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FIRST-PASS EFFECTS OF VERAPAMIL ON THE INTESTINAL ABSORPTION AND LIVER DISPOSITION OF FEXOFENADINE IN THE PORCINE MODEL

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Running title: Absorption and disposition of fexofenadine in the porcine model

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List of abbreviations:

ABCB1	ATP binding cassette B1, P-glycoprotein, MDR1
OATP2B1	Organic anion transport polypeptide 2B1, OATP-B, SLCO 2B1
BCS	Biopharmaceutical classification system
E_H	Liver extraction
P_{eff}	Jejunal effective permeability
f_{abs}	Fraction of the dose available that is absorbed
$A_{e,\ bile}$	Amount of dose excreted in the bile
$f_{e,\ bile}$	Fraction of dose excreted in the bile
$I_{in,\ max}$	Maximum concentration entering the liver
CL_{bile}	Biliary clearance

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ABSTRACT

The aim of this study in pigs was to investigate the local pharmacokinetics of fexofenadine in the intestine and liver by using the pig as model for drug transport in the entero-hepatobiliary system. A parallel group design including seven pigs (10-12 weeks, 22.2-29.5 kg) in three groups (G1, G2, G3) using a jejunal single-pass perfusion combined with sampling from the bile duct and the portal, hepatic and superior caval veins was performed. Fexofenadine was perfused through the jejunal segment alone (G1; 120 mg/L, total dose 24 mg) or with two different verapamil doses (G2; 175 mg/L, total dose 35 mg and G3; 1000 mg/L, total dose 200 mg). The animals were fully anaesthetized and monitored throughout the experiment. Fexofenadine had a low liver extraction (E_H; mean ± SEM) and neither the given doses of verapamil did affect the E_H (0.13 ± 0.04, 0.16 ± 0.03 and 0.12 ± 0.02 for G1, G2 and G3, respectively) or biliary clearance. The E_H for verapamil and antipyrine agreed well with human in vivo data. Verapamil did not increase the intestinal absorption of fexofenadine even though the jejunal permeability of fexofenadine, verapamil and antipyrine showed a tendency to increase in G2. This combined perfusion and hepatobiliary sampling method showed that verapamil did not affect the transport of fexofenadine in the intestine or liver. The model had a similar E_H for both verapamil and antipyrine as the corresponding values in vivo in humans.

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The effect of carrier-mediated membrane transport on absorption, first-pass liver extraction, distribution and elimination is not established, since the exact in vivo transport mechanisms and its relevance for many drugs that are transport substrates is far from completely understood. Although, our knowledge in the field has greatly increased, the integral view on the practical implications of the related findings for ADME research lacks the relevant human in vivo data and a stringent use of data from in vitro models (Meijer and Lennernas, 2005). In addition, investigations of the local liver exposure of drugs and metabolites are considered to be important when assessing dose dependent drug-induced hepatoxicity (Lee, 2003). Consequently, a model enabling direct determinations to be made of the in vivo kinetics of intestinal and hepatobiliary transport and the metabolism of drugs would be a valuable tool. The use of such a model should increase the understanding of the mechanisms underlying the processes determining the bioavailability and local liver exposure and have an impact on pharmacokinetics and the safety evaluation of drugs. This paper describes a novel pig model that combines an intestinal perfusion technique, previously validated in vivo in humans (Lennernas et al., 1992), with sampling from the portal and hepatic vein and bile collection.

The transport of a compound across biological membranes often involves multiple transport mechanisms. Hence, the intestinal absorption and disposition of drugs and metabolites is a complex process, governed by the composition of the membrane and physicochemical properties, affinity to membrane transporters and the local concentrations of the substances. The interpretation of the quantitive importance of membrane transporter on pharmacokinetics is also hard to distinguish from the impact of the metabolism of a substance.

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The low plasma protein bound histamine H_1 receptor antagonist fexofenadine is a drug with a negligible metabolism in humans (Lippert et al., 1999) and a low intestinal permeability in vivo, making it a suitable candidate as a molecular probe for the complex in vivo assessment of a drug transport protein (Hamman et al. 2001, Wang et al, 2002, Tannergren et al., 2003a; Tannergren et al., 2003b) Earlier in vitro results have shown that fexofenadine is transported by various mechanisms across biological membranes, including transcellular passive diffusion and, to some extent, P-glycoprotein (ABCB1) and organic anion transporting polypeptides (OATPs) (Cvetkovic et al., 1999; Dresser et al., 2002; Perloff et al., 2002; Kobayashi et al., 2003; Nozawa et al., 2004; Petri et al., 2004). Several clinical studies have reported drug-drug and drug-diet interactions between fexofenadine and substrates or inhibitors of both these transporters (Davit et al., 1999; Hamman et al., 2001; Dresser et al., 2002; Wang et al., 2002; Tannergren et al., 2003b).

Previously, we perfused the human jejunum in vivo and showed that concomitant administration of verapamil or ketoconazole with fexofenadine did not increase the low in vivo effective jejunal permeability (P_{eff}) of fexofenadine (Tannergren et al., 2003a; Tannergren et al., 2003b). However, the study conducted with verapamil demonstrated that the systemic exposure of fexofenadine increased 4-fold in the presence of the inhibitor (Tannergren et al., 2003b). It was suggested that this could be related to inhibition of verapamil on the OATP-mediated liver uptake from the sinusoids and/or secretion canalicular ABCB1 into the bile (Cvetkovic et al., 1999; Perloff et al., 2002; Petri et al., 2004). The absence of an effect on the in vivo jejunal permeability may be explained by dual inhibition of verapamil on the jejunal OATP uptake and on ABCB1 enterocyte secretion, as these are believed to be working in

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opposite directions in the intestine (Dresser et al., 2002; Tannergren et al., 2003b), although it could also be the case that only passive diffusion regulates the absorption. The pig has been used extensively in biomedical research and is commonly used for physiological studies of the digestive system. Pigs have received increased attention as potential models for investigating the impact of intestinal absorption and the first pass effects on drugs (Soucek et al., 2001; Tang et al., 2004). The expression and function of membrane transport proteins and enzymes have been explored less thoroughly in the pig than in the rat, but it has been reported that orthologs to ABCB1 and OATPs are present in the intestine and liver of pigs (Soucek et al., 2001; Goh et al., 2002; Tang et al., 2004).

The aim of the present study was to explore the entero-hepatic transport of fexofenadine by investigations of the local pharmacokinetics of fexofenadine when administered alone or together with verapamil.

Materials and Methods

Animals and experimental design. The study was of parallel group design and consisted of three groups (G1, G2 and G3) of pigs with seven animals in each group. 15 male and 6 female pigs of mixed breed (Hampshire, Yorkshire and Swedish Landrace) 10-12 weeks old and weighing 27.7 ± 0.4 kg (range 22.2-29.5 kg) were used.

The study was performed at the Clinical Research Department, Uppsala University Hospital, Sweden, and was approved by the local ethics committee for the use of laboratory animals in Uppsala, Sweden. Each perfusion was conducted for 100 minutes. The perfusate was sampled in 10-minute fractions and blood samples from the portal vein, the hepatic vein and the superior caval vein were withdrawn and bile

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collected every 20 minutes during the first 200 minutes and then 200, 240, 300 and 360 minutes after the start of the perfusion. A schematic drawing of the animal model is shown in Figure 1.

Investigational drugs. The perfusion solution consisted of potassium chloride 5.4 mM, sodium chloride 48 mM, mannitol 35 mM, D-glucose 10 mM and PEG 4000 1.0 g/L, all dissolved in a 70 mM phosphate buffer with pH 6.5 and an osmolality of 290 mOsm/kg. This perfusion buffer is validated and has been used as a vehicle in several perfusion studies (Lennernas et al., 1992; Sandstrom et al., 1999; Tannergren et al., 2003a; Tannergren et al., 2003b). In all the groups the jejunal segment was perfused with the perfusion solution containing 120 mg/L (223 µM) fexofenadine (TELFAST®; Aventis Pharma, Strasbourg, France). In the treatment groups, fexofenadine was co-administered with 175 mg/L (356 µM; G2) or 1000 mg/L (2030 µM; G3) of verapamil. Verapamil was the kind gift of Knoll AG, Ludwigshafen, Germany. The total dose of fexofenadine in the different groups was 24 mg and the doses given of verapamil were 35 mg and 200 mg in G2 and G3, respectively. The concentration of fexofenadine was the same as in an earlier study in humans and the verapamil had the same dose per kg in G2 and a higher intraluminal concentration in G3 (Tannergren et al., 2003b). Antipyrine (Astra Läkemedel AB, Södertälje, Sweden; 200 mg/L, 1.1 mM) was used as a marker for passive transcellular diffusion in the perfusion experiments and for first pass extraction in the liver. [^{14}C]-labeled polyethylene glycol 4000 (^{14}C-PEG 4000; 2.5 mCi/L, Amersham Pharmacia Biotech, Little Chalfont, England) was used as a non-absorbable volume marker.

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Experimental procedures. The animals were sedated by an intramuscular administration of tiletamine and zolazepam (ZOLETIL, Virbac S.A., Carros, France) 6 mg/kg, xylazin (ROMOPUN VET, 20 mg/mL, Bayer AG, Leverkusen, Germany) 2.2 mg/kg, Atropin (ATROPIN NM PHARMA 0.5 mg/mL, Merck NM AB, Stockholm, Sweden) 0.04 mg/kg in the transport box. Morphine (Morphine 10 mg/mL, Meda AB, Solna, Sweden) 1 mg/kg was administered intravenously in a peripheral vein in the ear to provide analgesia. A breathing tube was introduced through an incision in the throat. The pig was kept ventilated using an oxygen-air mix. A central venous catheter (CVC) was introduced into the jugular vein to be located in the superior caval vein of the pig. A mixture of ketamine (KETAMINOL VET, 100 mg/mL, Intervet, Stockholm Sweden) 20 mg/kg/hour, morphine (Morphine 10 mg/mL Meda AB, Solna, Sweden) 0.5 mg/kg/hour and pancuronium bromid (PAVULON 2 mg/mL, Organon AB, Gothenburg, Sweden) 0.25 mg/kg/hour were then given continuously intravenously through the CVC during the experiment to ensure that the animal was painless and anaesthetized. A second catheter was introduced into the other jugular vein and passed down into the hepatic vein; the location was verified by fluoroscopy (x-ray).

A midline laparotomy was performed and a catheter was placed in the portal vein for blood sampling. The bile duct was cannulated for the bile collection and a ligature was placed below the insertion of the catheter to prevent the bile from reaching the intestine. Drainage of urine and gastric fluids from the bladder and pylorus, respectively, was also provided. The Loc-I-Gut® perfusion tube (Synetics Medical, Stockholm, Sweden) was introduced through an incision in the duodenum and passed down into the jejunum. The multichannel tube is 175 cm long and made of polyvinylchloride with an external diameter of 5.3 mm (16 French). It contains six

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channels and is provided distally with two elongated 40 mm long elongated latex balloons, placed 10 cm apart, each separately connected to one of the smaller channels. The two wider channels located in the center of the tube are for infusion and aspiration of perfusate. The two remaining channels are intended for administration of a marker substance or for drainage. In this study, ligatures were placed around the jejunum at the normal position of the balloons on the tube, creating a 10 cm long segment. The segment was then rinsed with isotonic sodium chloride solution (37ºC) for at least 30 min allowing the animal to stabilize after the surgery. Stable perfusion conditions were usually attained within this 30 min period and the perfusion solution (37ºC) was pumped into the segment at a flow rate of 2 mL/min with a calibrated syringe pump (Model 355, Sage Instruments, Orion Research Inc, Cambridge, USA). The perfusate leaving the jejunal segment during the single-pass perfusion was quantitatively collected on ice at 10-minute intervals and immediately frozen at -20°C pending analysis. Immediately upon completion of the perfusion experiment (100 min), the jejunal segment was rinsed with 120 mL of isotonic saline to terminate the intestinal drug absorption process. Bile was collected and venous whole blood samples (5 mL) were drawn from the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.) at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 240, 300 and 360 minutes after the start of the perfusion. Blood samples were collected in glass tubes containing heparin and centrifuged at 1000 g for 10 minutes at 4°C. The plasma obtained was divided in two aliquots consisting of at least 1 mL each. The collected fractions of bile were weighed. All of the samples from the plasma, bile and perfusate were stored at –20°C pending analysis.

The status of the animal was continuously monitored during the experiment by recording the heart rate and blood pressure and blood-gas analyses. MACRODEX 60

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mg/ml (Pharmalink AB, Upplands Väsby, Sweden) was given when needed to retain the colloid osmotic pressure. The animal was terminated through the administration of potassium chloride, 10-15 ml (20-30 mmol), given in the superior caval vein.

Perfusate analysis. The fexofenadine and verapamil in the perfusate and perfusion solution were analyzed by HPLC with UV detection. The HPLC system consisted of a Shimadzu LC-9A pump (Shimadzu, Kyoto, Japan) and a Spectra 100 UV detector (Thermo Separation Products, San Jose, CA, USA) operated at 220 nm. The analytical column was a 5 µm Nucleosil 100 C18 column (150 x 4.6 mm length x inner diameter Chrompack). The mobile phase consisted of acetate buffer (pH 5.0, ionic strength 0.01): methanol: acetonitrile 42:35:23 % (v/v). The flow rate was 1 mL/min and the injection volume was 20 µL. The limit of quantification (LOQ) was set to 3.7 mg/L and 2.5 mg/L for fexofenadine and verapamil, respectively. The standard curves were linear in the range 3.7-149.6 mg/L and 2.5-199.6 mg/L. The CV of the inter-assay variability (N = 6; quality controls containing 6.5, 64.6 and 129.2 mg/L and 8.9, 88.8 and 177.6 mg/L for fexofenadine and verapamil, respectively) ranged between 0.7 and 3.6 % with an accuracy ranged between 1.1 and 5.2%. The concentrations of antipyrine were analyzed using an HPLC method with UV detection according to a previously validated method (Sandstrom et al., 1999). The total radioactivity of ^{14}C-PEG 4000 in the perfusion solution and the perfusate samples was determined by liquid scintillation counting (Mark III, Searle Analytic Inc., Des Plaines, IL, USA). The osmolality and pH of the perfusion solution and perfusate samples were measured with the vapor pressure method (5500 vapor pressure osmometer, Wescor Inc., Logan, UT, USA) and a pH meter (632 pH-Meter, Metrohm AG, Herisau, Switzerland), respectively.

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Plasma and bile analysis. Fexofenadine, verapamil and antipyrine were simultaneously quantified in the plasma samples. 100 µL of internal standard solution ([$^2H_6$]-fexofenadine 7.6 ng, [$^2H_3$]-verapamil 7.8 ng and propylphenazone 25 ng) and 1.0 mL of sodium acetate buffer pH 4.0 (0.2 M) were added to 1.0 mL of the plasma samples or blank plasma. The samples were vortex mixed and centrifuged for 10 minutes at 3500 rpm. The solid-phase extraction procedure was slightly modified from an earlier method (Tannergren et al., 2003b). The samples were applied to Isolute C18 solid phase extraction columns (3 mL, 500 mg, International Sorbent Technology Ltd., Mid Glamorgan, UK), which had been preconditioned sequentially with 2.0 mL methanol, 2.0 mL water and 1.5 mL of 0.2 M sodium acetate buffer at pH 4.0. After sample application, the columns were washed with 2.0 mL water and dried for 5 minutes with nitrogen. The analytes were subsequently eluted with 5.0 mL methanol containing 50 mM triethylamine. The eluates were collected and evaporated to dryness under a gentle stream of nitrogen at 50^{o}C. The residue in each vial was reconstituted in 50 µL of methanol and 0.1 % acetic acid in water (1:1, v/v) prior to analysis.

In the bile samples fexofenadine and verapamil were simultaneously quantified. 100 µL of the [$^2H_6$]-fexofenadine (7.6 ng) and [$^2H_3$]-verapamil (7.8 ng) and 800 µL of 0.1 % aqueous acetic acid/methanol (1:1, v/v) were added to 200 µL of the bile samples or blank bile. The samples were vortex mixed and centrifuged for 10 minutes at 3500 rpm and the supernatants were transferred to vials for analysis.

Both plasma and bile samples were analyzed with liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS) method, using an HP1100 liquid chromatograph with a binary pump (Hewlett-Packard, Waldbronn, Germany) and a

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Zorbax Eclipse XDB-C18 chromatographic column (50 x 2.1 mm length x inner diameter, particle diameter 5 µm, Agilent Technologies, Palo Alto, CA, United States). The samples were eluted using a gradient created by mixing phases from two reservoirs: A comprised of 0.1 % acetic acid in water and B consisting of methanol. The mobile phase composition was changed as follows: 0-3 minutes 30 % B, 3-4 minutes 30-90 % B, 4-10 minutes 90 % B. The injection volume was 20 µL and the volumetric flow-rate 0.2 mL/min. The chromatography was performed at ambient temperature. A Quattro LC (Micromass, Manchester, UK) quadrupole-hexapole-quadrupole mass spectrometer with an electrospray interface operating at positive potential was connected to the column outlet. The mass spectrometer was tuned manually for sensitivity during direct infusion of a standard solution of fexofenadine. The interfacial parameters used for analysis were: capillary 2.80 kV, cone 35 V and extractor 4 V.

The mass spectrometer was run in the Selected Reaction Monitoring (SRM) mode, switching between the transitions m/z 502.2 → 171.0 for fexofenadine $[M+H]^+$ and 508.2 → 177.0 for $[^2H_6]$-fexofenadine $[M+H]^+$ with a collision energy of 35 eV; the transitions 455.6 → 165.0 for verapamil $[M+H]^+$, 458.6 → 165.0 for $[^2H_3]$-verapamil $[M+H^+]$ and 189.0 → 130.2 for antipyrine $[M+H]^+$ were studied with a collision energy of 30 eV and, finally, the 231.2 → 145.2 transition for propylphenazone $[M+H]^+$ was investigated with a collision energy of 28 eV.

The limits of quantification for fexofenadine were 0.3 and 0.8 ng/mL for plasma and bile respectively, for verapamil they were 0.3 and 0.85 ng/mL in plasma and bile respectively and for antipyrine the limit was 13 ng/mL in plasma. The accuracy and repeatability parameters measured from the quality control samples are given in Table 1.

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Perfusate data analysis. All calculations from the single-pass perfusion experiment were made from steady-state concentrations of the outlet jejunal perfusate in the control and treatment groups. Each sample represents the mean concentration of the aliquots collected for each 10-minute interval (for the period 0–100 minutes). The net water flux (NWF, mL/h/cm) in the isolated jejunal segment was calculated according to equation 1

$$NWF = \left(1 - \frac{PEG_{out}}{PEG_{in}}\right) \cdot \frac{Q_{in}}{L} \qquad (1)$$

where PEG_{in} and PEG_{out} are the concentrations of ^{14}C-PEG 4000 (dpm/mL) entering and leaving the segment, respectively. Q_{in} (mL/min) is the flow rate of the perfusion solution entering the segment and L is the length of the perfused jejunal segment (10 cm). The concentration of each compound in the perfusate leaving the intestine is corrected for water flux and the fraction of drug being absorbed in the segment (f_{abs}) and the permeability (P_{eff}) are calculated.

The adsorption of fexofenadine and verapamil to the Loc-I-Gut® tube has been tested in previous studies (Sandstrom et al., 1999; Tannergren et al., 2003a; Tannergren et al., 2003b). Since the drugs did not bind to the perfusion tube and are stable in the perfusion solution, the amount that disappeared during the single passage through the jejunal segment is considered to be absorbed. The fraction of the drug absorbed in the segment during the perfusion (f_{abs}) is calculated from equation 2

$$f_{abs} = 1 - \left(\frac{C_{out} \cdot PEG_{in}}{C_{in} \cdot PEG_{out}}\right) \qquad (2)$$

where C_{in} and C_{out} are the concentrations entering and leaving the jejunal segment, respectively.

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The effective jejunal permeability (P_{eff}) of each drug is calculated according to a well-mixed tank model, as shown in equation 3 (Lennernas, 1997)

$$P_{eff} = \frac{C_{in} - C_{out}}{C_{out}} \cdot \frac{Q_{in}}{2\pi rL} \qquad (3)$$

where the cylindrical area representing the jejunal segment ($2\pi rL$) is calculated using the intestinal radius (r = 1.75 cm) and the length (L = 10 cm) of the segment. The value of the intestinal radius is approximated by the value obtained from a perfusion with barium conducting an x-ray analysis in humans (Knutson et al, unpublished data). P_{eff}, which is calculated from the disappearance of the drug from the jejunal segment, is a directly determined parameter of intestinal transport that can be used regardless of the mechanisms involved (Amidon et al., 1995).

Pharmacokinetic data analysis. The pharmacokinetic parameters for fexofenadine and verapamil were calculated using non-compartmental analysis and WinNonlin 4.0 (Pharsight Corp., Mountain View, CA, USA). The maximal peak plasma concentrations (C_{max}) and the times at which the maximum peaks occurred (t_{max}) were derived directly from the plasma-concentration-time profile and the bile-concentration-time profile. The areas under the plasma and bile concentration-time curves (AUC_{0-6}, AUC_{∞}) were calculated by using the linear/logarithmic trapezoidal rule. The area under the curve from the last measured concentration to infinity was obtained by extrapolation, by dividing the last predicted concentration by the terminal rate constant (k_e) obtained by log-linear regression analysis of the last three to five concentration-time points. The apparent terminal elimination half-life ($t_{½}$) was obtained from k_e. The apparent absorption rate coefficient (k_a) for the transport into the different plasma sampling sites was calculated by the Wagner-Nelson method (Wagner and Nelson, 1964).

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The liver extraction for the compounds was calculated according to equation 4

$$E_H = \frac{AUC_{V.P.} - AUC_{V.H.}}{AUC_{V.P.}} \qquad (4)$$

using the area under the curve from the portal ($AUC_{V.P.}$) and hepatic veins ($AUC_{V.H.}$) from 0-6 hours.

The maximum concentration entering the liver was calculated from equation 5 (modified from that in Ito et al (Ito et al., 1998)) as follows

$$I_{in,max} = I_{max} + \frac{Q_{in} \cdot C_{in} \cdot f_{abs}}{Q_H} \cdot (1 - E_G) \qquad (5)$$

in which I_{max} is the maximum plasma concentration that is systemic available. Q_{in} (2.0 mL/min) and C_{in} are the flow rate and the perfusion solution concentration entering the jejunal segment, respectively, f_{abs} is the fraction of the dose available that gets absorbed during the perfusion and Q_H is the hepatic blood flow in pigs (626 mL/min (Jiao et al., 2000)).

E_G is the metabolic first-pass extraction by the gut wall and for verapamil a value of 0.5 was chosen (Fromm et al., 1996; Sandstrom et al., 1999; von Richter et al., 2001).

The amount of dose excreted in the bile ($A_{e,\,bile}$) was calculated by taking the accumulated amount of the concentration in each sample multiplied by the volume of the bile collected for each time period. The fraction of dose excreted ($f_{e,\,bile}$) was the $A_{e,\,bile}$ divided by the dose given of each substance (equation 6).

$$f_{e,\,bile} = \frac{A_{e,\,bile}}{Dose} \qquad (6)$$

The biliary clearance (CL_{bile}) was calculated using the $A_{e,\,bile}$ divided by the portal vein plasma $AUC_{0\text{-}6}$ (equation 7).

$$CL_{bile} = \frac{A_{e,\,bile}}{AUC_{V.P.(0-6)}} \qquad (7)$$

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The bile flow was calculated using the total volume of bile collected during the experiment and dividing it by the time for the experiment.

Statistical analysis. The ANOVA general linear model (Minitab release 14, Minitab Inc., PA, USA) was used to evaluate the differences between the sampling in the different veins within the different animals, as well as the effects of co-administration of verapamil relative to the control group. All values are presented as mean ± standard error of mean unless otherwise stated. Differences between values were considered significant at $P < 0.05$.

Results

Intestinal perfusion and surgery. 20 of the 21 animals completed surgery and the perfusion tube was positioned in the proximal jejunum successfully during anesthesia. The effective jejunal permeability (P_{eff}) of fexofenadine was $0.02 \pm 0.01 \cdot 10^{-4}$ cm/s, $0.10 \pm 0.02 \cdot 10^{-4}$ cm/s ($P < 0.05$) and $0.04 \pm 0.01 \cdot 10^{-4}$ cm/s in G1, G2 and G3, respectively (Table 2). Accordingly, the fraction dose absorbed (f_{abs}) was 1 ± 0 % to 3 ± 1 % ($P < 0.05$) and 1 ± 0 % in G1, G2 and G3, respectively (Table 2). Individual P_{eff} values for fexofenadine are presented in Figure 2. Verapamil had a P_{eff} and f_{abs} of $0.64 \pm 0.14 \cdot 10^{-4}$ cm/s and 17 ± 3 % and $0.35 \pm 0.07 \cdot 10^{-4}$ cm/s and 9 ± 2 %, respectively in G2 and G3. The P_{eff} and f_{abs} of the passive transcellular marker antipyrine are given in Table 2.

The recovery of the nonabsorbable volume marker ^{14}C-PEG 4000 was 85 ± 7 %, 96 ± 2 % and 99 ± 6 % (Table 2) in G1, G2 and G3, respectively. The mean values of pH, osmolality, the flow rate leaving the segment (Q_{out}) and the net water flux are reported in Table 2.

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Plasma and bile data. The concentration time profiles of fexofenadine and verapamil from the different bile and plasma sampling sites are presented in Figures 3 and 4, respectively. The plasma pharmacokinetic parameters for fexofenadine and verapamil are displayed in Table 3.

The increased jejunal P_{eff} did not result in an increase of the exposure of fexofenadine at any of the three plasma sampling sites in G2. A difference was found in the plasma AUC for fexofenadine between the portal and hepatic sampling sites ($P < 0.001$), but not between the samples from the hepatic vein and superior caval vein (Table 3). The predictive value of the verapamil concentration entering the liver ($I_{in\ max}$) was 62.9 ng/mL and 199.8 ng/mL in G2 and G3, which was comparable with the observed portal vein concentration of 44.0 ± 12.9 ng/mL and 169.3 ± 22.3 ng/mL, respectively. The predicted values for fexofenadine were 17.7 ng/mL, 31.5 ng/mL and 17.2 ng/mL in G1, G2 and G3, for the respective portal vein concentrations 17.2 ± 7.5 ng/mL, 22.1 ± 8.0 ng/mL and 16.5 ± 2.8 ng/mL, respectively. Fexofenadine had a low liver extraction with a value of 0.13 ± 0.04, 0.16 ± 0.03 and 0.12 ± 0.02 in the G1, G2 and G3, respectively. Verapamil had a liver extraction of 0.82 ± 0.06 and 0.84 ± 0.06 in G2 and G3, respectively.

The bile AUC_{0-6} was 46-69 times higher than the portal plasma AUC_{0-6} for fexofenadine. The amount excreted in the bile represented 0.6 ± 0.3 %, 0.5 ± 0.1 % and 0.3 ± 0.0 % of the intestinal dose of fexofenadine administered in the G1, G2 and G3, respectively. The corresponding values for the bile clearance were 31.8 ± 6.2 mL/min, 33.2 ± 8.8 mL/min and 27.9 ± 3.3 mL/min. The bile flow was 0.54 ± 0.05 mL/min, 0.48 ± 0.05 mL/min and 0.67 ± 0.08 mL/min in G1, G2 and G3, respectively. Verapamil only exhibited a 2-4 fold increase in the AUC_{0-6} for bile,

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compared to the AUC_{0-6} for the portal plasma, and the amount excreted in bile were 0.009 ± 0.002 % and 0.086 ± 0.019 % of the dose administered in G2 and G3 which is in accordance with its almost complete metabolism. The bile clearance for unchanged verapamil were 1.0 ± 0.3 mL/min and 2.2 ± 0.5 mL/min in G2 and G3.

The plasma concentration time profiles for antipyrine at the different sampling sites are presented in Figure 5, corresponding to the pharmacokinetics of a drug with low liver extraction of 0.10 ± 0.06.

Discussion

The aim of the present study in pigs was to explore the drug transport mechanism in the entero-hepatic system for fexofenadine after oral administration.
Fexofenadine had a low jejunal P_{eff} and was classified as a low permeability compound according to the biopharmaceutical classification system (BCS) in the present pig absorption model. The value of P_{eff} for fexofenadine was similar to that obtained from perfusion data for humans in vivo (Tannergren et al., 2003b). The values of P_{eff} for verapamil and antipyrine in pigs were classified as a high permeability compounds. However, the human P_{eff} –values were 6-8 times higher then corresponding P_{eff} in pigs for these two drugs (Tannergren et al., 2003b). Similar model and/or species differences regarding intestinal permeability in rat and man has been reported by us previously (Lindahl et al., 1998; Sandstrom et al., 1998). A plausible explanation for the lower P_{eff} value seen in both the rat and the pig models are differences in the effective absorptive area within the perfused segment induced by the surgery and/or the anesthesia applied in both models. It might also be

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explained by species-related differences in physiology affecting partitioning into the membrane, diffusion coefficient and/or diffusion distance (Fagerholm et al., 1996). This might also partly be explained by the anesthesia applied in both models.

This study showed that verapamil increased the jejunal P_{eff} and the f_{abs} for fexofenadine, at the lower verapamil dose (G2) but not at the higher verapamil dose (G3). This observation might have several different explanations, but the most plausible explanation is that the animals in G2 for some reason had a general higher absorption capacity for fexofenadine, verapamil and antipyrine than both G1 and G3. Accordingly, it means that intestinal located P-gp (ABCB1) is not affected by verapamil and do not posses a major absorption barrier. This is in contrast to several reports concluding that intestinal ABCB1 is the major absorption barrier for fexofenadine (Tahara et al., 2005; Wu and Benet, 2005). The intestinal luminal concentration of verapamil 356µM (G2) and 2030µM (G3) are more than sufficient to inhibit intestinal ABCB1 since the IC_{50} has been determined to be in the range of 6.5-8.4µM (Perloff et al., 2002; Petri et al., 2004). Verapamil is not restricted to the same extent by the apical membrane since it is a highly permeable compound and intracellular/intermembrane concentration is well above the IC_{50} at the intralumen concentrations in the present study. However, the interpretation of our results may also be that verapamil possess one inhibitory profile at the lower concentration (G2) than at the higher concentration in G3. For instance, in G2 only efflux is inhibited and at G3 both absorption and efflux is inhibited, which leads to an unchanged intestinal permeability. The present study showed that fexofenadine still had a low permeability in all groups regardless of an effect of verapamil or not. This has also been demonstrated in earlier studies using the Caco-2 cell model (Petri et al., 2004) and a in vivo intestinal perfusion system (Tannergren et al., 2003b). In analogy with the

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conclusions drawn in the studies, the intestinal efflux is probably not the major reason for the low permeability of fexofenadine but the limitations in the absorption is instead related to the physicochemical properties of the compound.

A pharmacokinetic data analysis of the partial AUC values from the absorption phase (data not shown) of G2 and G3 did not display any statistical difference in the plasma AUC of fexofenadine in the portal vein related to the effect of verapamil. This supports further the standpoint that the intestinal absorption of fexofenadine is not dependent of ABCB1.

The function and transport capacity of different transport proteins in the pig is poorly understood. However, a western blot analysis has shown that a 170 kDa protein with functional characteristics of the human ABCB1 is expressed along the small intestine in pigs, with a distribution pattern similar to the one found in humans (Childs and Ling, 1996; Mouly and Paine, 2003; Tang et al., 2004; Zimmermann et al., 2005). However, information obtained about OATPs in pigs is scarce and, to our knowledge, no reports exist in which it is claimed that OATP is expressed in the small intestine of pigs. In humans, OATP1A2 and OATP2B1 could both transport fexofenadine (Cvetkovic et al., 1999; Kobayashi et al., 2003). Several groups have found that OATP2B1 is expressed in the small intestine (Kullak-Ublick et al., 2001; Kobayashi et al., 2003; Nozawa et al., 2004), and it has been suggested that OATP1A2 is also present in humans (Dresser et al., 2002; Smith et al., 2003; Dresser et al., 2005). A comparison between OATP1A2 in humans and pigs has shown that there is an 85 % similarity in the peptide sequence of the cDNA for the proteins (Goh et al., 2002). Hence, it is reasonable to assume that the transporter, if present, should function in a similar manner. However, species differences have been shown between rat oatp1a1 and OATP1A2, although they have a similarity of 67 % (Cvetkovic et al., 1999;

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Hagenbuch and Meier, 2003). Further investigations of the oatps in pigs are needed to be able to conclude if a difference in intestinal absorption of pigs and humans can be explained by a homologous transporter to the human OATP1A2, or OATP2B1 having a different substrate specificity for fexofenadine, or, alternatively, if the distribution of transporters is different in the different species.

Others have shown that the liver specific OATP1B1 and OATP1B3 have implications on the drug distribution of fexofenadine to the liver (Niemi et al., 2005; Shimizu et al., 2005). Among these it has been recently suggested that OATP1B3 has a more significant role at least in vitro (Shimizu et al., 2005). In the present study, verapamil neither affected the liver extraction, nor the biliary excretion of fexofenadine in the pig despite similar doses as in our human perfusion study (Tannergren et al., 2003b). This is an obvious difference to the reported fexofenadine-verapamil interaction that we suggested to be related to processes in the liver rather than in the intestine (Tannergren et al., 2003b). This might be caused by the fact that the portal concentration of the inhibitor verapamil was too low to efficiently inhibit the liver uptake and biliary excretion of fexofenadine in the pig model. Although, it is difficult to translate in vitro data from a human cell model and also to account for species differences, a rough comparison with the IC_{50} value of verapamil on fexofenadine transport can be made. The reported IC_{50} values for verapamil are between 6.5-8.4 µM on ABCB1-mediated transport (Perloff et al., 2002; Petri et al., 2004) and the C_{max} for verapamil in the portal vein and the bile in the present study was 0.09 µM (44 ng/mL) and 0.14 µM (70 ng/mL) in G2 and 0.34 µM (169 ng/mL) and 0.77 µM (383 ng/mL) in G3. The calculation of the maximum concentration of verapamil entering the liver (I_{max}) taking first-pass extraction in the gut wall into account the

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predicted value and the determined C_{max} value for the portal concentration of verapamil agreed very well. In humans, an estimated concentration of 207 ng/mL (0.42 µM) was obtained from a peripheral vein concentration of 49.5 ng/mL (0.10µM) using the equation (Tannergren et al., 2003b). Our previous human data indicates that verapamil can affect liver handling of fexofenadine at lower concentrations than the IC_{50} determined for the ABCB1 inhibition in vitro. One reason for this discrepancy between the in vitro determined IC_{50} and the human in vivo results could be the extensive metabolism of verapamil in vivo and if the metabolites are also ABCB1 inhibitors. It is expected that the liver concentrations of the metabolites from verapamil are similar for pigs and humans at comparable concentrations of verapamil. This assumption is based on previous reports that showed corresponding activities between the two species in major metabolizing cytochrome P450 enzymes (Anzenbacher et al., 1998; Soucek et al., 2001). However, there is still a possibility that verapamil have a different metabolic pattern in the two species that could lead to different levels of a potentially inhibiting metabolite. For the first time we showed by direct in vivo determination that fexofenadine has a low liver extraction (~15%) in the porcine model, which was not affected by verapamil. There present data challenge the quantitative importance of the OATP transporter for the uptake of fexofenadine to the liver in this model. The low transport across the sinusoidal hepatocyte membrane is most likely due to low passive diffusion, since the plasma protein binding is only 60-70%.

The absence of an effect of verapamil on the relative changes on fexofenadine transport may be interpretated as an already strongly inhibited ABCB1 activity by anesthesia. However, this is not a plausible explanation since the bile/portal vein concentration ratio is 65-70, clearly indicating a significant ABCB1-mediated

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secretion into the bile in the present model. This ratio also agrees with the in vitro cellular efflux ration of 28-85 at 50 to 1000 μM of fexofenadine in the donor chamber of the Caco-2 model (Petri et al., 2004). Further support for an absence of an significant effect of the main anesthetic agent applied is supported by the fact that morphine has been reported to have no effect of the ABCB1 mediated cellular transport of fexofenadine (a standard substrate for ABCB1) (Wandel et al., 2002).

In conclusion, the combined intestinal single-pass perfusion study with simultaneous hepatobiliary sampling showed that verapamil did not affect the intestinal permeability, liver extraction or biliary excretion of fexofenadine. It also clearly showed that the permeability across the intestinal barrier and sinusoidal membrane in the liver is low in vivo. The present study clearly shows that the main in vivo relevant mechanism responsible for the in vivo drug-drug interaction remains to be investigated. These findings emphasize the importance to validate in vitro data carefully and also take species differences in membrane transport into account. The future uses of the pig as a model for mechanistic in vivo pharmacokinetic studies is promising but require more validation regarding the function and expression of human enzymes and transporter orthologs.

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Footnotes

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Legends for Figures

Figure 1. A schematic drawing of the combined single pass perfusion and hepatobiliary sampling model. The intestinal segment is perfused with a drug solution using the Loc-I-Gut® instrument and plasma is taken from the sampling sites in the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.). The bile duct was cannulated and a ligature was placed below the insertion of the catheter to prevent bile from reaching the intestine. The primary bile (B.) was collected distally to the gall bladder to get a continuous flow.

Figure 2. Individual values for and the mean value of the effective jejunal permeability (P_{eff}) for fexofenadine in the study. The pigs in the control group (G1) were administered fexofenadine alone (120 mg/L, total dose 24 mg; N = 6), whilst those in the treatment groups also received verapamil (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7).

Figure 3. The plasma and bile concentration time profiles for fexofenadine from the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.), as well as the bile duct (B.). The pigs in the control group (G1) were administered fexofenadine alone (120 mg/L, total dose 24 mg; N = 6), whilst those in the treatment groups received verapamil as well (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7).

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Figure 4. The plasma concentration time profiles for verapamil (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7) from the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.), as well as the concentration time profiles from the bile duct (B.).

Figure 5. The plasma concentration time profiles for antipyrine from the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.).

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Table 1. Accuracy and repeatability of the analytical method based on quality control samples analyzed (n=8).

	Level (ng/mL)	Accuracy (%)	Repeatability (RSD%)		Accuracy (%)	Repeatability (RSD%)
Fexofenadine Plasma	186	98-102	1.8-3.3	Bile	92	6.2
	37	90-97	1.7-6.8		94	5.5
	3.7	92-100	1.7-4.4		99	14
	0.59	79-121	3.7-11.4			
Verapamil Plasma	196	97-99	1.6-2.8	Bile	96	6.8
	39	93-114	1.1-13		94	4.3
	3.9	88-99	2.9-10		96	13
	0.61	79-100	4.5-21			
Antipyrine Plasma	185	92-113	6.6-39			
	93	87-117	17-21			
	37	73-121	12-48			

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Table 2. Mean (± SEM) values of the effective jejunal permeability (Peff) and fraction absorbed of the dose available (fabs) for fexofenadine, verapamil and antipyrine together with other perfusion parameters for the group that was administered fexofenadine alone (G1; 120 mg/L, total dose 24 mg; N = 6) and for those who received fexofenadine together with verapamil (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7).

	Control (G1)	*Treatment (G2)*	*Treatment (G3)*
Fexofenadine			
P_{eff} (10^{-4} cm/s)	0.02 ± 0.01	0.10 ± 0.02*	0.04 ± 0.01
f_{abs} (%)	1 ± 0	3 ± 1*	1 ± 0
Verapamil			
P_{eff} (10^{-4} cm/s)	-	0.64 ± 0.14	0.35 ± 0.07
f_{abs} (%)	-	17 ± 3	9 ± 2
Antipyrine			
P_{eff} (10^{-4} cm/s)	0.49 ± 0.09	0.83 ± 0.17	0.50 ± 0.13
f_{abs} (%)	14 ± 2	20 ± 4	12 ± 3
PEG 4000 rec (%)	85 ± 7	96 ± 2	99 ± 6
NWF (mL/H * cm)	0.66 ± 0.31	0.20 ± 0.09	0.62 ± 0.28
Q_{out} (mL/min)	1.70 ± 0.11	1.84 ± 0.06	2.32 ± 0.20
pH	6.67 ± 0.05	6.63 ± 0.02	6.51 ± 0.01
Osmolality (mOsm/kg)	293 ± 10	287 ± 8	260 ± 5

* statistically different from control ($p<0.05$).

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Table 3. Pharmacokinetic parameters (± SEM) calculated from the plasma concentration time profiles for fexofenadine and verapamil from the portal vein (V.P.), the hepatic vein (V.H.) and the superior caval vein (V.). The pigs in the control group (G1) were administered fexofenadine alone (120 mg/L, total dose 24 mg; N = 6) and those in the treatment groups also received verapamil (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7).

	Sampling Site	Fexofenadine			Verapamil	
		Control (G1)	*Treatment (G2)*	*Treatment (G3)*	*Treatment (G2)*	*Treatment (G3)*
AUC_{0-6}						
(hr*ng/mL)	V.P.	64 ± 30	72 ± 23	42 ± 8	69 ± 16	256 ± 27
	V.H.	55 ± 25	59 ± 17	37 ± 6	13 ± 3	39 ± 8
	V.	56 ± 26	61 ± 18	36 ± 7	14 ± 5	41 ± 6
AUC_{∞}						
(hr*ng/mL)	V.P.	156 ± 79	94 ± 27	50 ± 26	71 ± 16	263 ± 27
	V.H.	115 ± 59	79 ± 21	44 ± 24	14 ± 3	42 ± 8
	V.	136 ± 60	80 ± 23	44 ± 25	20 ± 7	50 ± 7
E_H						
		0.13 ± 0.04	0.16 ± 0.03	0.12 ± 0.02	0.82 ± 0.06	0.84 ± 0.06
$t_{½}$						
(hr)	V.P.	5.1 ± 1.5	2.5 ± 0.3	1.9 ± 0.4	0.9 ± 0.2	1.5 ± 0.1
	V.H.	3.8 ± 0.7	2.4 ± 0.2	1.9 ± 0.2	0.9 ± 0.2	1.8 ± 0.2
	V.	5.5 ± 1.6	2.3 ± 0.2	2.0 ± 0.1	2.3 ± 0.2	2.2 ± 0.1
C_{max}						
(ng/mL)	V.P.	17.2 ± 7.5	22.1 ± 8.0	16.5 ± 2.8	44.0 ± 12.9	169.3 ± 22.3
	V.H.	14.6 ± 6.6	17.7 ± 5.4	13.4 ± 2.1	6.9 ± 1.7	20.7 ± 4.9
	V.	14.7 ± 6.8	18.3 ± 6.2	12.6 ± 2.0	5.0 ± 1.6	17.3 ± 2.9
T_{max}						
(hr)	V.P.	2.1 ± 0.3	1.8 ± 0.2	1.7 ± 0.0	1.3 ± 0.1	1.7 ± 0.0
	V.H.	2.3 ± 0.3	2.2 ± 0.4	1.7 ± 0.1	1.4 ± 0.2	1.8 ± 0.1
	V.	2.3 ± 0.3	2.2 ± 0.4	1.7 ± 0.0	1.8 ± 0.1	1.9 ± 0.1
k_a						
(1/hr)	V.P.	0.89 ± 0.23	1.19 ± 0.20	1.57 ± 0.29	-	-
	V.H.	1.02 ± 0.21	0.73 ± 0.11	1.22 ± 0.28	-	-
	V.	0.91 ± 0.19	0.82 ± 0.15	1.03 ± 0.29	-	-

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Table 4. Pharmacokinetic parameters (± SEM) calculated from bile concentration time profile for fexofenadine and verapamil. The pigs in the control group (G1) were administered fexofenadine alone (120 mg/L, total dose 24 mg; N = 6), whilst those in the treatment groups received fexofenadine with verapamil (G2; 175 mg/L, total dose 35 mg; N = 7, G3; 1000 mg/L, total dose 200 mg; N = 7).

	Sampling Site	*Contol (G1)*	**Fexofenadine** *Treatment (G2)*	**Verapamil** *Treatment (G2)*	**Fexofenadine** *Treatment (G3)*	**Verapamil** *Treatment (G3)*
AUC_{0-6} (hr*ng/mL)	B.	4420 ± 2194	4563 ± 972	164 ± 31	1950 ± 222	989 ± 212
AUC_{∞} (hr*ng/mL)	B.	12082 ± 4561	7917 ± 2085	186 ± 29	2685 ± 292	1076 ± 223
$A_{e, bile}$ (µg)	B.	146 ± 82	119 ± 33	3 ± 1	64 ± 8	30 ± 7
$f_{e, bile}$ (%)	B.	0.6 ± 0.3	0.5 ± 0.1	0.009 ± 0.002	0.3 ± 0.0	0.086 ± 0.019
CL_{bile} (mL/min)	B.	31.8 ± 6.2	33.2 ± 8.8	1.0 ± 0.3	27.9 ± 3.3	2.2 ± 0.5
$t_{½ (bile)}$ (hr)	B.	8.1 ± 4.8	3.4 ± 0.7	1.5 ± 0.3	2.7 ± 0.9	1.6 ± 0.3
C_{max} (ng/mL)	B.	1300 ± 608	1406 ± 333	70 ± 13	710 ± 112	383 ± 71
T_{max} (hr)	B.	2.7 ± 0.2	2.8 ± 0.4	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.2
Bile flow (mL/min)	B.	0.54 ± 0.05	0.48 ± 0.05		0.67 ± 0.08	

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Figure 1

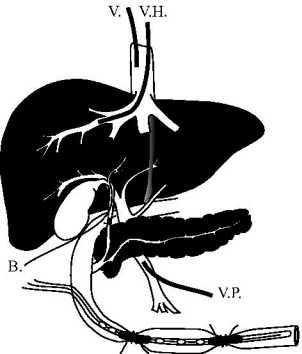


Figure 2

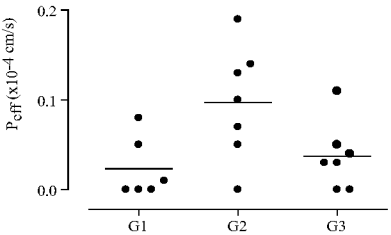


Figure 3

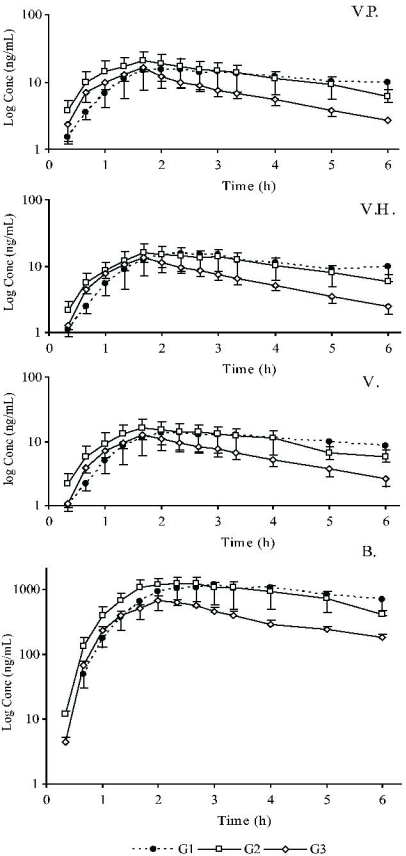
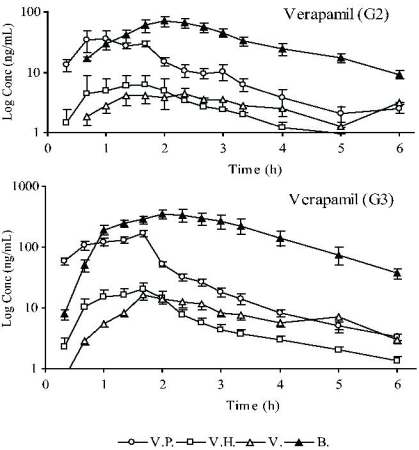

V.P.
Log Conc (ng/mL)
100
10
1
0
1
2
3
4
5
6
Time (h)
V.H.
Log Conc (ng/mL)
100
10
1
0
1
2
3
4
5
6
Time (h)
V.
log Conc (ng/mL)
100
10
1
0
1
2
3
4
5
6
Time (h)
B.
Log Conc (ng/mL)
1000
100
10
1
0
1
2
3
4
5
6
Time (h)
G1
G2
G3

Figure 4


Verapamil (G2)
Log Conc (ng/mL)
100
10
1
0
1
2
3
4
5
6
Time (h)
Verapamil (G3)
Log Conc (ng/mL)
1000
100
10
1
0
1
2
3
4
5
6
Time (h)
V.P.
V.H.
V.
B.

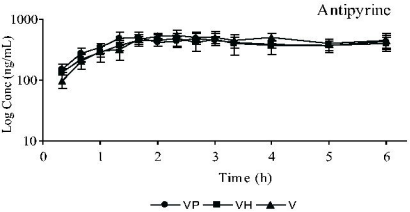


Figure 5