

FIRST-PASS HYDROLYSIS OF A PROPRANOLOL ESTER DERIVATIVE IN RAT SMALL INTESTINE

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Received October 3, 2005; accepted November 22, 2005

ABSTRACT:

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 that of 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 that 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, 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 μ M, 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 degradation clearance (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.

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 in the activation of prodrugs and are widely distributed in many tissues, including the intestines (Sato and Hosokawa, 1998; Zhang et al., 2002). Our understanding of the biochemistry and molecular biology of CES enzymes has recently increased dramatically (Sato and

Hosokawa, 1998; Wadkins et al., 2001; Bencharit et al., 2002, 2003). 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, whereas the major intestinal isoform is CES 2 isozymes. Therefore, the hydrolysis characteristics of small intestine are different from those of 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 (Prueksaritanont et al., 1998; Okudaira et al., 2000; 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 [pK_a : PL, 9.44; isovaleryl-PL, 8.59; log P (*n*-octanol/pH 4.0 buffer): PL, 0.38; isovaleryl-PL, 1.94]. In contrast, another hydrolysate, isovaleric acid (IVA), 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; Fagerholm et al., 1996) and maintains the intestinal architecture with respect to metabolism, absorption, and secretion (Pang et al., 1986; Zimmerman et al., 2000;

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

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.105.007682.

ABBREVIATIONS: CES, carboxylesterase; PL, propranolol; IVA, isovaleric acid; BSA, bovine serum albumin; FD-4, fluorescein isothiocyanate dextran 4000; HBSS, Hanks' balanced salt solution; HPLC, high performance liquid chromatography; AP, apical; BL, basolateral; AUC, area under the concentration curve; CL, clearance; Pgp, P-glycoprotein.

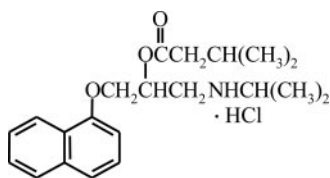


FIG. 1. Structure of *O*-isovaleryl-PL.

Tamura et al., 2003). 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. 2-(*N*-Morpholino)ethanesulfonic acid and HEPES were purchased from Wako Pure Chemical Industries, Ltd. Nobo heparin 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, 0.25% trypsin-EDTA, Dulbecco's phosphate-buffered saline, Hanks' balanced salt solution (HBSS), and Earle's balanced salt solution were purchased from Sigma (St. Louis, MO). Nonessential amino acid, penicillin-streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Cansera International Inc. (Rexdale, ON, Canada). All other chemicals were of analytical grade.

Preparation of Rat Jejunal 9000g 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 9000g for 20 min at 4°C to obtain the supernatant (S9) fraction. Protein contents were determined by the method of Bradford (1976) with BSA as the standard. These preparations were stored at –80°C until use.

Hydrolysis Experiments of Isovaleryl-PL in the Jejunal Mucosa S9 Fraction. The jejunal S9 fraction 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 dimethyl sulfoxide (final concentration, 4–200 µM). The final concentration of dimethyl sulfoxide was maintained at 0.5%, which had no effect on hydrolase activity. After 10 min of incubation, reactions were terminated by adding 5 ml of ethyl acetate and 1 ml of 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_m and V_{max} , were calculated by fitting the data to the Michaelis-Menten equation by nonlinear least-squares analysis, using the 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 h and then anesthetized by intraperitoneal injections of 2% sodium pentobarbital (0.5 ml). Vascular perfused intestinal loops were prepared as described previously (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 Krebs-Henseleit bicarbonate buffer (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. Krebs-Henseleit bicarbonate buffer 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 2-(*N*-morpholino)ethanesulfonic acid 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, respectively, for 60 min and immediately added to extraction solvent. The degradation of isovaleryl-PL was 1.5% and 8% after 1 h 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 3000 rpm for 10 min at 4°C, the supernatant was injected onto a HPLC column 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 of acetonitrile before injecting 30 µl onto the HPLC column. The luminal samples (100 µl) were deproteinized with 1 ml of acetonitrile and centrifuged at 3000 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 of 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 of NaCl and 10 M phosphoric acid (2 µl). The organic phases were concentrated under a stream of N_2 gas, and 5-µl aliquots were injected onto the gas chromatography 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 2.5×10^5 cells/ml onto polycarbonate filters (3-µm pores, 4.71-cm² growth area) and grown for 21 to 27 days in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) nonessential amino acid, 10% (v/v) fetal bovine serum, benzylpenicillin G (50 units/ml), streptomycin (50 µg/ml), and 2 mM L-glutamine at 37°C in a humidified air-5% CO₂ 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 Earle's balanced salt solution (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 volume of the same fresh medium. All samples were immediately assayed by HPLC. No damage to the Caco-2 cell membrane during the transport experiment was observed after staining with 0.1% trypan blue.

Assay. PL and isovaleryl-PL concentrations were determined by HPLC, consisting of a pump (Jasco PU-980; Jasco International Co. Ltd., Tokyo,

Japan), a data application apparatus (Shimadzu C-R4A; Shimadzu Corp., Kyoto, Japan), and a fluorescence detector (Jasco 820-FP; Jasco International Co. Ltd.). LiChrosorb RP-Select B columns (7 μm , 250 \times 4 mm i.d.; Shimadzu GLC Ltd., Tokyo, Japan) were used with a mobile phase of acetonitrile/20 mM KH_2PO_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 a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and a Shimadzu C-R6A data application apparatus (Shimadzu Corp.). A glass column packed with Gaskuropack 56 80/100 (3.2 mm i.d. \times 2.1 m; GL Sciences, Inc., Tokyo, Japan) was used under a carrier gas of N_2 at a flow rate 4.0 kgf/cm². Temperature set points 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 ($v_{1, \text{isovaleryl-PL}}$) and PL ($v_{1, \text{PL}}$) in the mesenteric vein was calculated according to eq. 1:

$$v_1 = Q_b \cdot C_b \quad (1)$$

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:

$$v_2 = Q_1 \cdot (C_{\text{in}} - C_{\text{out}}) \quad (2)$$

$$v_3 = Q_1 \cdot C_{\text{M,out}} \quad (3)$$

where Q_1 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 $C_{\text{M,out}}$ are the concentration of isovaleryl-PL and PL at the exit of the jejunal segment, respectively, and C_{out} and $C_{\text{M,out}}$ were corrected with the concentration of FD-4.

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

$$\text{CL}_{\text{app}} = \frac{\text{AUC}_b}{\text{AUC}_1} \cdot Q_b = \frac{\text{Absorbed amount}}{\text{AUC}_1} \quad (4)$$

AUC_b and AUC_1 are the areas under the curve of the administered compound in the mesenteric vein and in the intestinal lumen, respectively, at the steady state. The degradation clearance of isovaleryl-PL in the jejunal mucosa (CL_{deg}) was calculated by eq. 5,

$$\text{CL}_{\text{deg}} = \frac{\text{AUC}_{\text{M},1}}{\text{AUC}_{\text{P},1}} \cdot Q_1 + \frac{\text{AUC}_{\text{M},b}}{\text{AUC}_{\text{P},1}} \cdot Q_b = \frac{\text{Degraded amount}}{\text{AUC}_{\text{P},1}} \quad (5)$$

where $\text{AUC}_{\text{P},1}$ and $\text{AUC}_{\text{M},1}$ are the areas under the curve of isovaleryl-PL and PL in the intestinal lumen, respectively, at the steady state. $\text{AUC}_{\text{M},b}$ 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_{eff}) was calculated as:

$$P_{\text{eff}}(\text{cm/min}) = Q_1 \cdot \frac{1 - C_{\text{out}}/C_{\text{in}}}{2\pi RL} \quad (6)$$

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_{app}) across Caco-2 cell monolayers was calculated according to the following equation:

$$P_{\text{app}}(\text{cm/s}) = \frac{dQ/dt}{A \cdot C_0} \quad (7)$$

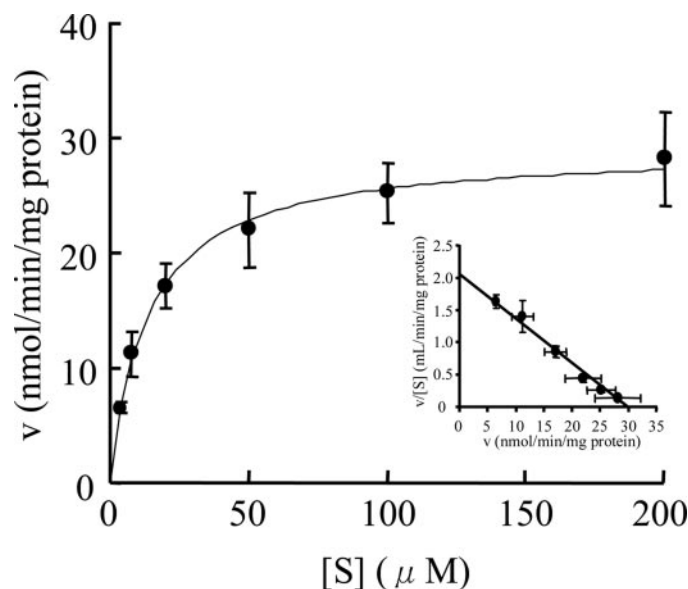


FIG. 2. Concentration dependence of hydrolysis of isovaleryl-PL in the jejunal S9 at 37°C. K_m and V_{max} were calculated to be $13.7 \pm 1.71 \mu\text{M}$ and $29.1 \pm 3.81 \text{ nmol/min/mg protein}$, respectively, by fitting the data to the Michaelis-Menten equation by nonlinear least-squares analysis.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

where dQ/dt is the rate of appearance of drugs in the basolateral compartment (steady-state flux, $\mu\text{mol/s}$), 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 (micromolar concentration).

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. Figure 2 shows the hydrolysis of isovaleryl-PL (final concentration, 4–200 μM) in the jejunal S9 fraction. The hydrolysis of isovaleryl-PL in the jejunum was a one-component reaction, and nonspecific hydrolysis was not observed. By fitting the data to the Michaelis-Menten equation, K_m and V_{max} values of $13.7 \pm 1.71 \mu\text{M}$ and $29.1 \pm 3.81 \text{ nmol/min/mg protein}$, respectively, were observed.

Absorption of PL in Rat Jejunal Single-Pass Perfusion. The appearance rate of PL in the mesenteric vein ($v_{1, \text{PL}}$, Fig. 3a) and the disappearance rate of PL in the jejunal lumen ($v_{2, \text{PL}}$, Fig. 3b) during perfusion with 300 μM PL are shown in Fig. 3. A steady state was achieved after perfusion for 20 min. The appearance and disappearance rates were $6.19 \pm 0.31 \text{ nmol/min}$ and $7.01 \pm 0.51 \text{ nmol/min}$, 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). Figure 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. Figure 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 those of PL, which represented 94% of total venous concentration of PL and isovaleryl-PL in the venous outflow. The appearance rate of PL at

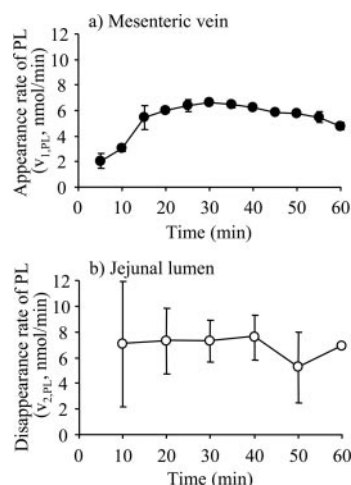


FIG. 3. Absorption of PL in the rat jejunal single-pass perfusion. a, an appearance rate of PL in the mesenteric vein ($v_{1, PL}$); b, a disappearance rate of PL in the jejunal lumen ($v_{2, PL}$). Rat jejunal loop (10 cm) was perfused with 300 μ M PL. The perfusion flow rate was 2.8 and 0.3 ml/min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

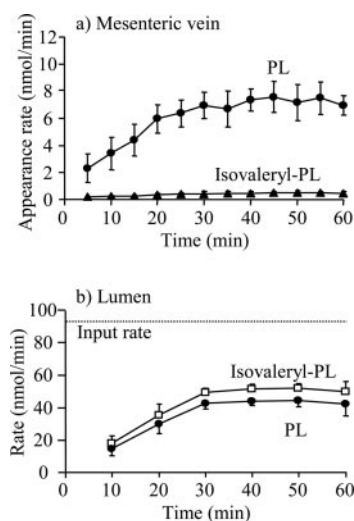


FIG. 4. Absorption of isovaleryl-PL in rat jejunal single-pass perfusion. a, the appearance rate of isovaleryl-PL and PL in the mesenteric vein ($v_{1, isovaleryl-PL}$ and $v_{1, PL}$, respectively); b, the disappearance rate of isovaleryl-PL and the appearance rate of PL in the jejunal lumen ($v_{2, isovaleryl-PL}$ and $v_{3, PL}$, respectively). The closed circle and triangle represent the appearance rate of PL and isovaleryl-PL, respectively. The 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 μ M 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 \pm S.D. ($n = 3$).

steady state was 7.20 ± 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 ± 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 ($v_{3, PL}$) was 43.2 ± 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 ± 1.95 nmol/min). Based on the hydrolysis experiment of isovaleryl-PL (300 μ M) in the perfusate maintained in the jejunal loop, it was estimated that 2.5% of isovaleryl-PL was hydrolyzed

TABLE 1

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

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

Perfusate	Recovery Percentage of Perfused Amount	
	PL	Isovaleryl-PL
PL		
Mesenteric blood	6.81 ± 0.21	
Intestinal fluid	92.1 ± 0.71	
Total ^a	98.9 ± 0.91	
Mucosal tissue ^b	4.76 ± 0.01	
Isovaleryl-PL		
Mesenteric blood	7.47 ± 1.33	0.51 ± 0.17
Intestinal fluid	44.6 ± 3.29	44.6 ± 7.48
Total ^a	97.4 ± 5.03	
Mucosal tissue ^b	7.14 ± 2.01	1.58 ± 0.20

^a Total recovery percentage represents the sum of recovery percentage of compounds in the mesenteric blood and intestinal fluid.

^b Recovery percentage of PL and isovaleryl-PL in the mucosal tissue was determined after washing of compounds from the jejunal lumen.

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, whereas 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. Because the amount of 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 with those of 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.

Figure 5 shows the appearance of IVA in the mesenteric vein ($v_{1, IVA}$) and jejunal lumen ($v_{3, IVA}$) during perfusion with 300 μ M 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

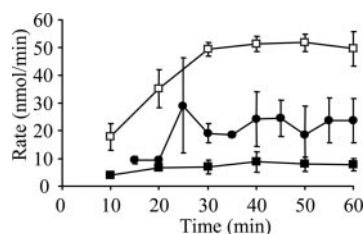


FIG. 5. Absorption and secretion of IVA derived from isovaleryl-PL in rat jejunal single-pass perfusion with isovaleryl-PL. Open squares represent the disappearance rate of isovaleryl-PL in the jejunal lumen. Closed circles and squares represent the appearance rate of IVA in the mesenteric vein and jejunal lumen, respectively. Rat jejunal loop (10 cm) was perfused with 300 μ M 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 \pm S.D. ($n = 3$).

retention of IVA in the intestinal mucosa and the quantitative limitation of IVA by gas chromatography.

Kinetic Analysis for Absorption of Isovaleryl-PL in Rat Jejunal Single-Pass Perfusion. Table 2 lists the apparent absorption clearance (CL_{app}) of PL and isovaleryl-PL, the degradation clearance (CL_{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_{eff}) for isovaleryl-PL was $16.6 \times 10^{-3} \pm 1.26 \times 10^{-3}$ cm/min. This value was 7-fold greater than that of PL due to its greater lipophilicity. However, CL_{app} of isovaleryl-PL was 2.42 ± 0.79 μ l/min, which was 9-fold lower than the CL_{app} of PL with PL perfusion. The disappearance rate of isovaleryl-PL (50.5 ± 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 that 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 μ l/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. 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 ($pK_a = 9.44$) 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)

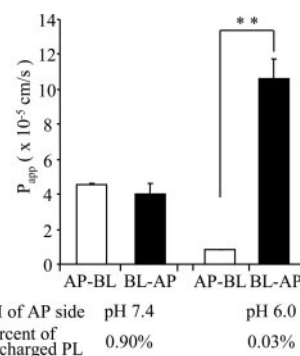


FIG. 6. Apparent permeability coefficients (P_{app}) of transport of PL across Caco-2 cell monolayers. Open and closed columns represent P_{app} of the transport of PL from AP to BL and from BL to AP, respectively. The transport experiment of PL (50 μ M) was carried out under conditions of pH 7.4 and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

experiments have shown the pH of this microclimate region to vary between 5.8 and 6.3, a range that is lower than the bulk solution in the intestinal tract. Therefore, pH 6.0 was selected as the apical medium pH in transport experiments across the Caco-2 cell monolayer.

Figure 6 shows the permeability constant (P_{app}) for apical to basolateral (AP-BL) and basolateral to apical (BL-AP) transport of PL through the Caco-2 cell monolayer at apical pH values of 6.0 and 7.4. When the apical pH was 7.4, P_{app} values for AP-BL and BL-AP transport of PL were 4.54×10^{-5} cm/s and 4.01×10^{-5} cm/s, respectively. This indicates that PL was transported by simple diffusion at an initial concentration of 50 μ M PL. In contrast, the AP-BL transport rate was lower (0.82×10^{-5} cm/s) and the BL-AP transport rate was higher (10.6×10^{-5} 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 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. Although 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

TABLE 2

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

Values represent mean \pm S.D. ($n = 3$).

Input Rate of Compounds		Absorption Rate (v_1)	Disappearance Rate (v_2)	Secretion Rate (v_3)	CL_{app}^a	P_{eff}^b	CL_{deg}^c
		nmol/min			μ l/min	$\times 10^{-3}$ cm/min	μ l/min
PL							
92.0 \pm 4.67 nmol/min	PL	6.19 \pm 0.31	7.01 \pm 0.51		21.6 \pm 0.16	2.20 \pm 0.16	
Isovaleryl-PL	Isovaleryl-PL	0.49 \pm 0.14	50.5 \pm 1.95		2.42 \pm 0.79	16.6 \pm 1.26	141 \pm 20.5
92.9 \pm 8.23 nmol/min ^d	PL	7.20 \pm 1.05		43.2 \pm 3.49			
	IVA	23.7 \pm 6.47		8.03 \pm 2.74			

^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 the initial concentration for 1 h in pH 6.5 luminal perfusate and pH 7.4 vascular perfusate, respectively.

to study transcellular transport in Caco-2 cell monolayers (Salama et al., 2004) and in vivo absorption experiments (Yamashita et al., 1997). As summarized in Table 2, although PL has sufficient lipophilicity to possess a high absorption rate, the P_{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_{eff} of PL ($2.20 \times 10^{-3} \pm 0.16 \times 10^{-3}$ cm/min) was lower than that value (ca. 8.5×10^{-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_{eff} was 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, 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 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 brush-border membrane, it is unlikely that isovaleryl-PL was hydrolyzed here. Moreover, PL levels in blood vessel and mucosal tissue cannot 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 $\mu\text{L}/\text{min}$) 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 seem 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 CL_{deg} (0.14 ml/min, Table 2) in the single-pass experiment. This result supports the view that CL_{deg} in the single-pass experiment is limited by the uptake of isovaleryl-PL.

Because 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 that of 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 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 was 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. In contrast, 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 at 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 pH of 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 monolayer 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 is controlled by their biological and physicochemical properties.

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