text{VER}}^{\text{max,met2}}} + f_{L}^{\text{VER}}
\]  

(C22)

For saturable verapamil and norverapamil biliary excretion, \(C_{\text{int,sec}}^{\text{VER}}\) and \(C_{\text{int,sec}}^{\text{NOR}}\) are:

\[
C_{\text{int,sec}}^{\text{VER}} = \frac{V_{\text{VER}}^{\text{max,sec}}}{V_{\text{VER}}^{\text{max,sec}} + f_{L}^{\text{VER}} C_{L}^{\text{VER}}}
\]  

(C23)

\[
C_{\text{int,sec}}^{\text{NOR}} = \frac{V_{\text{NOR}}^{\text{max,sec}}}{V_{\text{NOR}}^{\text{max,sec}} + f_{L}^{\text{NOR}} C_{L}^{\text{NOR}}}
\]  

(C24)

For rates of change of **preformed norverapamil** in the perfusate (B), tubing, liver blood (LB), liver (L) and bile compartments:

\[
\frac{dA_{\text{bile}}^{\text{NOR}}}{dt} = k_{t \rightarrow R}^{\text{NOR}} A_{\text{tubing}}^{\text{NOR}} - k_{R \rightarrow t}^{\text{NOR}} A_{B}^{\text{NOR}} + Q_L (C_{\text{LB}}^{\text{NOR}} - C_{B}^{\text{NOR}})
\]  

(C25)

\[
\frac{dA_{\text{tubing}}^{\text{NOR}}}{dt} = -k_{t \rightarrow R}^{\text{NOR}} A_{\text{tubing}}^{\text{NOR}} + k_{R \rightarrow t}^{\text{NOR}} A_{B}^{\text{NOR}}
\]  

(C26)
\[
\frac{dA_{\text{LB}}^{\text{NOR}}}{dt} = -Q_L \left(C_{\text{LB}}^{\text{NOR}} - C_B^{\text{NOR}}\right) - f_B^{\text{NOR}} C_{\text{LB}}^{\text{NOR}} \text{CL}_{\text{in}}^{\text{NOR}} + f_L^{\text{NOR}} C_L^{\text{NOR}} \text{CL}_{\text{ef}}^{\text{NOR}} \tag{C27}
\]
\[
\frac{dA_{\text{bile}}^{\text{NOR}}}{dt} = f_B^{\text{NOR}} C_{\text{LB}}^{\text{NOR}} \text{CL}_{\text{in}}^{\text{NOR}} - f_L^{\text{NOR}} C_L^{\text{NOR}} (\text{CL}_{\text{ef}}^{\text{NOR}} + \text{CL}_{\text{int,met}}^{\text{NOR}} + \text{CL}_{\text{int,sec}}^{\text{NOR}}) \tag{C28}
\]
\[
\frac{dA_{\text{TR1}}^{\text{NOR}}}{dt} = \frac{A_{\text{Dile}}^{\text{NOR}} - A_{\text{TR1}}^{\text{NOR}}}{\tau} \tag{C30}
\]
\[
\frac{dA_{\text{TR2}}^{\text{NOR}}}{dt} = \frac{A_{\text{TR2}}^{\text{NOR}} - A_{\text{TR1}}^{\text{NOR}}}{\tau} \tag{C31}
\]
\[
\text{CL}_{\text{int,sec}}^{\text{NOR}} = \frac{v_{\text{max,sec}}^{\text{NOR}}}{k_{\text{m,sec}}^{\text{NOR}} + f_L^{\text{NOR}} C_L^{\text{NOR}}} \tag{C32}
\]

For nonlinear biliary excretion of norverapamil, \(\text{CL}_{\text{int,sec}}^{\text{NOR}}\) is:

\[
\text{CL}_{\text{int,met}}^{\text{NOR}} \text{ may also be expressed as } (\text{CL}_{\text{int,met}}^{\text{NOR}} = \frac{v_{\text{max,met}}^{\text{NOR}}}{k_{\text{m,met}}^{\text{NOR}} + f_L^{\text{NOR}} C_L^{\text{NOR}}}); \text{ however in the preliminary study, incorporation of this nonlinear term failed to significantly improve the goodness-of-fitting when norverapamil data alone was fitted (data not shown), and only first-order conditions were assumed, and nonlinearity was not considered when all data was fitted simultaneously.}
Figure 3

A

B

Amount of Verapamil in Reservoir Perfusate (% dose) vs. Time (min)

Amount of Nonverapamil in Reservoir Perfusate (% dose) vs. Time (min)

1 µM
50 µM
100 µM

1.5 µM
5 µM