Title:
First-pass hydrolysis of a propranolol ester derivative in rat small intestine

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Text Statistics:
Number of text pages: 22
Words in Abstract: 206
Words in Introduction: 663
Words in Discussion: 1055
References: 36
Figures: 6
Tables: 2

Abbreviations: CES, carboxylesterase; PL, propranolol; IVA, isovaleric acid; HBSS, Hanks’ balanced salt solution; EBSS, Earle’s balanced salt solution; KHBB, Kerbs-Henseleit bicarbonate buffer; HPLC, high performance liquid chromatography; AP, apical; BL, basolateral; BBM, brush-border membrane.
ABSTRACT

In order to evaluate the first-pass hydrolysis of O-isovaleryl-propranolol (isovaleryl-PL), which was used as a model ester-compound, rat intestinal jejunum and blood vessels were perfused simultaneously. The membrane permeability of isovaleryl-PL was greater than PL because it was more lipophilic. Isovaleryl-PL was almost completely hydrolyzed to PL and isovaleric acid (IVA) in epithelial cells at a rate limited by its uptake. Based on pH-partitioning PL and IVA were transported into both vascular (pH 7.4) and luminal sides (pH 6.5). Therefore, when isovaleryl-PL was perfused into the jejunal lumen, more than 90% permeated into the blood vessel as PL. In addition, PL appeared in the lumen at a rate 6-fold greater than in blood vessels. When isovaleryl-PL was perfused its disappearance (50.5 ± 1.95 nmol/min) was the sum of the absorption and secretion rates of PL. In contrast, isovaleric acid (IVA) was transported into blood vessels rather than the jejunal lumen. In addition, the calculated degradation clearance from in vitro hydrolysis ($K_m$: 13.7 ± 1.71 µM, $V_{max}$: 29.1 ± 3.81 nmol/min/mg protein) was 3.42 mL/min/10 cm jejunum, which was 24-fold greater than the observed $CL_{deg}$ (0.14 ± 0.02 mL/min/10 cm jejunum). These findings indicate that in addition to the liver, the intestine markedly contributes to first-pass hydrolysis.
INTRODUCTION

The small intestine plays a significant role in the metabolism of orally administered xenobiotics, including therapeutic drugs, although it is regarded as an absorptive organ (Lin et al., 1999). Metabolic pathways found in the small intestine include both phase I and II reactions (Kaminsky and Zhang, 2003). Most drug-metabolizing enzymes present in the liver are also found in the small intestine; however, their levels are generally much lower in the small intestine (Lin et al., 1999). The importance of the small intestinal metabolizing enzymes arises from their location in the epithelial cells (enterocytes) of this organ, which can result in reduced systemic uptake of drugs. Clinical studies have shown that the small intestine contributes substantially to the overall first-pass cytochrome P450 mediated metabolism of drugs such as midazolam (Thummel et al., 1996), cyclosporine (Wu et al., 1995) and nifedipine (Holtbecker et al., 1996). In addition to oxidative metabolism by cytochrome P450, hydrolysis is also an important phase I reaction in the biotransformation of ester-containing drugs.

Carboxylesterase (CES; E.C. 3.1.1.1) is involved in the hydrolysis of a variety of ester- and amide-containing endogenous compounds. CESs are important both in the inactivation of drugs and the activation of prodrugs and are widely distributed in many tissues, including the intestines (Satoh and Hosokawa, 1998; Zhang et al., 2002). Our understanding of the biochemistry and molecular biology of CES enzymes has recently increased dramatically (Satoh and Hosokawa, 1998; Bencharit et al., 2002;
Bencharit et al., 2003; Wadkins et al., 2001). CESs are membrane-bound enzymes located in the endoplasmic reticulum. Like cytochromes P450, mammalian CESs comprise a multigene family, and the isozymes are classified into four main groups with several subgroups. The mammalian liver mainly expresses CES 1 and CES 2 group enzymes, while the major intestinal isoform is CES 2 isozymes. Therefore, the hydrolysis characteristics of small intestine are different from the liver. The intestinal hydrolysis may contribute to overall first-pass metabolism of an ester derivative that can improve membrane permeability by increasing the lipophilicity of their parent compounds (Mizen and Burton, 1998). However, only a few reports describe the intestinal first-pass hydrolysis and simultaneous analysis of disposition for ester compounds and their hydrolysates during intestinal absorption (Okudaira et al., 2000; Pruksaritanont et al., 1998; Ruiz-Balaguer et al. 2002).

The present study focused on the contribution of intestinal hydrolysis to the overall metabolism of the model ester-containing compound O-isovaleryl-propranolol (isovaleryl-PL; Fig. 1). Isovaleryl-PL and its hydrolysate, PL, are both weak basic and hydrophobic compounds [pKₐ: PL, 9.44; isovaleryl-PL, 8.59; logP (n-octanol / pH 4.0 buffer): PL, 0.38; isovaleryl-PL, 1.94]. In contrast, another hydrolysate, isovaleric acid, is an acidic and hydrophilic compound. Therefore, the hydrolysis of isovaleryl-PL in epithelial cells affects the absorption of PL and isovaleric acid. The absorption and hydrolysis of isovaleryl-PL was evaluated using in situ rat jejunal single-pass perfusion. This system correlates with the
fractional dose absorbed in humans (Amidon et al., 1988; Fragerholm et al., 1996) and maintains the intestinal architecture with respect to metabolism, absorption, and secretion (Pang et al., 1986; Tamura et al., 2003; Zimmerman et al., 2000). We reported previously that isovaleryl-PL was hydrolyzed in the rat intestine during absorption using an in situ closed loop preparation with infusion of fresh blood (Yoshigae et al., 1998). However, this method was inadequate for evaluating the pharmacokinetics of the intestinal first-pass hydrolysis of isovaleryl-PL at two points for the following reasons. First, an ester-compound is repeatedly taken up into the mucosa and returned to the intestinal lumen during the experiment. Second, esterases in rat blood can hydrolyze ester-compounds (Yoshigae et al., 1999). Therefore, the present study was performed using a rat jejunal single-pass perfusion system that simultaneously perfused blood vessels and the jejunum. We successfully characterized the intestinal absorption of isovaleryl-PL without hydrolysis in the gut luminal and vascular fluid.

Finally, it was demonstrated that the intestinal esterase significantly contributed to the first-pass hydrolysis of isovaleryl-PL and its hydrolysates were transported to both vascular and luminal sides according to their physical properties.
MATERIALS AND METHODS

Materials. O-isovaleryl-PL hydrochloride was synthesized from PL hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and isovaleryl chloride (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) as described previously (Shameem et al., 1993). The identity and purity of the synthesized isovaleryl-PL were confirmed by infrared, NMR, atomic analysis, and HPLC. MES and HEPES were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nobo heparine was purchased from Leo Pharmaceutical Products, Ltd. (Ballerup, Denmark). Bovine serum albumin (BSA, Fraction V), fluorescein isothiocyanate dextran 4000 (FD-4), Dulbecco’s modified Eagle’s medium (DMEM), 0.25% Trypsin-EDTA, Dulbecco’s phosphate buffered saline (D-PBS), Hanks’ balanced salts (HBS) and Earle’s balanced salts (EBS) were purchased from Sigma (St. Louis, MO). Nonessential amino acid (NEAA), Penicillin-Streptomycin and L-glutamine were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Cansera International Inc. (Rexdale, Ontario, Canada). All other chemicals were of analytical grade.

Preparation of rat jejunal 9,000g supernatant (S9). Male Wistar rats (250-300 g, 8 weeks) were used after overnight fasting with free access to water. Rats were anesthetized with ether and sacrificed by exsanguination from the abdominal aorta. Intestines were removed and rinsed with ice-cold 1.15% KCl. The jejunal segments were cut open and washed with ice-cold
1.15% KCl. The intestinal mucosa was stripped, minced, and homogenized with 3 volumes of 50 mM HEPES buffer (pH 7.4) containing 1.15% KCl using a Potter-Elvehjem Teflon pestle under ice-cold conditions. The homogenates were centrifuged at 9,000g for 20 min at 4˚C to obtain the supernatant (S9) fraction. Protein contents were determined by the method of Bradford with BSA as the standard (Bradford, 1976). These preparations were stored at -80˚C until use.

Hydrolysis experiments of isovaleryl-PL in the jejunal mucosa S9 fraction. The jejunal S9 was diluted with pH 7.4 HEPES buffer (50 mM) to 25 μg/mL. The S9 solution (400 μL) was preincubated at 37˚C for 5 min, and the reactions were started by adding 2 μL of racemic isovaleryl-PL dissolved in DMSO (final concentration: 4-200 μM). The final concentration of DMSO was maintained at 0.5%, which had no effect on hydrolase activity. After 10 min incubation, reactions were terminated by adding 5 mL of ethyl acetate and 1 mL saturated NaCl solution adjusted to pH 4.0 with phosphoric acid. After extraction of isovaleryl-PL and PL into ethyl acetate, the organic phase was evaporated to dryness. The resulting residues were redissolved in 250 μL of HPLC mobile phase before injecting 20 μL onto the HPLC column. The hydrolytic activity was evaluated by the formation of PL. Kinetic parameters, K<sub>m</sub> and V<sub>max</sub>, were calculated by fitting the data to the Michaelis-Menten equation by nonlinear least squares analysis, MULTI program (Yamaoka et al., 1981).
In situ intestinal single-pass perfusion. Male Wistar rats (250-300 g, 8 weeks of age) were housed in an air-conditioned room and given free access to commercial chow and tap water. Rats were fasted for 15 hr and then anesthetized by intraperitoneal injections of 2% sodium pentobarbital (0.5 mL). Vascular perfused intestinal loops were prepared as previously described (Yamashita et al., 1994). Briefly, a small intestinal loop (about 10 cm of upper jejunum) was isolated and both ends of the jejunal loop were cannulated with Teflon tubes (3 mm i.d.) after flushing out the intestinal contents with warmed physiological saline. The superior mesenteric artery and the portal vein were cannulated with polyethylene tubes (PE10 and PE15, respectively) for vascular perfusion. The cannulated intestinal segment was isolated from other portions and suspended in a serosal bath containing 150 mL of Kerbs-Henseleit bicarbonate buffer (KHBB, pH 7.4) warmed to 37°C.

Single-pass perfusion of the blood vessel was initiated after isolating the intestine and continued throughout the experiment. KHBB containing 3% BSA and 10 mM D-glucose was used as the vascular perfusate at a flow rate of 3.0 mL/min. The jejunal loop was perfused with MES buffer (pH 6.5) containing PL or isovaleryl-PL (300 µM) at a flow rate of 0.3 mL/min. FD-4 (0.1 mg/mL) as a nonabsorbable marker was added to the luminal perfusate and the volume of the luminal perfusate was corrected from the dilution of FD-4. The perfusates from the intestinal segment and the vascular outflow were collected at 10 min and 5 min intervals for 60 min, respectively, and immediately added to extraction solvent. The degradation
of isovaleryl-PL was 1.5% and 8% after 1 hr at 37°C in the luminal perfusate and vascular perfusate, respectively. Therefore, the degradation of isovaleryl-PL was negligible during sampling. After the perfusion period, the contents of the intestinal segment were drained with 10 mL of warmed saline. The intestinal mucosa was immediately stripped and homogenized with ultrasound in ice-cold acetonitrile. After the homogenized sample was centrifuged at 3,000 rpm for 10 min at 4°C, the supernatant was injected onto HPLC to determine the mucosal drug amount.

For determination of PL and isovaleryl-PL, aliquots of the vascular samples (6 mL) were adjusted to pH 4.0 by adding an equal volume of phosphate solution buffer saturated with NaCl and extracted with 10 mL of ethyl acetate. The organic phase was separated and evaporated to dryness. The resulting residue was redissolved in 200 µL acetonitrile before injecting 30 µL onto the HPLC column. The luminal samples (100 µL) were deproteinized with 1 mL acetonitrile, and centrifuged at 3,000 rpm for 5 min. The supernatant (30 µL) was injected onto the HPLC column. For the determination of isovaleric acid, vascular samples (6 mL) were extracted with 2.5 mL of ethyl acetate after adjusting pH to 4.0 by adding 2.5 g NaCl and 10 M phosphoric acid (30 µL). The luminal samples (2 mL) were extracted with 1.5 mL of ethyl acetate after adjusting pH to 4.0 with 1.0 g NaCl and 10 M phosphoric acid (2 µL). The organic phases were concentrated under a stream of N₂ gas, and 5 µL aliquots were injected onto the GC column. This extraction process caused less than 0.1% degradation of isovaleryl-PL. FD-4 (0.1 mg/mL) in luminal samples was determined by
fluorescence spectrometer.

**Transport across Caco-2 cell monolayers.** Caco-2 cells were seeded at a density of $2.5 \times 10^5$ cells/mL onto polycarbonate filters (3 µm pores, 4.71 cm$^2$ growth area) and grown for 21 to 27 days in DMEM supplemented with 1% (v/v) NEAA, 10% (v/v) FBS, benzylpenicillin G (50 Units/mL), streptomycin (50 µg/mL) and 2 mM L-glutamine at 37°C in a humidified air-5% CO$_2$ atmosphere. Cell passage 34 was used in the experiments. For the drug transport experiment across Caco-2 cell monolayers, the apical (AP) medium (1.5 mL) was either HBSS (pH 7.4) or EBSS (pH 6.0). The cell monolayers were equilibrated with basolateral (BL) medium (pH 7.4 HBSS, 2.6 mL) and AP medium for 30 min at 37°C. Hereafter, the donor and receptor media were replaced with PL containing (50 µM) medium and fresh medium, respectively. At appropriate intervals, samples (150 µM) were taken from the receiver compartment and replaced with an equal the same volume of fresh medium. All samples were immediately assayed by HPLC. No damage to Caco-2 cell membrane during transport experiment was observed after staining with 0.1% trypan blue.

**Assay.** PL and isovaleryl-PL concentrations were determined by HPLC consisting of pump (Jasco PU-980; Jasco International Co. Ltd., Tokyo, Japan), data application apparatus (Shimadzu C-R4A; Shimadzu corp., Kyoto, Japan) and a fluorescence detector (Jasco 820-FP; Jasco International Co. Ltd., Tokyo, Japan). LiChrosorb RP-select B column
(7μm, 250 x 4 mm i.d.; Shimadzu GLC Ltd., Tokyo, Japan) were used with a mobile phase of acetonitrile/20 mM KH$_2$PO$_4$ [1 : 1 (v/v)] at a flow rate of 1.0 mL/min. Both PL and isovaleryl-PL were detected with excitation and emission wavelengths of 285 and 340 nm, respectively. The quantitative limitation of both compounds was 30 pmol of PL and 60 pmol of isovaleryl-PL as the injected amounts.

Isovaleric acid concentration was determined by Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and Shimadzu C-R6A data application apparatus (Shimadzu corp., Kyoto, Japan). A glass column packed with Gaskuropack 56 80/100 (3.2 mm i.d. x 2.1 m; GL Sciences, Inc., Tokyo, Japan) was used under a carrier gas of N$_2$ at flow rate 4.0 kgf/cm$^2$. Temperature setpoints of vaporization, column and detector compartment were 220, 200 and 230˚C, respectively. The quantitative limit of isovaleric acid was 1 nmol as the injected amount.

**Data analysis.** Absorption parameters in the *in situ* perfusion experiment were obtained as shown below. The appearance rate ($v_1$) of isovaleryl-PL ($v_1$, isovaleryl-PL) and PL ($v_1$, PL) in the mesenteric vein was calculated according to eq. 1:

$$v_1 = Q_b \cdot C_b$$

where $Q_b$ and $C_b$ are the flow rates of vascular perfusion and the concentration of isovaleryl-PL or PL in the mesenteric vein, respectively.
The disappearance rate ($v_2$) of isovaleryl-PL and the appearance rate ($v_3$) of PL in the intestinal lumen were calculated as follows:

$$v_2 = Q_l \cdot (C_{in} - C_{out})$$

$$v_3 = Q_l \cdot C_{M, out}$$

where $Q_l$ is the flow rate of intestinal perfusion. $C_{in}$ is the concentration of isovaleryl-PL at the entrance to the jejunal segment. $C_{out}$ and $C_{M, out}$ are the concentration of isovaleryl-PL and PL at the exit of the jejunal segment, respectively. $C_{out}$ and $C_{M, out}$ were corrected with the concentration of FD-4.

The apparent absorption clearance into the mesenteric vein ($Cl_{app}$) was calculated by eq. 4.

$$Cl_{app} = \frac{AUC_b}{AUC_l} \cdot Q_b = \frac{\text{Absorbed amount}}{AUC_l}$$

$AUC_b$ and $AUC_l$ are the areas under the curve of the administered compound in the mesenteric vein and in the intestinal lumen at the steady-state, respectively. The degradation clearance of isovaleryl-PL in the jejunal mucosa ($Cl_{deg}$) was calculated by eq. 5.

$$Cl_{deg} = \frac{AUC_{M, l}}{AUC_{P, l}} \cdot Q_l + \frac{AUC_{M, b}}{AUC_{P, l}} \cdot Q_b = \frac{\text{Degraded amount}}{AUC_{P, l}}$$

Where $AUC_{P, l}$ and $AUC_{M, l}$ are the areas under the curve of isovaleryl-PL and PL in the intestinal lumen at the steady state, respectively. $AUC_{M, b}$ is
the area under the curve of PL in the mesenteric vein. AUC in the intestinal lumen was obtained by assuming that the concentration of isovaleryl-PL or PL in the intestinal loop decreased according to first-order kinetics.

For comparison of the absorption parameter with the reported value, the permeability rate constant ($P_{\text{eff}}$) was calculated as:

$$P_{\text{eff}} \text{ (cm/min)} = Q_l \cdot \frac{1 - C_{\text{out}}/C_{\text{in}}}{2\pi RL}$$  \hspace{1cm} (6)

where $R$, the radius of the segment, was assumed to be 0.178 cm (Yamashita et al., 1997) and $L$ is the length of the segment (i.e. 10 cm).

The apparent permeability coefficient ($P_{\text{app}}$) across Caco-2 cell monolayers was calculated according to the following equation:

$$P_{\text{app}} \text{ (cm/sec)} = \frac{dQ/dt}{A \cdot C_0}$$  \hspace{1cm} (7)

where $dQ/dt$ is the rate of appearance of drugs in the basolateral compartment (steady-state flux, $\mu$mol/sec), $A$ is the surface area of cell monolayer (i.e. 4.71 cm$^2$) and $C_0$ is the initial drug concentration in the donor compartment ($\mu$M).
RESULTS

Hydrolysis of isovaleryl-PL in jejunal S9. Kinetics experiments using the jejunal S9 fraction showed that mucosal tissue contributes to the hydrolysis of isovaleryl-PL. Fig. 2 shows the hydrolysis of isovaleryl-PL (final concentration: 4-200 µM) in the jejunal S9. The hydrolysis of isovaleryl-PL in the jejunum was a one component reaction and non-specific hydrolysis was not observed. By fitting the data to the Michaelis-Menten equation, $K_m$ and $V_{max}$ values of $13.7 \pm 1.71 \mu M$ and $29.1 \pm 3.81$ nmol/min/mg protein were observed, respectively.

Absorption of PL in rat jejunal single-pass perfusion. The appearance rate of PL in the mesenteric vein ($v_{1,PL}$, Fig. 3a) and disappearance rate of PL in the jejunal lumen ($v_{2,PL}$, Fig. 3b) during perfusion with 300 µM of PL are shown in Fig. 3. A steady-state was achieved after perfusion for 20 min. The appearance and disappearance rates were $6.19 \pm 0.31$ nmol/min and $7.01 \pm 0.51$ nmol/min, respectively. Both rates, being approximately equal, suggested that PL was not metabolized during absorption.

Absorption of isovaleryl-PL in rat jejunal single-pass perfusion. Isovaleryl-PL is a good substrate for evaluating intestinal first-pass hydrolysis, since it is hydrolyzed in the intestinal mucosa (Fig. 2) and further metabolism of PL is not observed during absorption (Fig. 3). Fig. 4a shows the appearance rate of PL and isovaleryl-PL in the mesenteric vein ($v_1$) when 300 µM isovaleryl-PL was perfused in the luminal side. Fig. 4b
shows the disappearance rate ($v_2$) of isovaleryl-PL and the appearance rate ($v_3$) of PL in the rat luminal perfusate. Steady-state was achieved after perfusion for 30 min. Interestingly, the venous levels of isovaleryl-PL were lower than PL, which represented 94% of total venous concentration of PL and isovaleryl-PL in the venous outflow. The appearance rate of PL at steady-state was $7.20 \pm 1.05$ nmol/min, which was similar to the appearance rate of PL under perfusion with PL (Fig. 3). In contrast, the appearance of isovaleryl-PL in the mesenteric vein ($v_1$, isovaleryl-PL) at steady-state was $0.49 \pm 0.14$ nmol/min, which was 15-times lower than the appearance of PL. Furthermore, PL appeared not only in the mesenteric vein but also in the jejunal lumen. The appearance rate of PL in the jejunal lumen ($v_3$) was $43.2 \pm 3.49$ nmol/min, which was much faster than its appearance in the mesenteric vein. The appearance rate of PL in the jejunal lumen contributed to about 85% of the disappearance rate of isovaleryl-PL in the jejunal lumen ($50.5 \pm 1.95$ nmol/min). Based on the hydrolysis experiment of isovaleryl-PL (300 µM) in the perfusate maintained in the jejunal loop, it was estimated that 2.5% of isovaleryl-PL was hydrolyzed during its passage through the intestinal segment at a flow rate of 0.3 mL/min. Therefore, it seemed that PL appeared in the jejunal lumen and the mesenteric vein after isovaleryl-PL was hydrolyzed in epithelial cells.

**Recovery of PL and isovaleryl-PL at steady-state in rat jejunal single-pass perfusion.** Table 1 lists the recovery of PL and isovaleryl-PL in the mucosa, blood vessel and intestinal lumen at steady-state. About 90%
of the input amount was present in the jejunal fluid in perfusion experiments with PL and isovaleryl-PL. The amount of PL in the mucosal tissue was 7.14% and 4.76% under isovaleryl-PL and PL perfusion, respectively, despite a 2-fold lower concentration of PL in the intestinal fluid under isovaleryl-PL perfusion. This finding supports the view that the hydrolysis of isovaleryl-PL was predominantly catalyzed by intracellular esterase. Interestingly, 52% of perfused isovaleryl-PL was converted to PL and only 7.47% was transported into the mesenteric vein while 44.6% was secreted into the luminal side. These results indicate that PL is easily transported into the intestinal lumen rather than the mesenteric vein after hydrolysis in epithelial cells. In addition, total recovery was nearly 100% with isovaleryl-PL perfusion, which suggests that the metabolic pathway of isovaleryl-PL in the intestine only involves hydrolysis of the ester-bond.

**Absorption and secretion of isovaleric acid.** Since the amount of isovaleric acid (IVA) converted from isovaleryl-PL during absorption in the jejunal loop was equal to the amount of PL, the absorption and secretion of IVA were compared to PL. Although endogenous fatty acid leakage from mucosal cells was monitored during the perfusion experiment, no interference peak was observed for the analysis of IVA, which had a retention time of 23.5 min.

Fig. 5 shows the appearance of IVA in the mesenteric vein (v_{1, IVA}) and jejunal lumen (v_{3, IVA}) during perfusion with 300 µM isovaleryl-PL. Steady-state was achieved after about 30 min. In contrast to PL, the
appearance rate of IVA in the mesenteric vein was 3-fold greater than in the jejunal lumen. These results suggest that isovaleryl-PL was hydrolyzed in mucosal cells and IVA and PL were transported at rates based on their physical properties. The total appearance rate of IVA in the mesenteric vein and intestinal lumen was about 60% of the disappearance rate of isovaleryl-PL in the jejunal lumen (50.5 nmol/min). This discrepancy might be caused by retention of IVA in the intestinal mucosa and the quantitative limitation of IVA by GC.

**Kinetic analysis for absorption of isovaleryl-PL in rat jejunal single-pass perfusion.** Table 2 lists the apparent absorption clearance (CL\text{app}) of PL and isovaleryl-PL, the degradation clearance (CL\text{deg}) of isovaleryl-PL in rat jejunal single-pass perfusion, and the absorption, degradation and secretion rates of each compound. The calculated permeability rate constant (P\text{eff}) for isovaleryl-PL was $16.6 \times 10^{-3} \pm 1.26 \times 10^{-3}$ cm/min. This was 7-fold greater than that of PL because of its greater lipophilicity. However, CL\text{app} of isovaleryl-PL was $2.42 \pm 0.79$ µL/min, which was 9-fold lower than CL\text{app} of PL with PL perfusion. The disappearance rate of isovaleryl-PL ($50.5 \pm 1.95$ nmol/min) was comparable to the sum of absorption and secretion rates of PL when isovaleryl-PL was perfused. These findings indicate that the isovaleryl-PL which disappeared from the jejunal segment was completely absorbed and hydrolyzed to PL, which was then transported to both jejunal and vascular sides. In addition, the degradation clearance of isovaleryl-PL at steady-state
was 141 ± 20.5 µL/min, which was calculated from the total amount of PL that appeared in both the blood vessel and the intestinal lumen (eq. 5).

**Effect of apical pH on transport of PL across Caco-2 cell monolayers.**

In order to clarify why PL was transported to the luminal side rather than the vascular side of epithelial cells, we investigated the effect of apical and basolateral pH on the transport of PL (pKa = 9.44) across Caco-2 cell monolayers. The basolateral medium pH was fixed at 7.4 and apical medium pH was varied between 6.0 and 7.4. *In situ* rat intestinal single-pass perfusion was performed with intestinal fluid (pH 6.5) under physiological conditions. However, it has been reported that an acid microclimate is present just above the epithelial cell layer in the upper small intestine. The acid microclimate is maintained by the secretion of hydrogen ions from epithelial cells and the restriction of ion diffusion within the mucus layers (Shiau et al., 1985). Both *in vitro* (Lucas et al, 1975) and *in vivo* (Lucas, 1983) experiments have shown the pH of this microclimate region to vary between 5.8 and 6.3, a range which is lower than the bulk solution in the intestinal tract. Therefore, a pH 6.0 was selected as the apical medium pH in transport experiments across the Caco-2 cell monolayer.

Fig. 6 shows the permeability constant (P_app) for apical to basolateral (AP-BL) and basolateral to apical (BL-AP) transport of PL through Caco-2 cell monolayer at apical pH values of 6.0 and 7.4. When the apical pH was 7.4, P_app for AP-BL and BL-AP transport of PL were 4.54 x 10^{-5} cm/s and
4.01 x 10^{-5} \text{ cm/s}, respectively. This indicates that PL was transported by simple diffusion at an initial concentration of 50 \mu M PL. In contrast, the AP-BL transport rate was lower (0.82 x 10^{-5} \text{ cm/s}) and the BL-AP transport rate was higher (10.6 x 10^{-5} \text{ cm/s}) when a pH gradient (apical: 6.0; basolateral: 7.4) was present. The efflux ratio of PL was 13 under this pH gradient condition.
DISCUSSION

Cloning and \textit{in vitro} expression of molecular targets can rapidly be optimized using the powerful drug discovery tools currently available, thereby facilitating the drug discovery process. Thus, the new essence of the drug design question becomes the efficient delivery of the biologically active compounds to the target site. While oral delivery is the preferred route of administration, it is the most difficult route to optimize. The prodrug approach can address poor intestinal absorption by improving passive transport. Lipophilicity, which is a major determinant of membrane permeation, is often correlated with the partition coefficient when aqueous solubility is not exceeded and the unstirred water layer is not an imposing barrier (Ungell et al., 1998). In general, PL has been used as a marker of passive diffusion to study transcellular transport in Caco-2 cell monolayers (Salama et al., 2004) and \textit{in vivo} absorption experiments (Yamashita et al., 1997). As summarized in Table 2, although PL has sufficient lipophilicity to possess a high absorption rate, $P_{\text{eff}}$ of isovaleryl-PL was about 7-fold greater. This arises from the greater lipophilicity of isovaleryl-PL (log $PC$: 1.94) over that of PL (log $PC$: 0.38). The observed $P_{\text{eff}}$ of PL ($2.20 \times 10^{-3} \pm 0.16 \times 10^{-3}$ cm/min) was lower than that value (ca. $8.5 \times 10^{-3}$ cm/min) reported by Yamashita et al. (1997). This discrepancy may be explained by the pH value of the luminal perfusate as the concentration of uncharged PL, which is the absorbed form, was lower in the current study (pH 6.5 of luminal fluid) than in previous studies (pH 7.0 of luminal fluid).

The disappearance rate of isovaleryl-PL that correlated with $P_{\text{eff}}$ was
ID#: DMD/2005/007682

comparable to the sum of the absorption and secretion rates of PL derived from isovaleryl-PL (Fig. 4, Table 2). The hydrolysis of isovaleryl-PL to PL probably occurred in the luminal fluid, the brush-border membrane (BBM) and enterocytes. The hydrolysis of isovaleryl-PL in the luminal fluid (pH 6.5) was negligible with only 2.5% of isovaleryl-PL being hydrolyzed across the length of the jejunal segment. The hydrolysis rate of isovaleryl-PL in the brush-border membrane vesicle (BBMV) of rat intestine was 10-fold lower than in the intestinal S9 fraction (Yoshigae et al., 1998). Since membrane proteins levels are low at the BBM, it is unlikely that isovaleryl-PL was hydrolyzed here. Moreover, PL levels in blood vessel and mucosal tissue can not be explained by PL concentrations in the jejunal lumen if isovaleryl-PL was converted to PL only in the luminal side. These results suggest that isovaleryl-PL was hydrolyzed by intracellular esterase in the mucosa. The degradation clearance of isovaleryl-PL in the single-pass experiment (CL$_{deg}$; 141 µL/min) was 6.5-fold larger than the absorption clearances of PL (Table 2). Taking into account the difference of P$_{eff}$ between PL and isovaleryl-PL, CL$_{deg}$ might be comparable to the uptake clearance of isovaleryl-PL into the jejunum. Therefore, isovaleryl-PL might be hydrolyzed at a rate limited by uptake into epithelial cells.

Jejunal epithelial cells appear to have a great ability to hydrolyze isovaleryl-PL. The degradation clearance of isovaleryl-PL in the jejunal S9 fraction was based on the concentration of isovaleryl-PL in the mucosa (97.7 nmol/g tissue, Table 1), the S9 protein concentration (about 28 mg
S9/ g mucosal tissue) and the mucosal tissue weight (0.47 g) of 10 cm of jejunal segment. The calculated degradation clearance based on in vitro hydrolysis was 3.42 mL/min/10 cm jejunum, which was 24-fold greater than the observed \( \text{CL}_{\text{deg}} \) (0.14 mL/min; Table 2) in the single-pass experiment. This result supports the view that \( \text{CL}_{\text{deg}} \) in single-pass experiment is limited by the uptake of isovaleryl-PL.

Since PL and IVA are formed by hydrolysis of isovaleryl-PL in the mucosa, their concentrations are higher in mucosa than in the vascular and luminal sides. They are then transported from the mucosa to both vascular and luminal sides by passive diffusion. Lower lipophilicity of IVA compared with PL would suggest that absorption of IVA should not exceed absorption of PL. However, the absorption rate for IVA into the mesenteric vein was larger than PL (Fig. 5, Table 2). These data indicate that the absorption rate of an acidic compound (IVA) is increased by forming the prodrug, even if the prodrug is hydrolyzed in the mucosa. In contrast to IVA, the absorption rate of PL, a weak base with \( \text{pK}_a \) 9.44, is not as readily increased as it is more easily transported into the intestinal lumen than the blood vessels.

The transport of PL by P-glycoprotein (Pgp) mediated efflux was reported previously (Yang et al., 2000, D’Emanuele et al., 2004). However, we found that PL transported by simple diffusion without the participation of Pgp across Caco-2 cell monolayers at apical pH 7.4. Collett et al. (2004) previously reported that PL stimulated ATPase for Pgp but, because of its lipophilicity, showed no asymmetric permeability due to high membrane
permeability. On the other hand, the efflux ratio of PL was 13 in Caco-2 cell monolayer at apical pH 6.0 (Fig. 5). The pH-dependent passive efflux across Caco-2 cell monolayers was also reported by Neuhoff et al. (2003). The concentration of uncharged PL, a basic drug, was lower at pH 6.0 (0.03%) than pH 7.4 (0.90%). The pH-partitioning theory predicts that the permeability of PL in the AP-BL direction should be lower when there is an apical of pH 6.0 than when it is at a pH of 7.4 and the BL-AP permeability of PL should be higher at pH 6.0. Our results in Caco-2 cell monolayers experiments agree with this prediction. The pH-dependent passive permeability of weak bases into the luminal side may be observed in the perfused rat intestine in situ, as reported by Taylor et al. (1985). Therefore, it is necessary to consider the physicochemical properties of the parent drug when designing a prodrug that is converted in the intestinal mucosa.

Consequently, we used isovaleryl-PL as a model ester-compound to evaluate intestinal first-pass hydrolysis. Interestingly, the capacity of intestinal hydrolysis was remarkable and the degradation clearance was limited by the uptake rate of isovaleryl-PL. Isovaleryl-PL was taken up into mucosal tissue was hydrolyzed to PL and IVA and each metabolite was transported by passive diffusion, according to pH-partition theory, into blood vessel, and the intestinal lumen. This indicates that in addition to the liver, the intestine markedly contributes to first-pass hydrolysis and the absorption of parent drugs are controlled by their biological and physicochemical properties.
ID#: DMD/2005/007682

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Footnote

This work was supported in part by a Grant-in-Aid for Scientific Research (16590085) from the Japan Society for the Promotion of Science.
Figure Legends

Fig. 1. Structure of O-isovaleryl-propranolol (isovaleryl-PL).

Fig. 2. Concentration-dependence of hydrolysis of isovaleryl-PL in the jejunal S9 at 37°C. \( K_m \) and \( V_{\text{max}} \) were calculated to be 13.7 ± 1.71 \( \mu \text{M} \) and 29.1 ± 3.81 nmol/min/mg protein by fitting the data to the Michaelis-Menten equation by nonlinear least squares analysis, respectively. The inset is an Eadie-Hofstee plot of the same data. The jejunal S9 was diluted with pH 7.4 HEPHS buffer (50 mM) at 25 \( \mu \text{g/mL} \). Isovaleryl-PL concentrations were 4 \( \mu \text{M} \) to 200 \( \mu \text{M} \). Each point represents mean ± S.D. (\( n = 3 \)).

Fig. 3. Absorption of PL in the rat jejunal single-pass perfusion. a) An appearance rate of PL in the mesenteric vein (\( v_{1,\text{PL}} \)); b) a disappearance rate of PL in the jejunal lumen (\( v_{2,\text{PL}} \)). Rat jejunal loop (10 cm) was perfused with 300 \( \mu \text{M} \) PL. The perfusion flow rate was 2.8 and 0.3 mL/min for the mesenteric vein and jejunal lumen, respectively. Each point represents mean ± S.D. (\( n = 3 \)).

Fig. 4. Absorption of isovaleryl-PL in rat jejunal single-pass perfusion. a) The appearance rate of isovaleryl-PL and PL in the mesenteric vein (\( v_{1,\text{isovaleryl-PL}} \) and \( v_{1,\text{PL}} \)), respectively); b) the disappearance rate of isovaleryl-PL and the appearance rate of PL in the jejunal lumen (\( v_{2,\text{isovaleryl-PL}} \) and \( v_{2,\text{PL}} \)),
Isovaleryl-PL and PL', respectively. Closed circle and triangle represent the appearance rate of PL and isovaleryl-PL, respectively. Open square represents the disappearance rate of isovaleryl-PL. The dotted line represents the input rate of isovaleryl-PL into the entrance of the jejunal loop. Rat jejunal loop (10 cm) was perfused with 300 μM isovaleryl-PL. The perfusion flow rate was 2.8 and 0.3 mL/min for the mesenteric vein and the jejunal lumen, respectively. Each point represents mean ± S.D. (n = 3).

**Fig. 5.** Absorption and secretion of isovaleric acid (IVA) derived from isovaleryl-PL in rat jejunal single-pass perfusion with isovaleryl-PL. Open squares represent the disappearance rate of isovaleryl-PL in the jejunal lumen. Closed circles and squares represent the appearance rate of IVA in the mesenteric vein and jejunal lumen, respectively. Rat jejunal loop (10 cm) was perfused with 300 μM isovaleryl-PL. The perfusion flow rate was 2.8 and 0.3 mL/min for the mesenteric vein and the jejunal lumen, respectively. Each point represents mean ± S.D. (n = 3).

**Fig. 6.** Apparent permeability coefficients (P_{app}) of transport of PL across Caco-2 cell monolayers. Open and closed column represent P_{app} of the transport of PL from AP to BL and from BL to AP, respectively. The transport experiment of PL (50 μM) was carried out under conditions of pH 7.4 and pH 6.0 in the AP side. The pH of BL side was maintained at pH 7.4 throughout. Each point represents mean ± S.D. (n = 3). **P < 0.01.
TABLE 1

Recovery percentage of PL and isovaleryl-PL at steady-state in rat jejunal single-pass perfusion

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>Recovery % of perfused amount</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PL</td>
<td>Isovaleryl-PL</td>
<td></td>
</tr>
<tr>
<td>PL</td>
<td>Mesenteric blood 6.81 ± 0.21</td>
<td>9.21 ± 0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intestinal fluid 92.1 ± 0.71</td>
<td>92.1 ± 0.91</td>
<td></td>
</tr>
<tr>
<td>Total&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.9 ± 0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal tissue&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.76 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isovaleryl-PL</td>
<td>Mesenteric blood 7.47 ± 1.33</td>
<td>0.51 ± 0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intestinal fluid 44.6 ± 3.29</td>
<td>44.6 ± 7.48</td>
<td></td>
</tr>
<tr>
<td>Total&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.4 ± 5.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucosal tissue&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.14 ± 2.01</td>
<td>1.58 ± 0.20</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Total recovery percentage represents the sum of recovery percentage of compounds in the mesenteric blood and intestinal fluid.

<sup>b</sup> Recovery percentage of PL and isovaleryl-PL in the mucosal tissue were determined after washing of compounds from the jejunal lumen.

The mucosal concentration of PL was 376 ± 15.0 nmol/g tissue under PL perfusion, and that of PL and isovaleryl-PL was 436 ± 89.2 and 97.7 ± 11.0 nmol/g tissue respectively under isovaleryl-PL perfusion. 300 µM PL or isovaleryl-PL was perfused in the jejunal lumen at 0.3 mL/min. The flow rate of vasculare perfusate was 2.8 mL/min. The samples of luminal and vascular perfusate were collected at 10 and 5 min intervals for 60 min, respectively. Values represent mean ± S.D. (n = 3).
**TABLE 2**

Kinetic parameters for absorption of PL and isovaleryl-PL in rat jejunal single-pass perfusion

<table>
<thead>
<tr>
<th>Input rate of compounds</th>
<th>Absorption rate ($v_1$)</th>
<th>Disappearance rate ($v_2$)</th>
<th>Secretion rate ($v_3$)</th>
<th>CL$_{app}^a$</th>
<th>P$_{eff}^b$</th>
<th>CL$_{deg}^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/min</td>
<td>nmol/min</td>
<td>nmol/min</td>
<td>µL/min</td>
<td>µL/min</td>
<td>µL/min</td>
</tr>
<tr>
<td>PL</td>
<td>92.0 ± 4.67</td>
<td>6.19 ± 0.31</td>
<td>7.01 ± 0.51</td>
<td>21.6 ± 0.16</td>
<td>2.20 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Isovaleryl-PL</td>
<td>0.49 ± 0.14</td>
<td>50.5 ± 1.95</td>
<td></td>
<td>2.42 ± 0.79</td>
<td>16.6 ± 1.26</td>
<td>141 ± 20.5</td>
</tr>
<tr>
<td>Isovaleryl-PL$^d$</td>
<td>92.9 ± 8.23</td>
<td>7.20 ± 1.05</td>
<td></td>
<td>43.2 ± 3.49</td>
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<tr>
<td>IVA</td>
<td>23.7 ± 6.47</td>
<td></td>
<td></td>
<td>8.03 ± 2.74</td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ CL$_{app}$ was calculated using eq. 4.

$^b$ P$_{eff}$ was calculated using eq. 6. The radius of segment was assumed to be 0.178 cm (Yamashita et al., 1997).

$^c$ CL$_{deg}$ was calculated using eq. 5.

$^d$ Degradation of isovaleryl-PL was 1.5% and 8% of initial concentration for 1 h in pH 6.5 luminal perfusate and pH 7.4 vascular perfusate, respectively.

Values represent mean ± S.D. ($n = 3$).
Fig. 1.
Fig. 2.
Fig. 3.

a) Mesenteric vein

b) Jejunal lumen
Fig. 4.

![Graph showing appearance rate of PL and Isovaleryl-PL in the mesenteric vein and lumen over time.](image-url)
Fig. 5.
Fig. 6.

<table>
<thead>
<tr>
<th>pH of AP side</th>
<th>Percent of uncharged PL</th>
<th>$P_{\text{app}} \times 10^5 \text{ cm/s}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>0.90%</td>
<td>AP-BL: 4.0, BL-AP: 5.0, AP-BL: 10.0, BL-AP: 14.0</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>0.03%</td>
<td><strong>”</strong></td>
</tr>
</tbody>
</table>