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Title Page

Stereoselective first-pass metabolism of verapamil in the small intestine and liver in rats

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Running title: Stereoselective first-pass metabolism of verapamil

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List of abbreviations:

VP: verapamil, NVP: norverapamil, AUC: area under the drug concentration-time curve, CLtot: total clearance, CLh: hepatic clearance, CLint: intrinsic clearance, F: bioavailability

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Abstract

Verapamil is used as a racemate, but shows stereoselective pharmacokinetics and pharmacodynamics. It undergoes extensive first-pass metabolism. Stereoselective first-pass metabolism in the intestine and liver was investigated *in vivo* and *in vitro* to determine its impact on the disposition of verapamil and its main metabolite, norverapamil. Verapamil racemate was administered to rats intravenously, orally and via the portal vein. The formation rates of the main metabolites of the verapamil enantiomers were estimated in an *in vitro* intestinal microsomal study. The hepatic bioavailability of verapamil showed saturable metabolism and the hepatic bioavailability of R- verapamil was higher than that of S- verapamil. Conversely, the intestinal bioavailability of R-verapamil was lower than that of S- verapamil, resulting in a higher systemic bioavailability of S- verapamil. The pharmacokinetics of the norverapamil enantiomers were similar. These results suggest that the stereoselectivity of the total bioavailability of verapamil is determined by first-pass metabolism in the small intestine and liver, and that the norverapamil enantiomers observed in the systemic circulation after oral administration of verapamil racemate originate mainly from the liver in rats.

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Introduction

The first-pass metabolism of drugs in the small intestine and liver limits their bioavailability to the systemic circulation. Although the effects of first-pass metabolism in the liver after oral administration have been well studied, it is now known that many drugs also undergo first-pass metabolism in small intestine. Reductions in the first-pass metabolism of these drugs, caused by drug-drug interactions or disease states, may occur to a different extent in the small intestine and liver. It is therefore very important to elucidate the mechanisms underlying first-pass metabolism, and the separate contributions of metabolism in the small intestine and liver, in order to be able to predict changes in oral bioavailability.

Verapamil (VP) is a calcium antagonist used clinically for the treatment of hypertension, and for prophylaxis of supraventricular and ventricular arrhythmias. VP has a relatively narrow therapeutic plasma concentration range, and shows relatively large interindividual variations in its pharmacokinetics and pharmacodynamics (Vogelgesang et al., 1984; Echizen et al., 1985ab, 1988). Although VP is commercially available as a racemic mixture, its pharmacological effects and disposition have been reported to show stereoselectivity in both humans and animals. The antiarrhythmic effect of S-VP, as estimated by ECG, is 10-20 times higher than that of R-VP in humans

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(Echizen et al., 1985ab). The oral bioavailability of S-VP is about 20% in humans, whereas that of R-VP is about 50% (Vogelgesang et al., 1984; Echizen et al., 1985ab, 1988). The plasma protein binding of R-VP is higher than that of S-VP (free fractions: 7% and 12%, respectively) (Gross et al., 1988; Robinson and Mehvar, 1996). We have previously reported that VP binds enantioselectively to α1-acid glycoprotein and phosphatidylserine, and shows an interaction in the binding between the enantiomers (Hanada et al., 1998b, 2000).

Norverapamil (NVP) is the main metabolite of VP. The area under the plasma concentration-time curve values for both VP (AUCvp) and NVP (AUCnvp) show enantiomeric differences; those for the R-isomer are greater than those for the S-isomer after oral administration in humans (Echizen et al., 1988). If S-VP is metabolized to a much greater extent than R-VP, and the clearances of the NVP enantiomers are assumed to be the same, the AUCnvp for the S-isomer would be greater than that for the R-isomer. A similar relationship between VP and NVP is observed in rats, although the AUCvp and AUCnvp values for the S-isomer are greater than those for the R-isomer (Bhatti and Foster, 1997). These differences may be due to either stereoselective first-pass metabolism in the small intestine and liver or the disposition of NVP.

The contribution of the small intestine to first-pass metabolism after oral

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administration has not yet been directly estimated, and the pharmacokinetic interactions of the VP enantiomers remain unclear. We therefore investigated the stereoselective first-pass metabolism of verapamil in the small intestine and liver using previously reported *in vivo* dosing technique and *in vitro* metabolism experiments with intestinal microsomes. In particular, we aimed to elucidate the reason for the differences between the AUCvp and AUCnvp values for the two enantiomers on the basis of stereoselective first-pass metabolism in the small intestine and liver, and plasma protein binding. Although the relative relationships of the AUCs for the two enantiomers are opposite to those observed in humans, rats were used as the animal model for elucidating the mechanisms underlying the stereoselectivity of VP and NVP metabolism in the small intestine and liver.

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Materials and Methods

Materials:

Racemic VP hydrochloride and NVP hydrochloride were kindly provided by Eisai Co. (Tokyo, Japan). N-dealkylated VP hydrochloride and O-demethylated VP hydrochloride were kindly provided by Knoll AG (Ludwigshafen, Germany). R-Propranolol hydrochloride was obtained from Aldrich Chem. Co. (Milwaukee, WI). [N-methyl-^{3}H]Verapamil hydrochloride (specific activity, 2.2 TBq/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). The S- and R-enantiomers of VP were separated using a previously reported enantioselective high-performance liquid chromatography (HPLC) method (Hanada et al., 1998ab; Hashiguchi et al., 1996) and their stereochemical purities were ascertained by stereospecific HPLC resolution (the stereochemical purities of S-VP and R-VP were 98.6% and 99.3%, respectively). All other reagents used were of analytical grade, unless stated otherwise.

Animals:

The male Wistar rats (230 – 300 g) were handled in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and as approved by Meiji Pharmaceutical University Institutional

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Animal Care and Use Committees. The rats were kept under stable humidity and temperature conditions.

Blood to plasma partition ratio:

One hundred μL VP or NVP solution was added to freshly isolated rat blood (final concentrations of VP and NVP were 0.1, 0.5 and 2.0 μg/mL) and incubated for 1 h at 37ºC. A sample of the incubated blood was taken for determination of the drug concentrations in whole blood, and a further aliquot was centrifuged at 1,500 x g for 5 min at 37ºC to produce plasma. The concentrations of the VP and NVP enantiomers in the blood and plasma samples were determined by the HPLC method described below.

Protein Binding:

The protein binding of the VP enantiomers was evaluated using equilibrium dialysis (Hanada et al., 1988b; Hanada et al., 2000). Briefly, 5 μL ^{3}H-labeled R- or S-VP (2.5 GBq/mmol) was added to 0.5 mL freshly isolated serum. The solutions were dialyzed using a 5-cell equilibrium dialyzer (10 rpm, Spectrum Medical Industries, Inc. TX, USA) against phosphate buffer (0.113 M Na_2HPO_4 and 0.017 M KH_2PO_4, pH 7.4) for 2 h at 37°C. The membrane used was a Spectra/Por-2, MW cut-off 12,000-14,000 (Spectrum

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Inc.). Once equilibrium had been reached, the radioactivities of the solutions (inside and outside the dialysis tube) were determined using a liquid scintillation counter (Aloka Co., Ltd., Tokyo, Japan). Sample volume alteration during dialysis was corrected according to the change in the protein concentration. The concentration dependency of VP binding to rat serum protein was also studied (the final drug concentration in serum ranged from 0.06 to 3.0 μg/mL).

In vivo experiments:

The *in vivo* experiments for determining separate intestinal and hepatic bioavailabilities were performed using a previously reported procedure (Mihara et al., 2001). The drug was administered via three different routes: infusion into the femoral vein (i.v.), into the portal vein (p.v.), and into the duodenum as a substitute for per-oral (p.o.) administration. Male Wistar rats (230-250 g) maintained on standard food and water were fasted for 18 h prior to the experiment. Intraperitoneal sodium pentobarbital (50 mg/kg) was administered to each rat and an abdominal incision was made to expose the distal ileum. Heparinized polyethylene tubes (PE-10) were inserted into the mesenteric vein and the femoral artery and vein. The three cannulations were carried out in all rats for either drug administration or as a sham operation.

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In the intravenous (1 mg/kg) and oral administration (5, 10 mg/kg) groups, racemic VP solution (5 mg/mL) was administered as a bolus via the femoral vein or duodenal cannula, respectively. In the portal vein administration group, racemic VP (0.31, 0.63, 1.25, 2.5 or 5.0 mg/mL) was infused for 20 min (corresponding to the normal absorption time) through the mesenteric cannula (total dose 0.62-10 mg/kg). Blood samples (about 0.2 mL) were obtained from the femoral artery at 1, 5, 15, 30, 60, 90, 120 and 180 min for the intravenous and oral administration groups, and at 10, 20, 30, 40, 60, 90, 120 and 150 min for the portal vein administration group.

In order to investigate the stereoselectivity of NVP pharmacokinetics, NVP racemate (2.5, 5 or 10 mg/kg) was administered intravenously to rats under sodium pentobarbital anesthesia. After administration, blood samples were withdrawn from the femoral artery at 5, 10, 15, 30, 45, 60, 90 and 120 min. All blood samples were heparinized and were immediately centrifuged at 1,500 x g for 5 min. The plasma samples were then stored at -20°C until required for analysis.

Preparation of intestinal microsomes:

The rats were decapitated, then the upper jejunums (30 cm from the pyloric valve) and livers were removed and immediately placed into ice-cold 0.9% (w/v) NaCl. The

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intestinal segments were then flushed with 50 mL ice-cold 0.9% (w/v) NaCl to remove mucus and food residues (Mihara et al., 2001). Intestinal cells were obtained by shaving with a glass slide, placed in ice-cold 50 mM phosphate buffer (pH 7.4) and dispersed by sonicating (5 s, 3 times). Sixty µl phenylmethylsulfonylfluoride (10 mg/mL in acetone) was added and the mixture was homogenized (500 rpm, 6 strokes). The homogenate was centrifuged at 9,000 x g for 30 min at 4°C. The supernatant was centrifuged at 105,000 x g for 65 min at 4°C. The final pellet was suspended in ice-cold 50 mM phosphate buffer (pH 7.4) and homogenized (500 rpm, 4 strokes).

VP metabolism by microsomes:

The metabolism of VP by the microsomes was assessed using each enantiomer separately because of analytical difficulties when all the metabolites were mixed. Eight hundred µL 50 mM phosphate buffer (pH 7.4), 100 µL of the relevant VP enantiomer (0.05, 0.1, 0.5, 1, 1.5, 2, 3 and 4 mM) and 100 µL of the microsomal suspension (protein concentration was 1.0 mg/mL) were mixed. The reaction was started by adding an NADPH generating system. After incubation for 10 min at 37°C, the reaction was stopped by adding 1 mL 5% trichloroacetic acid and the concentrations of N-dealkylated VP, O-demethylated VP and NVP were determined by HPLC as described below. The effect of rabbit antiserum

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against rat CYP1A, 2B, 2C and 3A on the formation of these metabolites was also studied using a previously reported method (Mihara et al., 2001). The binding of the VP enantiomers to the microsomes was determined by equilibrium dialysis as described above.

Analytical Methods:

The concentrations of the VP and NVP enantiomers in the plasma and blood were determined using a previously reported enantioselective HPLC method (Hashiguchi et al., 1996; Hanada et al., 1998a). The HPLC system consisted of a Shimadzu HPLC apparatus (Shimadzu, Kyoto, Japan), an LC-9A HPLC pump and a C-R6A Chromatopac integrator. VP was detected by an RF-535 fluorescence detector which was operated at excitation and emission wavelengths of 272 and 312 nm, respectively. The enantiomers were separated using a ChiralPak AD column (250 x 4.6 mm I.D., Daicel, Tokyo, Japan) at 40°C. The mobile phase comprised hexane-isopropanol-diethylamine (94:6:0.1 (v/v)) at a constant flow rate of 1.2 mL/min. The drugs were extracted from the biological fluids using an organic solvent (ethyl ether) under alkaline conditions. With regard to analytical accuracy, the within- and between-day coefficients of variation were less than 9.2%.

The concentrations of N-dealkylated and O-demethylated VP and NVP in the

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microsomal suspension were determined under the following HPLC conditions. The HPLC system consisted of a Shimadzu HPLC apparatus, an LC-6A HPLC pump and a C-R6A Chromatopac integrator. These metabolites were detected by an RF-535 fluorescence detector which was operated at excitation and emission wavelengths of 272 and 312 nm, respectively. The metabolites were separated using an L-column ODS (250 x 4.6 mm I.D., Chemical Inspection and Testing Institute, Japan) at 51°C. The mobile phase comprised 6 mM ammonium acetate: acetonitrile: methanol (70: 25: 5, v/v; pH 4.2) at a constant flow rate of 1.2 mL/min. Two hundred µL 1 M NaOH and 6 mL ethyl-ether were added to 1 mL microsomal suspension and shaken for 10 min. The organic layer was evaporated to dryness under a stream of nitrogen gas in a water-bath at 50°C, the residue was reconstituted with 0.1 mL mobile phase and a 50-µl aliquot was injected into the HPLC system. With regard to analytical accuracy, the recovery was greater than 91% and the within- and between-day coefficients of variation were less than 6.7%.

Data analysis:

The areas under the plasma concentration versus time curves after VP administration into the femoral vein (AUCiv), the portal vein (AUCpv) and the duodenum (AUCpo) were calculated by the trapezoidal and log-trapezoidal rules using the computer program

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WinNonlin (Pharsight, IL, USA). Total bioavailability (Ft), hepatic bioavailability (Fh) and gastrointestinal bioavailability (Fg) were calculated using the following equations:

$$Ft = \frac{AUC_{po}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{po}}; Fh = \frac{AUC_{pv}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{pv}}; Fg = \frac{1}{Fa} \cdot \frac{Ft}{Fh} = \frac{AUC_{po}}{AUC_{pv}} \times \frac{Dose_{pv}}{Dose_{po}}$$

where Fa is the fraction of absorption and assumed to be 1.

The total and hepatic clearances (CLtot and CLh, respectively) were calculated using the following equations:

CLtot=Dose/AUCiv and *CLh=Qh x Eh*

where Qh and Eh are the hepatic blood flow rate and extraction ratio (1-Fh) and a liver blood flow rate of 60 mL/min/kg was used (Davies and Morris, 1993). Extra-hepatic clearance was estimated as CLtot – CLh.

In the microsomal experiments, data points for the formation velocities (v) of each metabolite at varying concentrations of the substrate verapamil (S) were fitted by the non-linear least-squares regression program WinNonlin to a one-enzyme model yielding the maximum velocity (Vmax) and affinity constant (Km), using the following equation:

$$v = \frac{Vmax \cdot S}{Km + S}$$

The differences between means for the enantiomers were tested using Student's paired *t*-test or unpaired *t*-test. A p value less than 0.05 was considered to indicate a significant difference.

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Results

Both the blood to plasma concentration (B/P) ratio and the plasma protein binding of VP and NVP were concentration-independent within the concentration range studied (VP: 100-1000 ng/mL; NVP: 500-2000 ng/mL). The B/P ratio of R-VP was significantly higher than that of S-VP (Table 1). Similarly, the unbound fraction of R-VP was significantly higher than that of S-VP. After correction for the unbound fraction, the B/P ratio did not differ significantly between the enantiomers (S-VP: 8.3 vs R-VP: 7.6), indicating that the apparent stereoselectivity of distribution into the blood can be accounted for by plasma protein binding in rats. On the other hand, the B/P ratios of the NVP enantiomers were not significantly different.

The plasma concentrations of S-VP were higher than those of R-VP and NVP was not detected after intravenous administration (Fig. 1). On the other hand, the concentrations of both enantiomers in whole blood were comparable. The plasma clearance was comparable to that obtained in our previously reported study using constant intravenous infusion (Hanada et al., 1998), indicating that the clearance of VP was linear at this dose. In this study, further data obtained were based on the concentrations in blood. The mean AUC values for S-VP and R-VP were 11864 and 7619 ng/mL/min, respectively, after intravenous administration (1 mg/kg racemate).

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The concentrations of S-VP and S-NVP were higher than those of the R-isomers after oral administration of VP racemate (Fig. 2), as has been reported previously (Bhatti and Foster, 1997). The oral clearance obtained at a dose of 5 mg/kg dose (S-VP; 406, R-VP; 936 mL/min/kg) was comparable to that at 10 mg/kg (S-VP; 479, R-VP; 965 mL/min/kg). The mean AUC values for S-VP and R-VP were 10440 and 5181 ng/mL/min, respectively, after oral administration (10 mg/kg racemate). On the other hand, after intraportal vein administration of VP racemate, the concentration of R-VP was higher than that of S-VP, whereas the concentration of S-NVP was higher than that of R-NVP. The relationship between the dose administered via the portal vein and the AUC showed that the AUC increased disproportionately at racemate doses of more than 5.0 mg/kg (Fig. 3), indicating that first-pass metabolism in the liver is nonlinear within the dose range studied. In order to calculate each bioavailability value within the linear range, therefore, a similar AUC for VP (obtained by portal vein administration at dose of 3.0 mg/kg racemate) was used for calculation of hepatic bioavailability (Fh).

The Ft of S-VP was higher than that of R-VP (Table 2). However, the Fh of R-VP was almost double that of S-VP and the Fg of S-VP was higher than that of R-VP. These results indicate that systemic bioavailability is determined by first-pass metabolism in

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both the small intestine and liver in rats, and that the stereoselectivity of Ft appears to result from the relative contributions of both organs to first-pass metabolism.

The calculated hepatic blood clearances of S- and R-VP were 52.2 and 47.0 mL/min/kg, respectively (Table 2). The calculated extra-hepatic clearances of S- and R-VP were 21.7 and 31.1 mL/min/kg, respectively, suggesting that VP may also be eliminated by extra-hepatic routes. The hepatic intrinsic clearance of S-VP was higher than that of R-VP.

Neither the blood clearance nor the volume of distribution at steady state of the NVP enantiomers was dose-dependent at the doses used (data not shown). The blood clearance and volume of distribution at steady state of S- and R-enantiomers were 257±53 and 279±54 mL/min/kg, and 7409±3450 and 6935±3865 mL/kg, respectively (n=4). These parameters did not differ significantly between the enantiomers, indicating that distribution and elimination of NVP are not stereoselective.

We performed an *in vitro* study of the metabolism of VP in the intestine to confirm the enantioselectivity of VP metabolism (Fig. 4). The intrinsic clearances of R-VP to

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N-dealkylated VP and NVP in the intestine were significantly higher than those of S-VP. However, intrinsic clearance to O-demethylated VP did not differ significantly between the enantiomers (Table 3). The effects of CYP antibodies on the formation rates of N-dealkylated VP, O-demethylated VP and NVP from S-VP in rat intestinal microsomes were also investigated. The formation rates of N-dealkylated VP and NVP were significantly depressed in the presence of anti-CYP3A2 in an antibody-concentration-dependent manner. On the other hand, the formation rates of O-demethylated VP were decreased in the presence of anti-CYP1A2 (data not shown).

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Discussion

The partition of R-VP into blood cells was higher than that of the S-isomer but this apparent stereoselectivity could be accounted for by a difference in plasma protein binding, as has been reported by others (Robinson and Mehvar, 1996). The hepatic clearances of both VP enantiomers were considerably limited by blood-flow. The calculated extra-hepatic clearance of VP was 22-31 mL/min/kg, suggesting that VP may be eliminated by extra-hepatic metabolism as well as hepatic metabolism. Because the urinary excretion of both enantiomers is negligible (Eichelbaum et al., 1979), the small intestine may be one of the organs responsible for extra-hepatic metabolism. Sandstrom et al. reported significant efflux of NVP into the lumen after intravenous administration of VP to rats and suggested that VP was metabolized in the enterocytes after intravenous administration (Sandstrom et al., 1998).

In this experiment, the infusion time of VP into the portal vein was set at 20 min because the maximum plasma concentration of VP after oral administration occurs at about 20 min. However, the AUCpv showed dose-dependency, so the bioavailability was calculated using the AUC obtained under apparent linear conditions. The Ft of S-VP was higher than that of R-VP. This result was comparable to that reported others (Bhatti and

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Foster, 1997). However, the Fh of R-VP was almost double that of S-VP, whereas the Fg of S-VP was higher than that of R-VP. These results suggested that the stereoselectivity of the AUC of VP appears to be determined by first-pass metabolism in both the intestine and the liver (Fig. 5).

Efflux of NVP into the jejunum has been reported after intravenous administration of VP racemate to rats (Sandstrom et al., 1998). Both VP and NVP are known substrates for and inhibitors of P-glycoprotein (Sandstrom et al., 1998). Our preliminary in situ small intestinal perfusion study (an experimental procedure described previously by Mihara et al., 2001) showed that NVP was detectable in the outlet perfusate (data not shown). These results indicate that the NVP formed in the intestinal lumen and/or intestinal wall may be excreted into the lumen.

Although VP underwent first-pass metabolism in the intestine, the extent of its metabolism was smaller than that in the liver. The NVP metabolized in the intestine did not appear in the systemic circulation. Furthermore the pharmacokinetics of NVP enantiomers was non-stereoselective. These results suggest that the reason why the AUC of S-NVP is higher than that of R-NVP, despite the AUC of S-VP being higher than that of R-VP after oral administration, is that hepatic first-pass metabolism of VP is the major factor determining the AUC of NVP (Fig. 5).

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In order to confirm the stereoselective metabolism of VP in the intestine, we conducted an *in vitro* microsomal study. The concentrations of VP enantiomers used to investigate the kinetic parameters were corrected for the unbound fractions in the reaction mixture. The intrinsic clearances (Vmax/Km) of the R-isomer to N-dealkylated VP and NVP were significantly higher than those of the S-isomer. This stereoselectivity was corresponded to that of first-pass metabolism in the small intestine observed *in vivo* experiments (Table 2). The CYP enzyme governing N-dealkylated VP and NVP formation was CYP3A2, whereas that responsible for O-demethylated VP formation was CYP1A2. Interestingly, S-VP was preferentially metabolized to norverapamil and O-demethylated VP in rat liver microsomes (Nelson and Olsen, 1988; Nelson et al., 1988) and this stereoselectivity also corresponded to the Fh values observed in our *in vivo* experiments (Table 2).

In previous studies, the AUCs of S- and R-VP in blood obtained after oral administration of VP (80 mg/kg) to healthy volunteers (Hashiguchi et al., 1996), corrected for the blood to plasma concentration ratio (Robinson and Mehvar, 1996), were 29 and 121 ng h/mL, respectively, and the corresponding AUCs of the NVP enantiomers were 80 and 168 ng h/mL. First-pass metabolism of VP in enterocytes has also been reported in human (Sandstrom et al, 1999; von Richter et al, 2001). The extraction ratios of R- and S-VP by enterocytes were 0.49 and 0.68, respectively, and those by liver were

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0.63 and 0.79, respectively (Sandstrom et al, 1999). These results indicate that S-VP undergoes extensive first-pass metabolism with the same direction of stereoselectivity in both organs.The species differences seen in the *in vitro* intestinal microsomal metabolic activity and the unbound fraction in blood also support this theory. Even if it is assumed that all the NVP formed in the intestine is excreted into the intestinal lumen and does not enter the systemic circulation, this does not explain the stereoselectivity and higher AUC level of R-NVP in human. One possible explanation may be a difference in the elimination clearance of NVP, because the unbound fraction of R-NVP in human blood was approximately half that of S-NVP (Robinson and Mehvar, 1996). Further studies will therefore be required to investigate the species difference and stereoselectivity of first-pass metabolism in humans.

In summary, the Fh of R-VP was higher than that of S-VP. Conversely, the intestinal bioavailability of R-VP was lower than that of S-VP, resulting in a higher absolute bioavailability of S-VP. The Ft therefore appears to be determined by first-pass metabolism in both organs, and the NVP enantiomers observed in the systemic circulation after oral administration of VP racemate originate from stereoselective hepatic first-pass metabolism in rats.

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Footnotes

a) This work was supported in part by a grant from the Japan Research Foundation for Clinical Pharmacology.

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c) Numbered footnotes

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Legends for Figures

Figure 1 Comparison between the time courses of concentrations of verapamil (VP) in plasma and blood after intravenous (1.0 mg/kg) administration of racemic VP to rats.

Each data represents mean ± SD (n=4).

Figure 2 Time courses of verapamil (VP) and norverapamil (NVP) concentrations in whole blood after oral (10 mg/kg; A) and intraportal vein (5 mg/kg; B) administration of racemic VP to rats.

Each data point represents the mean ± SD (n=4).

Figure 3 Time courses of verapamil (VP) concentrations in whole blood after intraportal vein (A) and relationship between doses and the area under the concentration time curve of verapamil (VP) administration of racemic VP to rats.

Each data point represents the mean ± SD (n=3-8).

Figure 4 Time courses of formation rate of verapamil metabolites in rat intestinal microsomes (A) and Eadie-Hofstee plots for metabolite formation from S-VP (B) and R-VP (C) in rat intestinal microsomes.

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Figure 5 Suggested scheme of the first-pass metabolism of verapamil enantiomers in rats.

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Table 1 Whole blood to plasma concentration (B/P) ratio and plasma protein binding of verapamil (VP) and norverapamil (NVP) enantiomers in rats

	S	R
B/P ratio		
VP	0.57 ± 0.10	0.84 ±0.07 *
NVP	1.25 ±0.18	1.42 ±0.19
Unbound fraction in plasma		
VP	0.069 ±0.009	0.110 ±0.018 *

All data are expressed as mean ± S.D. (n=4)

* significantly different from S-isomer ($p<0.05$)

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Table 2 Bioavailability and clearances of verapamil after administration of racemic verapamil to rats

	S-VP	R-VP
Total (Ft)	0.088	0.068
Liver (Fh)	0.130	0.217
GI tract (Fg)	0.677	0.313
Total blood clearance (mL/min/kg)	73.9±18.4	78.1±15.7
Hepatic blood clearance (mL/min/kg)	52.2	47.0
Hepatic intrinsic clearance (mL/min/kg)	10117	6998

Calculation of each bioavailability (n=4) from the *in vivo* study are given in detail in Method section

Hepatic blood clearance was calculated from the hepatic blood flow (Q_{liver}; 60 mL/min/kg) and the hepatic extraction ratio (1-Fh)

Hepatic intrinsic clearance was calculated by a parallel tube model ($Fh=EXP(-fub\times CL_{int}/Q_{liver})$.

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Table 3 Metabolic parameters for N-dealkylated VP, O-demethylated VP and norverapamil (NVP) in rat small intestinal microsomes

	N-dealkylated VP		O-demethylated VP		NVP	
	S	R	S	R	S	R
Km	125.3±18.2	65±11.2 *	140±52.2	74.4±11.3	79.6±18.6	59.5±11.6
Vmax	11.2±1.9	13.8±5.2	6.7±2.2	8.7±2.7	13.1±3.4	16.0±3.4
Vmax/Km	0.09±0.02	0.21±0.08 *	0.06±0.03	0.12±0.06	0.07±0.03	0.27±0.06 *

Each value represents the mean ± SD (n=4)

* $p<0.05$ (vs S-enantiomer)

Abbreviations and units: Km, affinity constant (μM); Vmax, maximum velocity of formation (pmol/min/mg protein); Vmax/km, intrinsic clearance (μL/min/g protein)

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Figure 1

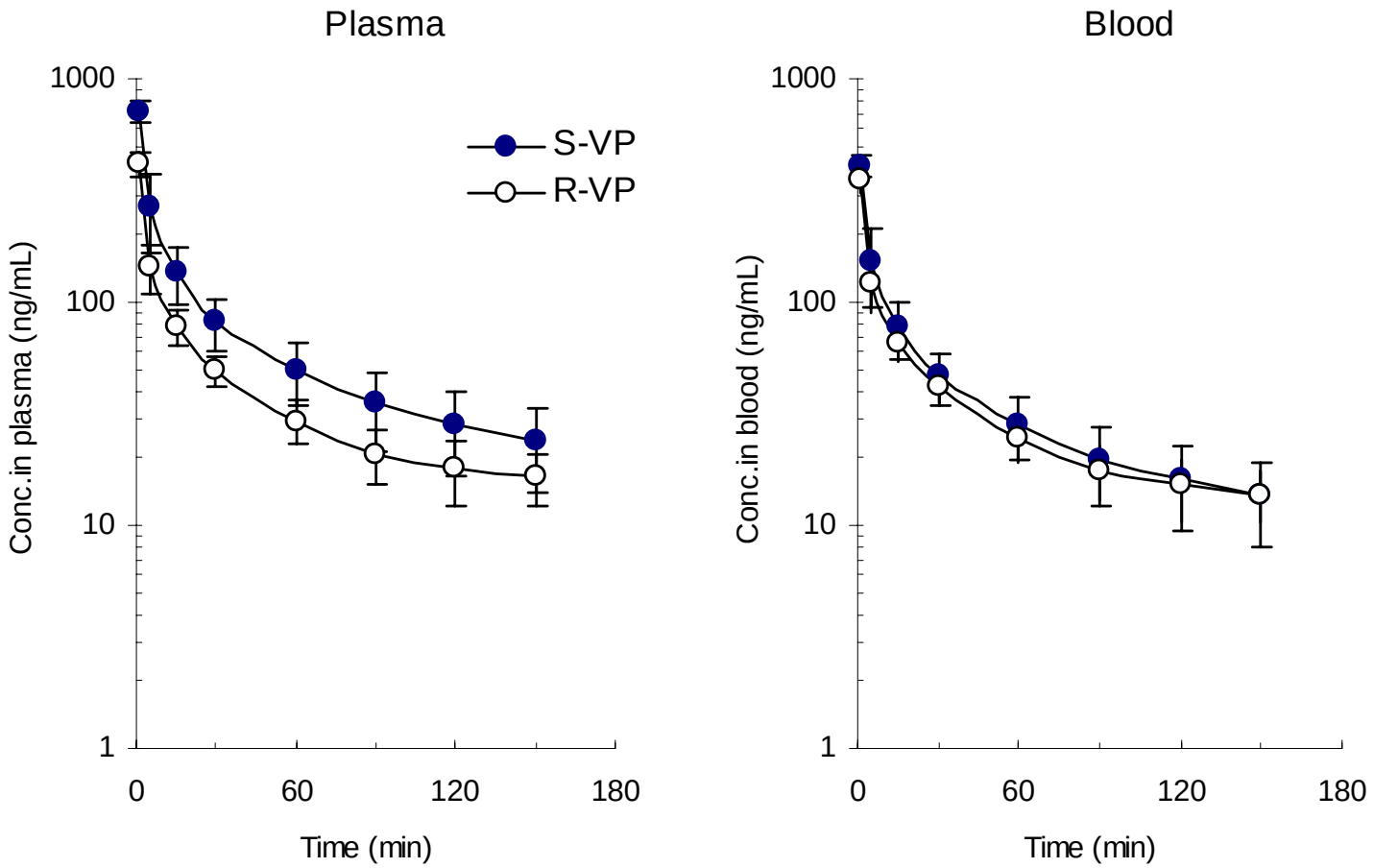

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Figure 2

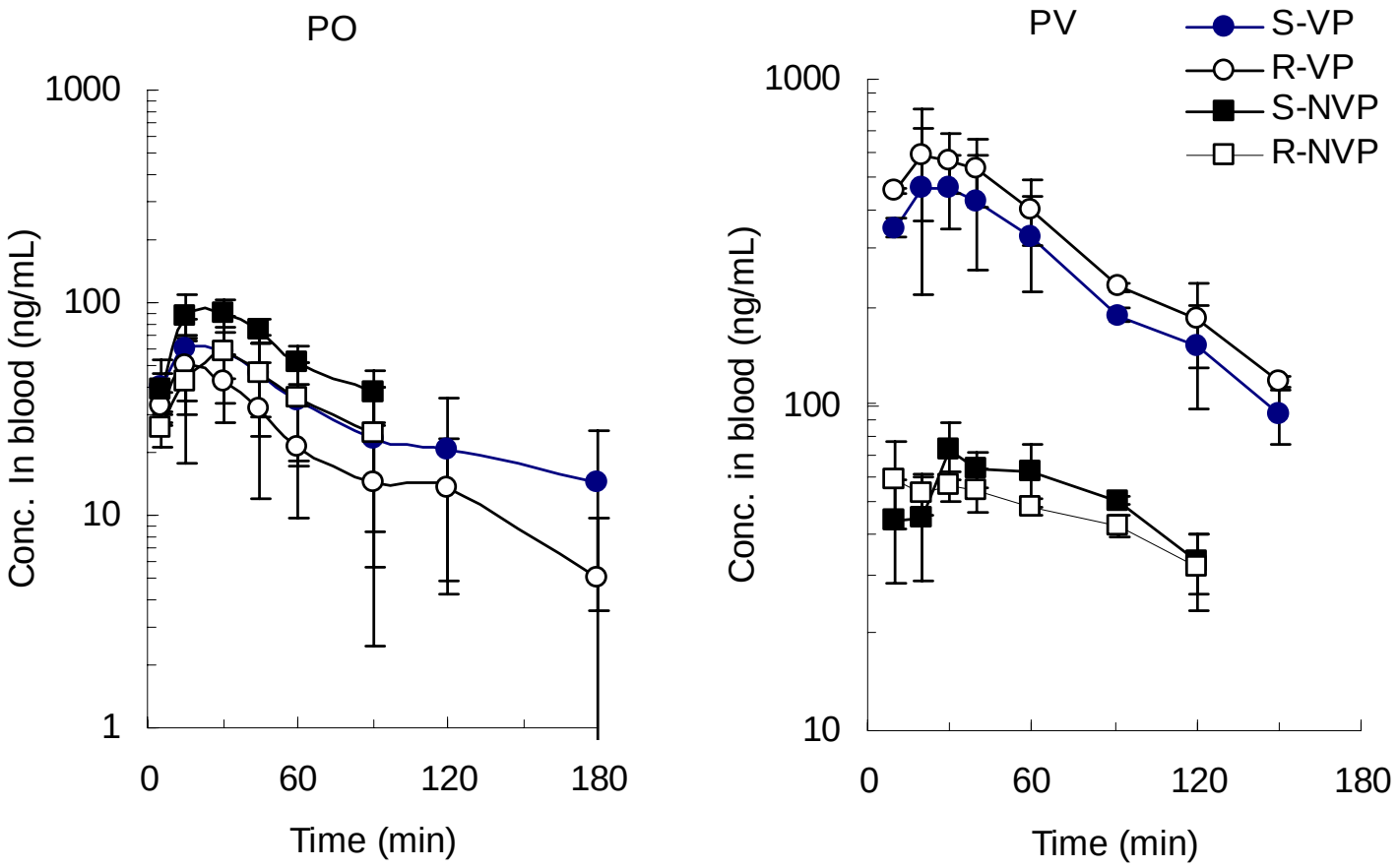

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Figure 3

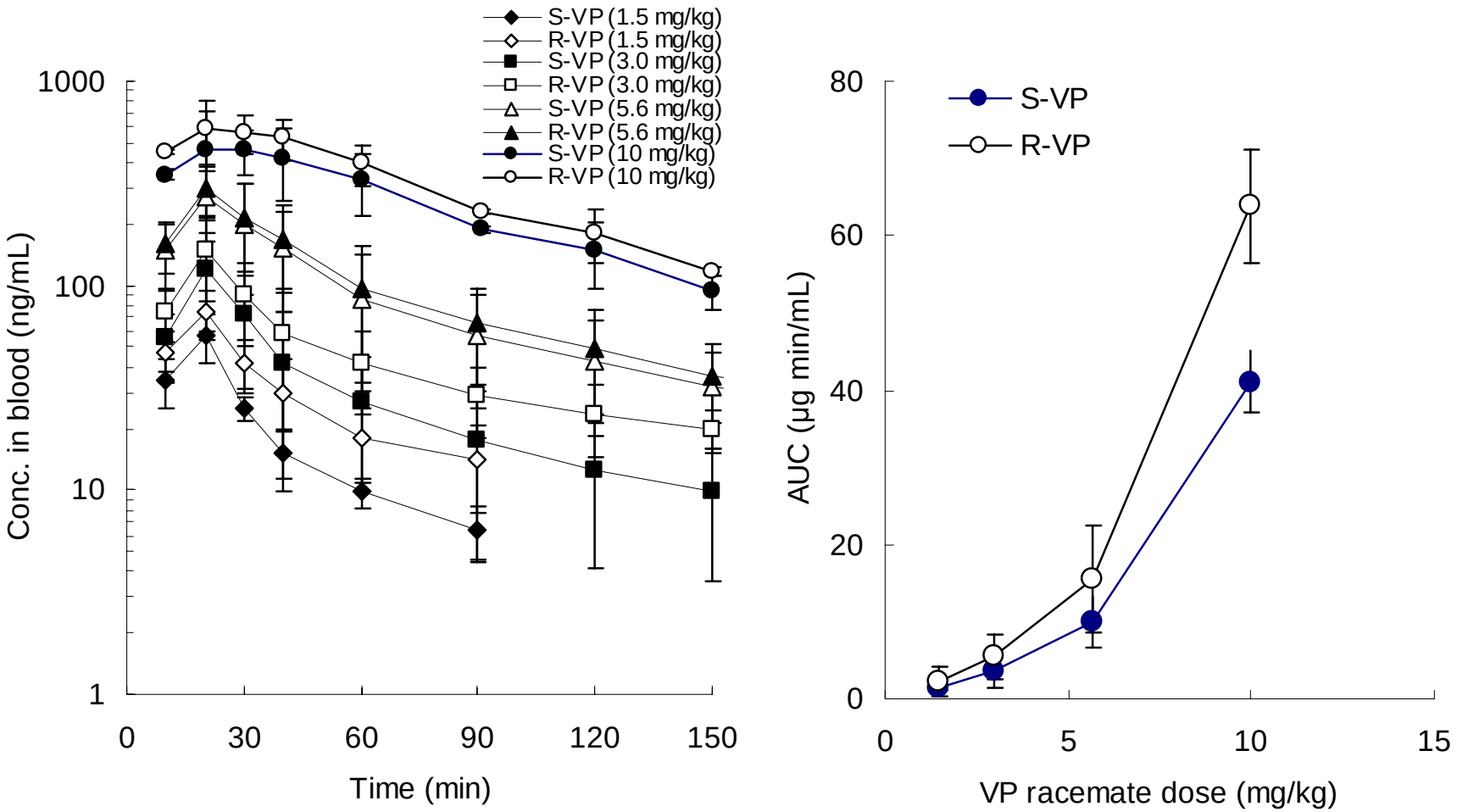

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Figure 4

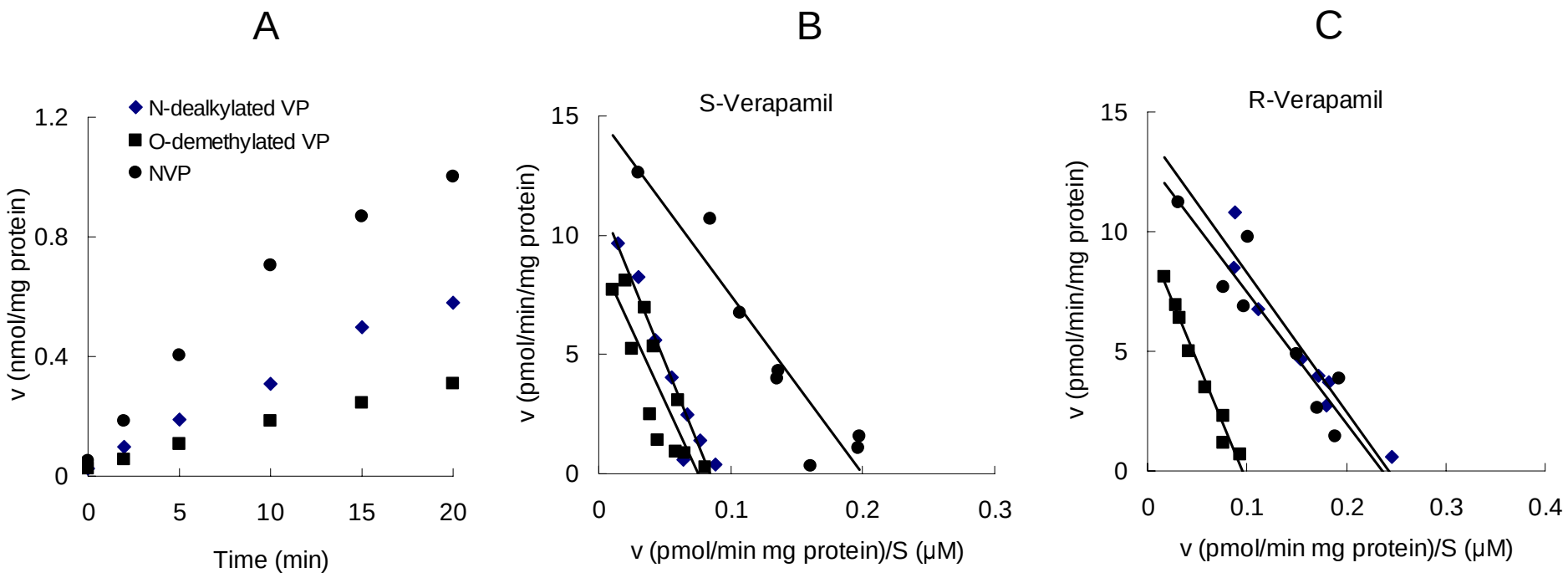

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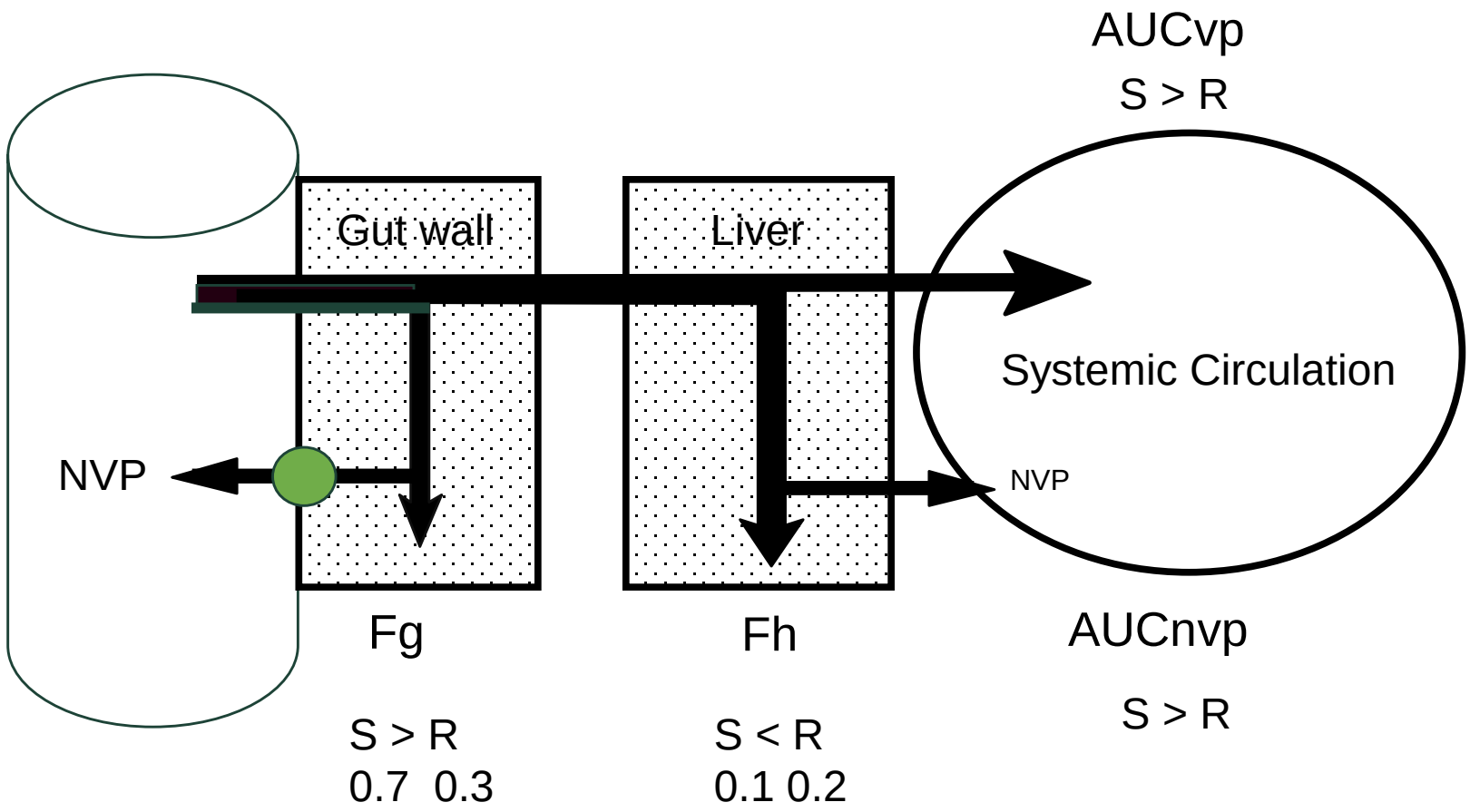
Figure 5



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