Different Effects of Ketoconazole on the Stereoselective First-Pass Metabolism of R/S-Verapamil in the Intestine and the Liver: Important for the Mechanistic Understanding of First-Pass Drug-Drug Interactions

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ABSTRACT:

In this acute study a pig jejunal intestinal perfusion model with multiple plasma sampling sites and three different administration routes was used to investigate the quantitative contribution of the intestine versus liver to the first-pass extraction of each enantiomer of verapamil (VER). A subclinical dose of ketoconazole (8 mg) was coadministered in the perfusion solution to selectively inhibit gut wall CYP3A. Both enantiomers of VER and its main metabolite norverapamil (NOR) as well as the inhibitor ketoconazole were quantified in all plasma compartments by liquid chromatography-tandem mass spectrometry. The overall first-pass metabolic extraction of VER and the metabolite NOR was shown to be stereoselective with the S-isomer being more extensively extracted. For VER the ratio of R- enantiomer to S-enantiomer was greater in the hepatic vein than in the portal vein (2.2 versus 1.4), indicating that the stereoselective metabolism of VER in pigs mainly occurs on the first pass through the liver and not in the intestine. Ketoconazole increased the area under the curve from time 0 to 6 h and Cmax of R- and S-VER at least 3-fold in the portal vein, most likely explained by inhibition of gut wall metabolism. Conversely, hepatic extraction was increased because the effect of gut wall metabolism was not observed at the peripheral sampling sites. In conclusion, this study provided novel and more direct information on the contribution of the intestine and the liver, respectively, to the overall first-pass extraction of racemic VER.

First-pass gut wall metabolism of drugs has, during the last few decades, been recognized as an important determinant of bioavailability, a source for variability, and a site for numerous drug interactions. Although the total content of CYP3A in the entire human small intestine is only 1% of that in the liver (Paine et al., 1997), intestinal first-pass extraction of CYP3A substrates is often reported to be high (Kolars et al., 1991; Paine et al., 1996; Sandström et al., 1998; von Richter et al., 2001). The most direct evidence for a significant contribution of intestinal metabolism in humans came from studies during the anhepatic phase in patients undergoing liver transplantations, in which drug and metabolite concentrations were determined in portal venous blood after oral dosing of the parent compound (Regårdh et al., 1989; Kolars et al., 1991; Paine et al., 1996). These studies reported that approximately 50% of the luminal administered amounts of the CYP3A4 substrates felodipine, cyclosporine, and midazolam was metabolized when they reached the portal vein. However, because of ethical and technical limitations with sampling of portal venous blood in humans, the indirect method by comparison of plasma AUCs after intravenous and oral administration has been more frequently used to gain knowledge of intestinal metabolism in humans. On the basis of this method the gut wall mucosa has been suggested to contribute to the same extent as the liver to verapamil (VER) first-pass metabolism (Sandström et al., 1999; von Richter et al., 2001). An alternative approach to provide more detailed information on first-pass metabolism in the intestine and liver is the use of animal models. With an in vivo intestinal and vascular access-ported model the extraction of VER was determined to be high (79%) in dogs (Lee et al., 2001) but negligible in rabbits (Kunta et al., 2004). In rats the pharmacokinetics of VER and norverapamil (NOR) was studied using multiple administration routes (Hanada et al., 2008). The results indicated that systemic bioavailability of VER was stereoselective and determined by first-pass metabolism in both the small intestine and the liver. However, the distribution and elimination of NOR were suggested to be nonstereoselective.

ABBREVIATIONS: AUC, area under the plasma concentration-time curve; VER, verapamil; NOR, norverapamil; PEG 4000, polyethylene glycol 4000; T, treatment group; LC, liquid chromatography; MS/MS, tandem mass spectrometry; VER-d3, trideuterated verapamil; NOR-d3, trideuterated norverapamil; HPLC, high-performance liquid chromatography; QC, quality control; P450, cytochrome P450.
VER (α-isopropyl-α-[(N-methyl-N-homoveratryl)-α-amino propyl]-3,4-dimethoxyphenyl acetoneitril) is a well known nonselective calcium channel blocker with antiangiogenic, antihypertensive, and antiarrhythmic properties (Eichelbaum et al., 1979). The commercial brand consists of a racemic mixture of equal amounts of two optical isomers; i.e., (+)-(R)-VER and (−)-(S)-VER, with different pharmacokinetic and pharmacodynamic properties. Both enantiomers have high human in vivo jejunal permeability and are mainly transported by passive transcellular diffusion (Sandström et al., 1998, 1999; Engman et al., 2003). The metabolism of VER is stereoselective and extensive, which classifies S- and R-VER as both high and intermediate hepatic extraction compounds, respectively. After a single oral immediate dose (160 mg) in humans the bioavailability is 20 and 45% for S- and R-VER, respectively (Vogelgesang et al., 1984). The enzyme primarily involved in the metabolism of VER and NOR is CYP3A4 (Kromer et al., 1992, 1993; Busse et al., 1995; Tracy et al., 1999).

In this study, we used pig as a model for absorption and first-pass metabolism. The pig has physiological characteristics similar to those of humans (Kararli, 1995) and seems to be a good test species for prediction of human drug metabolism (Skaanild and Friis, 1999; Bogaards et al., 2000; Skaanild, 2006). Several different human CYP3A4 test substrates have been used to estimate the activity of pig CYP3A. The testosterone 6-hydroxylase activity measured in pigs compared well with the reported average activity in humans (1.69 nmol/min/mg protein) (Skaanild and Friis, 1999; Bogaards et al., 2000). Furthermore, ketoconazole, a strong inhibitor of human CYP3A4 activity, could inhibit testosterone hydroxylation up to 80%, and it was found to be just as potent in pigs as in humans (Bogaards et al., 2000). Similar results were found for the human CYP3A4 substrate nifedipine (Skaanild and Friis, 1999) and inhibition studies with troleandomycin, another human CYP3A4 inhibitor (Anzenbacher et al., 1998).

In the present study, we aimed to assess the quantitative contribution of the intestine versus liver to the first-pass extraction of racemic VER by applying a pig intestinal single-pass perfusion model with simultaneous sampling from the portal, hepatic, and systemic blood vessels. With the purpose of local inhibition of pig CYP3A in the gut wall a low dose of ketoconazole (in total 8 mg) was coadministered with verapamil in the perfusion solution.

### Materials and Methods

**Animals.** This acute study was performed at the Clinical Research Department, Uppsala University Hospital, Uppsala, Sweden, and was approved by the local ethics committee for the use of laboratory animals in Uppsala, Sweden (application number C 257/6). In total, 15 animals (12 male and 3 female) were included in the study. The pigs were of mixed breed (Hampshire, Yorkshire, and Swedish landrace), were 10 to 12 weeks old, and had a mean weight of 23.7 ± 1.28 kg (range 21.8–25.9 kg). Food was withheld overnight before the acute experiment, but water was allowed ad libitum.

**Anesthesia.** The pigs were sedated during transport to the hospital by intramuscular administration of a mixture of 3 mg/kg ketamine and 3 mg/kg zolazepam (Zoletil; Virbac S.A., Carros, France), 2.2 mg/kg xylazine (20 mg/ml, Rompun Vet; Bayer AG, Leverkusen, Germany), and 0.04 mg/kg atropine (0.5 mg/ml, Atropin NM Pharma; Merck NM AB, Stockholm, Sweden). To ensure that the pigs were pain-free and anesthetized throughout the whole acute experiment they were continuously given morphine 1 mg/kg i.v. (10 mg/ml Morfin Meda; Meda AB, Solna, Sweden), 20 mg/kg/h ketamine (100 mg/ml, Ketaminol Vet; Intervet, Stockholm, Sweden), and 0.25 mg/kg/h pancuronium bromide (2 mg/ml Pavulon; Organon AB, Gothenburg, Sweden).

To keep overall body fluid balance and retain osmotic pressure the pigs were administered 10 ml/kg/h Rehydrex (Fresenius Kabi AB, Uppsala, Sweden), 8 ml/kg/h Ringer-acetate (Fresenius Kabi AB) and 60 mg/ml dextan 40 (Macrodex) in NaCl (Meda AB), in total approximately 250 ml. The pigs were kept ventilated with an oxygen-air mix by means of a Servo 900C ventilator (Siemens-Elema, Solna, Sweden) that was introduced through an incision in the throat.

**Surgery.** The surgery was performed using a previously reported procedure (Petri et al., 2006; Persson et al., 2008; Sjödin et al., 2008). The abdominal cavity was opened through a midline incision. The Loc-I-Gut perfusion tube (Synectics Medical, Stockholm, Sweden) was introduced through an incision in the duodenum and passed down into the jejunum. Once in the right position the two balloons were inflated (20–25 ml of air), and ligatures were placed on the outside of the balloons to create a 10-cm-long segment. Catheters for blood sampling were introduced in a portal vein, hepatic vein, and femoral vein. Drainage of urine and gastric fluids from the bladder and pylorus, respectively, was provided. The body temperature of the animals was maintained by using a thermostat-controlled heating pad. Blood gases, electrocardiograms, heart rate, and arterial and central venous pressures were monitored throughout the experiment to ensure normal physiological values.

To allow the animal to stabilize after the surgery the segment was rinsed with isotonic sodium chloride solution at 37°C for at least 20 min. When stable perfusion conditions were attained, a perfusion solution (37°C) containing 5.4 mM potassium chloride, 48 mM sodium chloride, 35 mM mannitol, 10 mM D-glucose, and 1 g/l 14C-labeled polyethylene glycol 4000 (PEG 4000), all dissolved in 70 mM phosphate buffer with pH 6.5 and an osmolality of 290 mOsmol/kg, was pumped through the segment during, in total, 360 min at a flow rate of 2 ml/min (single-pass) with a calibrated syringe pump (model 355; Sage Instruments, Orion Research Inc., Cambridge, MA). PEG 4000 (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used a nonabsorbable volume marker. Immediately at the end of the acute experiment each pig was sacrificed with administration of 20 to 30 nmol of potassium chloride given as a bolus dose in a superior caval vein.

**Treatment.** The study had a parallel group design and consisted of four treatment groups (Table 1). The pigs in treatment groups 1 (T1; n = 4) and 2 (T2; n = 4) received racemic VER (Knoll AG, Darmstadt, Germany) dissolved in the intestinal perfusion solution (1200 mg/l; 240-mg dose) and single-pass perfused through the segment during 100 min. In T2 the same perfusion was used but with a low dose of ketoconazole (33 mg/l, 65 μM; 8-mg dose; Sigma-Aldrich, Stockholm, Sweden). Ketoconazole was administered 20 min before VER and also together with VER during the 100-min perfusion. After the 100-min drug perfusion period was over, the jejunal segment was rinsed with 120 ml of isotonic saline within 2 min to terminate the intestinal drug absorption process. During the following 260 min of the experiment the

### Table 1

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of Animals</th>
<th>Study Drugs</th>
<th>Total Dose</th>
<th>Site of Administration</th>
<th>Sampling Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>R/S-verapamil</td>
<td>240 mg</td>
<td>Jejunal segment</td>
<td>Plasma (VP, VH, VF)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>R/S-verapamil</td>
<td>240 mg</td>
<td>Jejunal segment</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>R/S-verapamil</td>
<td>33 mg</td>
<td>Portal vein</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>R/S-verapamil</td>
<td>3 mg</td>
<td>Ear vein</td>
<td>Yes</td>
</tr>
</tbody>
</table>

VP, portal vein; VH, hepatic vein; VF, femoral vein.
segment was perfused with perfusion solution without drugs. An overview of the perfusion schedule is shown in Fig. 1.

The pigs in treatment groups 3 (T3; n = 4) and 4 (T4; n = 3) were given racemic VER intravenously (Isoptin, 2.5 mg/ml; Abbott Scandinavia AB, Solna, Sweden). T3 received 33 mg during a 100-min continuous infusion (1 mg/ml, 20 ml/h) into the portal vein and T4 in received a total of 3 mg as a 5-min continuous infusion (0.5 mg/ml, 72 ml/h) into the ear vein.

Sampling. The perfuse leaving the jejunal segment was collected on ice at 20-min intervals, mixed, weighed, and immediately frozen at −20°C pending analysis in T1 to T4. Blood was withdrawn (5 ml) from the portal vein, hepatic vein, and femoral vein just before the start of the experiment and at 10, 20, 50, 80, 100, 120, 140, 180, 240, and 360 min after the administration of VER in T1 to T3. In T4 the 20-min sample was replaced with two other time points: 10 and 30 min. To avoid cross-contamination when sampling from the same site as the dose was given (the portal vein in T3) the infusion pump used for administration was stopped during each sampling. Blood samples were collected in Vacutainers containing lithium and heparin (BD Biosciences, Plymouth, UK) and centrifuged at 3500 rpm for 10 min at 4°C (Universal 16R; MSE, Leicester, UK). The supernatants were measured with the vapor pressure method and a pH meter, respectively.

Perfusate Analysis. R-VER, S-VER, R-NOR, and S-NOR were simultaneously quantified in the perfuse samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the selected reaction monitoring mode. The internal standards used were trideuterated verapamil (VER-d3) and trideuterated norverapamil (NOR-d3). The perfusate samples (100 ml) were mixed with 100 μl of MiliQ water and 100 μl of VER-d3 solution (internal standard for VER) and 100 μl of NOR-d3 solution (internal standard for NOR) after which they were injected into the LC-MS/MS system. When necessary, the samples were diluted in water before analysis.

The LC-MS/MS method has been described previously for plasma samples (Hedeland et al., 2004). The analytical column was a Chiral AGP (150 x 4.0 mm length x inner diameter; ChromTech AB, Hägersten, Sweden). The LC-MS/MS system consisted of an Agilent 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) and a Quattro LC quadrupole-hexapole-quadrupole mass spectrometer (Micromass, Manchester, UK). The mobile phase was a mixture of 85% aqueous ammonium acetate buffer (20 mM) at pH 7.4 and 15% acetonitrile. The volumetric flow rate was 0.6 ml/min. The ionization mode was positive electrospray and the selected reaction monitoring transitions used were m/z 455 [M + H]+ → 165 for VER, m/z 458 [M + H]+ → 165 for VER-d3, m/z 444 [M + H]+ → 165 for NOR, and m/z 444 [M + H]+ → 165 for NOR-d3.

The calibration equations for quantification were constructed as the chromatographic peak area ratio (analyte/internal standard) as a function of analyte concentration. Linear regression with the weighting factor 1/x^2 was used for curve fitting. The relative standard deviations of the quality-control samples are shown in Table 2.

Total radioactivity of [14C]PEG 4000 in the perfusion solution and the perfusate samples was determined by liquid scintillation counting (Packard Instruments 1900 CA TriCarb; Canberra Industries, Meriden, CT) after addition of 10 ml of Ultima Gold liquid scintillation cocktail to a 0.5-ml perfusate sample. The osmolality and pH of the perfusion solution and perfusate samples were measured with the vapor pressure method and a pH meter, respectively.

Plasma Analysis. Verapamil and norverapamil. The plasma sample preparation was obtained via liquid-liquid extraction with a slightly modified procedure compared with the one described previously (Hedeland et al., 2004). To 500 μl of plasma, 100 μl of internal standard solution was added (VER-d3 and NOR-d3 at 300 ng/ml). The plasma was made alkaline by addition of 100 μl of 2 M NaOH (aqueous) and was thereafter extracted with 6 ml of hexane-2-butanol (98:2, v/v) for 20 min. After 10 min of centrifugation, the organic phase was transferred to a borosilicate glass tubes (Fisher Scientific, Loughborough, UK), and centrifuged at 3500 rpm for 10 min at 4°C (Universal 16R; Hettich, Tuttlngen, Germany). The plasma obtained was immediately frozen and stored at −20°C until analyzed.

Stereoselective quantification of the enantiomers of VER and NOR was then performed with the same LC-MS/MS method as described for the perfusate samples above. The relative standard deviations of the quality-control samples are shown in Table 2.

Ketoconazole. The ketoconazole concentration in pig plasma was measured, after its extraction, by HPLC with electrospray ionization tandem mass spectrometry using econazole as the internal standard. The analyte and the internal standard were extracted as follows: 200 μl of each plasma sample was transferred to borosilicate glass tubes (Fisher Scientific, Loughborough, UK), and 50 μl of econazole-water (1:1, v/v) was added, followed by the internal standard (25 μl of a 50 ng/ml econazole solution), followed by 400 μl of acetonitrile. The mixtures were vortexed and thereafter centrifuged at 4500 rpm for 10 min at 4°C (Mistrall 3000i; MSE, Leicester, UK). The supernatants were transferred into 100-μl glass vial inserts, which were placed in 2-ml amber glass auto-injector vials. Calibration standards were prepared by adding 50 μl of an analyte solution of appropriate concentration to 50 μl of naive pig plasma, after which the analyte and the internal standard were extracted as described above.

An HPLC system (Agilent Series 1100; Agilent Technologies), equipped with a HTC PAL autosampler (LEAP Technologies, Carboro, NC), was used. A 3.5-μm C18 Atlantis HPLC column (50 x 4.6 mm i.d.; Waters Ltd., Elstree, Herts, UK) was used in conjunction with a Phenomenex MAX RP guard column (4 x 3 mm) at a temperature of 40°C. The mobile phase comprised water (solvent A), acetonitrile (solvent B), and a 2.5% v/v aqueous formic acid

<table>
<thead>
<tr>
<th>Substance</th>
<th>Sample Matrix</th>
<th>Concentration (ng/ml)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-VER</td>
<td>Plasma</td>
<td>1.2</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.5</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>237</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Per fusate</td>
<td>22.5</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>10.1</td>
</tr>
<tr>
<td>R-NOR</td>
<td>Plasma</td>
<td>1.2</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>236</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Per fusate</td>
<td>6</td>
<td>4.3</td>
</tr>
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<td></td>
<td></td>
<td>21</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>1.3</td>
</tr>
<tr>
<td>S-NOR</td>
<td>Plasma</td>
<td>1.2</td>
<td>13.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Per fusate</td>
<td>6</td>
<td>4.0</td>
</tr>
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<td></td>
<td></td>
<td>21</td>
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<td></td>
<td></td>
<td>95</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>1180</td>
<td>5.8</td>
<td></td>
</tr>
</tbody>
</table>

RSD, relative standard deviation.

* n = 6.
* n = 7.
* n = 3.
solution (solvent C). The composition of the mobile phase (with respect to percentage of solvents A, B, and C) was controlled using a quaternary pump over the course of each run. Solvent C was maintained at 2% throughout. At 0 to 0.2 min, isocratic conditions were run at 20% B. Solvent B was increased from 20 to 80% in the time range from 0.2 to 2.0 min (linear gradient) and held at 80% from 2.0 to 2.5 min. Re-equilibration was performed by a step change to 10% B at 2.5 min that was held there till 3.5 min. A sample volume of 20 µl and a mobile phase flow rate of 1.1 ml/min with a total run time of 3.5 min were used.

Data were collected using a PE SCIEX API 4000 triple quadrupole mass spectrometer (ABI-SCIEX, Toronto, ON, Canada) operated in multiple reaction monitoring mode. Atmospheric pressure ionization was performed, using a TurboIonSpray source, in the positive ion mode. The vaporizer temperature was set to 650°C, and the ionization voltage was 3500 V. Nitrogen was used as the curtain gas (setting 10 psi), nebulizer gas (40 psi), and heater gas (55 psi). The ion source temperature was set to 650°C, and the ionization voltage was 3500 V. Nitrogen was used as the curtain gas (setting 10 psi), nebulizer gas (40 psi), and heater gas (55 psi). The CAD gas setting was 5 psi, and the declustering potential was 115 V. The ions monitored were ketoconazole, m/z 531.3 [M + H]+ → 489.3, and econazole, m/z 381.0 [M + H]+ → 125.2. Fragmentation of these ions using collision-activated dissociation and a collision energy of approximately 40 eV in the Q2 region of the mass spectrometer resulted in strong product ions for the analytes and the internal standard.

To validate the assay, the linearity of the standard curve was determined by plotting the area ratios of analyte to internal standard against the actual concentration using 1/x weighted least-squares regression over a calibration range of 0.5 to 20 ng/ml. Six quality control (QC) standards (2 each of low 2 ng/ml, medium 5 ng/ml, and high 10 ng/ml concentrations) were analyzed over the course of each run. The acceptability of each batch of test samples depended on the data from the calibration standards and the QC samples, fulfilling the following requirements: 1) a minimum of four of six QC samples being within ±15% of their respective calculated concentrations and no more than one QC samples being of greater than ±15% at any one concentration and 2) at least five calibration samples being within ±15% of their respective target concentrations or ±20% at the lower limit of quantification. To minimize the possibility of carryover effects, mobile phase injections were run between samples when it was necessary to follow a high concentration sample with a low concentration sample. The liquid chromatography-tandem mass spectrometry method developed gave good levels of specificity and sensitivity for the quantitative determination of analytes and internal standard in 200 µl of pig plasma. Retention times for ketoconazole and econazole were 2.2 and 2.48 min, respectively, and no interference from the pig plasma was observed. The assay showed excellent linearity, and the calibration curves had regression coefficients greater than 0.999. The lower limit of quantification was 0.5 ng/ml, whereas the lower limit of detection was in the region of 0.1 ng/ml.

**Ratio of Plasma and Blood Concentrations.** Pig whole blood was collected at the local slaughter house and transported on ice to the laboratory in glass tubes containing lithium and heparin. The ratio of the plasma (C_p) and blood (C_b) concentrations was determined according to principles presented previously (Hinderling, 1997). Racemic VER was added at seven different concentrations in blood (10, 50, 100, 150, 200, 250, and 300 ng/ml), and the samples were incubated for 30 min at 37°C. A control experiment, in which NaCl was used instead of blood, was performed to determine unspecific binding to the tubes and devices. After centrifugation (4°C, 1000g, 10 min) the R- and S-VER concentrations were determined with LC-MS/MS, using the method described above.

**Stability and Adsorption Test of R/S-Verapamil.** Stability and adsorption of R/S-VER have been tested previously in our group by in vitro perfusion using the Loc-I-Gut tube inside a glass tube at 37°C for 200 min (Sandström et al., 1998). R/S-VER was stable in the perfusion solution at 37°C for at least 200 min. Approximately 5% of R/S-VER was adsorbed to the perfusion instrument during the in vitro perfusion. The adsorption of R/S-NOR was assumed to be the same because of the structural similarities with R/S-VER.

**Intestinal Perfusate Data Analysis from Single-Pass Perfusion.** The net water flux (NWF) per centimeter in the single-pass perfused jejunal segment was calculated according to eq. 1 for each perfusate sample:

\[
NWF = \frac{1}{L} \left( \frac{PEG_{\text{out}}}{PEG_{\text{in}}} \right) Q_{\text{in}}
\]

where \( PEG_{\text{in}} \) and \( PEG_{\text{out}} \) are the concentrations of \(^{14}\text{C}\)PEG 4000, a nonabsorbable volume marker (disintegrations per milliliter), entering and leaving the jejunal segment, respectively. \( Q_{\text{in}} \) is the flow rate of the perfusion solution (2 ml/min) and \( L \) is the length of the perfused jejunal segment (10 cm). For the pigs to be included in the study the total PEG 4000 recovery (PEG4000 rec.), calculated as cumulative \( PEG_{\text{in}} \) divided by cumulative \( PEG_{\text{out}} \), had to be 80% or more. The concentration of each compound in the collected perfusate was corrected for the NWF before fraction absorbed (\( f_{\text{abs}} \)) and intestinal effective permeability (\( P_{\text{eff}} \)) were calculated by using the perfusate concentration of \(^{14}\text{C}\)PEG 4000. The amount that disappeared during single passage of the jejunal segment was assumed to have been absorbed (eq. 2):

\[
f_{\text{abs}} = 1 - \left( \frac{C_{\text{in}} \times PEG_{\text{in}}}{C_{\text{out}} \times PEG_{\text{out}}} \right)
\]

where \( C_{\text{in}} \) and \( C_{\text{out}} \) are the inlet and outlet concentrations of each compound, respectively. Intestinal \( P_{\text{eff}} \) was calculated according to a well-mixed tank model (Lennmäki et al., 1997) (eq. 3):

\[
P_{\text{eff}} = \frac{C_{\text{in}} - C_{\text{out}}}{C_{\text{in}} \times 2 \times \text{ml} / \text{L}}
\]

The surface of the cylinder (2πrL) of the jejunal segment was calculated using the intestinal radius (\( r = 1.25 \) cm) and the length (\( L = 10 \) cm) of the jejunal segment in these pigs.

**Pharmacokinetic Data Analysis.** The pharmacokinetic variables for R/S-VERapamil and ketoconazole were calculated using noncompartmental analysis (extravascular input for T1 and T2 and constant infusion for plasma data for T3 and T4) in WinNonlin 5.2 (Pharsight, Mountain View, CA). The maximal peak plasma concentrations (\( C_{\text{max}} \)) and the times at which the maximum peaks occurred (\( t_{\text{max}} \)) were derived directly from each plasma concentration-time profile in all three compartments. The area under the plasma concentration-time curve (AUC) was estimated using the linear/log trapezoidal method (linear/logarithmic trapezoidal rule up to/after \( C_{\text{max}} \), respectively).

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th><strong>P</strong> and <strong>f</strong> for R- and S-VER together with other perfusion parameters for the group that was administered racemic VER alone (T1) and for the group that received racemic VER together with ketoconazole (T2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R-VER</strong></td>
<td><strong>T1</strong></td>
</tr>
<tr>
<td>( P_{\text{eff}} ) (10^{-4} cm/s)</td>
<td>0.97 ± 0.50</td>
</tr>
<tr>
<td>( f_{\text{abs}} ) (%)</td>
<td>19 ± 9</td>
</tr>
<tr>
<td><strong>S-VER</strong></td>
<td><strong>T1</strong></td>
</tr>
<tr>
<td>( P_{\text{eff}} ) (10^{-4} cm/s)</td>
<td>1.15 ± 0.98</td>
</tr>
<tr>
<td>( f_{\text{abs}} ) (%)</td>
<td>21 ± 15</td>
</tr>
<tr>
<td>PEG 4000 recovery (%)</td>
<td>90 ± 9</td>
</tr>
<tr>
<td>NWF (ml/h/cm)</td>
<td>1.8 ± 1.6</td>
</tr>
<tr>
<td>Q_{\text{out}} (ml/min)</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>pH</td>
<td>6.53 ± 0.03</td>
</tr>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>287 ± 19</td>
</tr>
</tbody>
</table>
was calculated from time 0 to the last measurable concentration point ($C_{\text{last}}$). AUC$_{0-\text{last}}$ was calculated by extrapolating the curve to infinity by adding $C_{\text{last, extrapolated}}$ to AUC$_{0-\text{tmax}}$. $A_t$ is the first-order elimination rate constant estimated from at least three of the last measurable concentrations after $C_{\text{max}}$. The terminal half-life ($t_{1/2}$) was obtained from the elimination rate constant as $\ln(2)/A_t$.

**Determination of intestinal and hepatic extraction using systemic blood concentrations (approach 1).** The total clearance (CL) for each enantiomer of VER was calculated by dividing the dose by the AUC$_{0-\text{last}}$ measured in the femoral vein after T4 (eq. 4):

$$\text{CL} = \frac{\text{Dose}_T}{\text{AUC}_T}$$  \hspace{1cm} (4)

The total blood clearance (CL$_b$) was estimated by multiplying CL by the ratio of $C_p$ and $C_o$ determined in this study (eq. 5):

$$\text{CL}_b = \text{CL} \times \frac{C_p}{C_o}$$  \hspace{1cm} (5)

The hepatic extraction ($E_H$) of (R/S)-VER was estimated by dividing CL$_b$ by the hepatic blood flow ($Q_H$) (52 ml/min/kg; Nordgren et al., 2002) (eq. 6):

$$E_H = \frac{\text{CL}_b}{Q_H}$$  \hspace{1cm} (6)

An alternative way of calculating the hepatic extraction from systemic blood concentrations without using CL in the equation was also used (eq. 7):

$$E_H = \frac{\text{AUC}_{T3}}{\text{AUC}_{T4}}$$  \hspace{1cm} (7)

where AUC$_{T3}$ and AUC$_{T4}$ are the AUCs received in the femoral vein after administration of VER in the portal vein (T3) and ear vein (T4), respectively.

The bioavailability ($F$) was calculated using the dose-corrected AUC$_{0-6\text{ h}}$ determined in the femoral vein after administration of the dose in the perfused intestinal segment (T1/T2) or after infusion into the ear vein (T4) (eq. 8):

$$F = \frac{\text{AUC}_{0-6\text{ h},T1/T2} \times \text{Dose}_{T4}}{\text{AUC}_{0-6\text{ h},T1/T2} \times F_{\text{abs}} \times \text{Dose}_{T1/T2}}$$  \hspace{1cm} (8)

$f_{\text{abs}}$ (from eq. 2) is added in the denominator to include the dose that disappeared (absorbed) across the single-pass jejunal segment. To get a mean and variation value the bioavailability was calculated with a mean value of AUC$_{0-6\text{ h},T1/T2}$ (intravenous reference) but with individual values of AUC$_{0-6\text{ h},T1/T2}$.

Once the estimates of $F$, $E_H$, and $E_T$ were obtained, the intestinal extraction ratio ($E_I$) was derived from eq. 9:

$$F = f_{\text{abs}} \times (1 - E_H) \times (1 - E_T)$$  \hspace{1cm} (9)

**Determination of intestinal and hepatic extraction using plasma concentrations from multiple plasma sampling sites (approach 2).** The hepatic extraction in T1 and T2 was calculated based on the differences in AUC$_{0-6\text{ h}}$ between the portal vein (VP) and hepatic vein (VH) (eq. 10):

$$E_H = \frac{\text{AUC}_{0-6\text{ h},VP} - \text{AUC}_{0-6\text{ h},VH}}{\text{AUC}_{0-6\text{ h},VP}}$$  \hspace{1cm} (10)

The $E_I$ was calculated using the dose corrected hepatic vein AUC$_{0-6\text{ h}}$ after administration of the dose (D) in the perfused intestinal segment (T1/T2) or after infusion into the portal vein (T3):

$$1 - E_I = \frac{(1 - f_{\text{abs}}) \times \text{AUC}_{0-6\text{ h},T1/T2} \times \text{Dose}_{T1/T2}}{\text{Dose}_{T1/T2}}$$  \hspace{1cm} (11)

To get a mean and variation value the intestinal extraction was calculated with a mean value of AUC$_{0-6\text{ h},T1/T2}$ but with individual values of AUC$_{0-6\text{ h},T1/T2}$.

**Statistical Analysis.** For the pharmacokinetic statistical analysis, AUC and $C_{\text{max}}$ were assumed to be log-normally distributed and are presented as geometric means with a 95% confidence interval and $t_{1/2}$ was assumed to be normally distributed and is presented as arithmetic mean ± S.D. $t_{\text{max}}$ was assumed to be a nonparametric parameter and is presented as median and range. The differences between T1 and T2 or the two enantiomers were evaluated by a Student’s two-sample equal variance (homoscedastic) or paired $t$ test, with a two-tailed distribution. Differences between mean values were considered significant at $p < 0.05$.

**Results**

**Intestinal Perfusion.** All 15 pigs in this acute study completed surgery and the perfusion tube was successfully positioned in the proximal jejunum. However, one pig in T2 was excluded from all calculations because the recovery of the nonabsorbable volume marker [$^{14}$C]PEG 4000 was too low (65% in ID 15, cutoff value was 80%).

Perfusion and absorption variables are shown in Table 3. $P_{\text{eff}}$ and $f_{\text{abs}}$ of the two enantiomers of VER were slightly increased in T2.
compared with T1, but the effect was not significant (p values are reported in Table 3).

**Plasma Data for Verapamil after Intestinal Administration (T1/T2).** The mean plasma concentration-time profiles and pharmacokinetic variables (with and without ketoconazole) are shown in Fig. 2, A to C, and Table 4. AU C_{0–6 h} (R-VER) in the portal vein was significantly increased 3.3 ± 1.3-fold (p < 0.01) and 4.3 ± 2.4-fold (p < 0.05) for R- and S-VER, respectively, when VER was coadministered with ketoconazole (T2). AU C_{0–6 h} in the other two plasma sampling sites as well, but the effect was not significant. The increase was 2.1 ± 0.9-fold (p = 0.055) and 2.0 ± 1.2-fold (p = 0.18) for R-VER in the hepatic vein and femoral vein, respectively, and 2.4 ± 1.2-fold (p = 0.070) and 1.9 ± 1.0-fold (p = 0.21) for S-VER. AU C_{max} increased accordingly to the AU C_{0–6 h}, whereas \( t_{max} \) and \( t_{1/2} \) were unaffected in all plasma sampling sites by administration of ketoconazole (T2).

As expected, the metabolism of racemic VER was stereoselective with the S-isomer being more extensively metabolically extracted. The AU C_{0–6 h} (R-VER/AU C_{0–6 h} (S-VER) ratio was measured to be 1.4 ± 0.4, 2.2 ± 0.6, and 1.9 ± 0.3 in the portal vein, hepatic vein, and femoral vein, respectively. Ketoconazole did not significantly change this ratio (1.1 ± 0.0, 1.9 ± 0.3, and 1.9 ± 0.2 in the portal vein, hepatic vein, and femoral vein, respectively). It is interesting to note that the enantioselectivity was not as pronounced in the portal vein as in the hepatic vein [AU C_{0–6 h} (R-VER/AU C_{0–6 h} (S-VER) ratio 1.4 versus 2.1 in T1 (p = 0.05) and 1.1 versus 1.9 in T2 (p < 0.05)], indicating that the stereoselective metabolism of VER in pigs is located mainly in the liver and not the intestine.

**Plasma Data for Norverapamil after Intestinal Administration (T1/T2).** The plasma concentration-time profiles and pharmacokinetic variables of both enantiomers of NOR before and after treatment with ketoconazole are shown in Figs. 3, A to C, and Table 4. AU C_{0–6 h} of NOR was increased in all plasma sampling sites when ketoconazole was added. R-NOR was significantly increased 2.5 ± 0.5-fold (p < 0.05), 2.5 ± 0.6-fold (p < 0.05), and 2.6 ± 0.5-fold (p < 0.05) in the portal vein, hepatic vein, and femoral vein, respectively. S-NOR increased 2.3 ± 1.3-fold (p = 0.075), 2.7 ± 0.9-fold (p = 0.050), and 2.4 ± 1.4-fold (p = 0.26) in the portal vein, hepatic vein, and femoral vein, respectively. In addition, AU C_{max} increased for both enantiomers. \( t_{max} \) and \( t_{1/2} \) were unaffected by treatment with ketoconazole.

The plasma concentration of R-NOR was found to be much higher than that for S-NOR despite more rapid metabolism of S-VER, which most likely is due to a higher metabolic clearance ratio (S-NOR/R-NOR). The AU C_{0–6 h} (R-NOR/AU C_{0–6 h} (S-NOR) ratio was 10.5 ± 5.1, 16.8 ± 1.6, and 14.8 ± 6.9 in the portal vein, hepatic vein, and femoral vein, respectively. In T2 the ratio was found to be 13.1 ± 2.8, 12.3 ± 0.9, and 12.0 ± 1.8 in the respective vein. This result indicates that the plasma pharmacokinetics of the phase I metabolites R/S-NOR is more stereoselective than those of the parent drug R/S-VER.

**Pharmacokinetics of Verapamil and Norverapamil after Intravenous Infusion in Portal Vein (T3) and Ear Vein (T4).** The plasma concentration-time profiles of each enantiomer of VER and NOR after intravenous infusion of VER are presented in Figs. 4 and 5, and the pharmacokinetic variables are shown in Table 5. After a continuous 100-min portal vein infusion, the AU C_{0–6 h} and \( C_{max} \) were higher for R-VER and R-NOR than for S-VER and S-NOR in all plasma sampling sites. The AU C_{0–6 h} (R-NOR/AU C_{0–6 h} (S-NOR) ratio was 1.5 ± 0.4, 2.1 ± 0.8, and 1.7 ± 0.7 in the portal vein, hepatic vein, and femoral vein, respectively. Accordingly the AU C_{0–6 h} (R-NOR/AU C_{0–6 h} (S-NOR) ratio was 12.3 ± 6.2, 22.3 ± 9.0, and 14.3 ± 8.5, which further supports the fact that the metabolism of R/NOR is more stereoselective than that of the parent drug R/S-VER. After T4 there was no difference observed between the R- and S-isomers, which demonstrates that the first-pass extraction in the liver was a major determinant of the strong enantioselective plasma pharmacokinetics of VER and its active metabolite.

**Organ Extraction of Verapamil Using Approach 1.** The bioavailability (based on peripheral plasma concentrations) of VER after enteral administration was calculated to be 11 ± 6 and 6 ± 3% for R- and S-VER, respectively, after single-pass perfusion of the drug in the portal vein, hepatic vein, and femoral vein, respectively.
In T2 the bioavailability (based on peripheral concentrations) was not changed and was 11 ± 5 and 6 ± 3% for R- and S-VER, respectively. CL (in T4) was 691 ± 156 and 688 ± 104 ml/min for R- and S-VER, respectively. CLb was, accordingly, 621 ± 140 and 757 ± 115 ml/min. CLb is based on the measured in vitro Cb/Cp ratio values for R- and S-VER of 0.9 ± 0.08 (n = 3) and 1.1 ± 0.08 (n = 3), respectively. By using eq. 6, the EHT of R- and S-VER was calculated to 50 ± 9 and 61 ± 6%, respectively (Table 6).
second approach, the n0.5 mg/ml, 72 ml/h, total dose 3 mg; and S after dose adjustment, the systemic concentration of VER should be the intestine (T1/T2) or in the portal vein (T3) (eq. 11). Theoretically determined in the hepatic vein after administration of VER either in Equation 7 gave similar results, and the fabs for other substances in this model (Petri et al., 2006; Persson et al., 2008), it classifies both enantiomers of VER as high-permeability drugs in pigs. In general, the Peff values obtained in the pig model are lower than reported jejunal Peff values (in vivo) in humans. For VER the Peff has been reported to be approximately 5 to 7 times higher in humans than in pigs (Sandström et al., 1999; Tannergren et al., 2004). It is likely that species differences affecting partitioning into the membrane, intramembrane diffusion coefficient, and/or diffusion distance have an effect on the value obtained (Fagerholm et al., 1996). In addition, differences in effective absorptive area within the perfused segment and the surgery and/or anesthesia applied in the pig model could explain the inconsistency and may have affected the processes above.

AUC0–6 h and Cmax of the two enantiomers of VER increased significantly at least 3-fold in the portal vein when ketoconazole was added in the perfusion solution. This increase was probably mainly due to inhibition of CYP3A-mediated metabolism in the enterocyte. In comparing E3 in T1 and T2, the metabolism was decreased approximately 25% after inhibition with ketoconazole (from 61 to 47% for R-VER and from 74 to 55% for S-VER). Because of a tendency (no significant effect) for a higher Peff and fab for VER in T2 compared with T1 in this study, we cannot exclude the possibility that the increased concentration in the portal vein was also a consequence of an increased absorption rate resulting from inhibition of P-glycoprotein in the intestine. However, because VER is a highly permeable drug and is transported mainly by passive diffusion (Sandström et al., 1998, 1999; Engman et al., 2003), it is likely that this would affect the absorption only to a minor extent.

A drug must pass sequentially from the intestinal lumen through the gut wall and liver. Based on this sequence it is expected that a change in the portal compartment would transfer along to the hepatic vein and finally the femoral vein in accordance with the quantitative relationship: F = fab · Peff · fabs (Rowland and Tozer, 1995). We were surprised to find that this was not the case in this study. The significant 3- to 4-fold increase of R/S-VER in AUC0–6 h and Cmax seen in the portal vein after ketoconazole treatment was only a maximum 2-fold in the hepatic vein and femoral vein, respectively. In comparison with the higher in T3 because the drug is escaping the intestinal extraction and is only affected by the metabolism in the liver. E3 in T1 was calculated to 61 ± 17 and 74 ± 13% for R- and S-VER, respectively (Table 6). After coadministration of ketoconazole the extraction was 47 ± 10% (p = 0.25) and 55 ± 15% (p = 0.13), respectively (Table 6). E3 was measured to be 49 ± 21 and 68 ± 13% for R- and S-VER, respectively, in T1 and was 65 ± 20 (p = 0.24) and 80 ± 11% (p = 0.29), respectively, in T2 (Table 6).

**Plasma Data for Ketoconazole.** The inhibitor ketoconazole was analyzed in the three different plasma sampling sites, and the plasma concentration-time profile and the pharmacokinetic variables are reported in Fig. 6 and Table 7, respectively. The highest plasma concentration of ketoconazole was received in the portal vein (AUC0–6 h 10.4 h · ng/ml; Cmax 3.8 ng/ml). During liver passage 58% was extracted, and the AUC0–6 h in the hepatic vein and femoral vein was 4.2 and 2.5 h · ng/ml, respectively.

**Discussion**

In this acute nonclinical study we have used a combined jejunal single-pass perfusion and multiple blood sampling model in pigs to investigate the relative role of intestine and liver in the first-pass metabolism of each enantiomer of VER. The two enantiomers of VER had a jejunal Peff of approximately 1 × 10−5 cm/s, which is in agreement with jejunal Peff data from a previous perfusion study in the same pig model (Petri et al., 2006). Compared with the Peff values for other substances in this model (Petri et al., 2006; Persson et al., 2008), it classifies both enantiomers of VER as high-permeability drugs in pigs. In general, the Peff values obtained in the pig model are lower than reported jejunal Peff values (in vivo) in humans. For VER the Peff has been reported to be approximately 5 to 7 times higher in humans than in pigs (Sandström et al., 1999; Tannergren et al., 2004). It is likely that species differences affecting partitioning into the membrane, intramembrane diffusion coefficient, and/or diffusion distance have an effect on the value obtained (Fagerholm et al., 1996). In addition, differences in effective absorptive area within the perfused segment and the surgery and/or anesthesia applied in the pig model could explain the inconsistency and may have affected the processes above.
The lack of an inhibitory effect on R-VER and N-OH-R-VER metabolism in the intestine resulted in unchanged overall bioavailability compared to T1. However, in the liver, R-VER and N-OH-R-VER metabolism was significantly inhibited by ketoconazole coadministration, as indicated by the values for $E_{G}$, $E_{h}$, and $E_{H}$ in T3. The decrease in $E_{G}$ and $E_{h}$ and increase in $E_{H}$ indicate a decrease in hepatic extraction and an increase in intestinal extraction, respectively.

**TABLE 5**

<table>
<thead>
<tr>
<th>Substance</th>
<th>E&lt;sub&gt;g&lt;/sub&gt;</th>
<th>E&lt;sub&gt;h&lt;/sub&gt;</th>
<th>E&lt;sub&gt;H&lt;/sub&gt;</th>
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<tr>
<td><strong>R-VER</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Approach 1</td>
<td>77 ± 4</td>
<td>50 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Approach 2</td>
<td>61 ± 17</td>
<td>47 ± 10</td>
<td>49 ± 21</td>
</tr>
<tr>
<td><strong>S-VER</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approach 1</td>
<td>86 ± 2</td>
<td>61 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Approach 2</td>
<td>74 ± 13</td>
<td>55 ± 15</td>
<td>68 ± 13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated according to eq. 6.
<sup>b</sup>Calculated according to eq. 7.

The mean plasma concentration-time profiles of ketoconazole in the portal vein (VP), hepatic vein (VH), and femoral vein (VF) after coadministration of a low dose of ketoconazole (33 mg, total dose 8 mg; n = 3) given 20 min before VER and also together with VER during a 100-min jejunal perfusion.

**Fig. 6.** The mean plasma concentration-time profiles of ketoconazole in the portal vein (VP), hepatic vein (VH), and femoral vein (VF) after coadministration of a low dose of ketoconazole (33 mg, total dose 8 mg; n = 3) given 20 min before VER and also together with VER during a 100-min jejunal perfusion.

The maximal concentration of ketoconazole in the portal vein. The $K_{i}$ value for the competitive inhibition of CYP3A4-mediated testosterone 6β-hydroxylation by ketoconazole has been determined to be 0.10 μM in humans (Lillibridge et al., 1998). One plausible explanation for the increased liver extraction is increased uptake of VER into the liver hepatocyte during the first pass of the liver. For drugs with intermediate and high liver extraction during first pass, this explanation is valid. (Rowland and Tozer, 1995).

The AUC<sub>0–6h</sub> of NOR was surprisingly increased 2- to 2.5-fold in the plasma sampling sites when ketoconazole was coadministered with VER. The only possible explanation for this observation is that
not only the formation of NOR but also the further metabolism of the metabolite has been inhibited by ketoconazole. A similar result was found in a clinical study, which reported that St. John’s wort induced the metabolism of both VER and NOR (Tannergren et al., 2004). The inhibition in this pig study was most effective in the intestine because ketoconazole on the jejunal permeability and CYP3A metabolism of (S)-verapamil in humans.


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