Altered Diurnal Rhythm of Intestinal Peptide Transporter by Fasting and Its Effects on the Pharmacokinetics of Ceftibuten

XIAOYUE PAN, TOMOHIRO TERADA, MASAKIHO OKUDA, and KEN-ICHI INUI

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan
Abbreviations: AUC, area under the plasma concentration-time curve from 0 h to 3 h; CL\text{tot}, total body clearance; C_{max}, peak plasma concentration; K_e, elimination rate constant; T_{max}, time to peak plasma concentration; V_d, volume of distribution; T_{1/2}, half-life; PEPT, peptide transporter; Gly-Sar, glycylsarcosine; CETB, ceftibuten; HPLC, High performance liquid chromatograph.

Recommended Section: Absorption, Distribution, Metabolism, & Excretion
Abstract

We previously demonstrated that H+/peptide cotransporter PEPT1 shows a diurnal rhythm in the rat small intestine. In the present study, we examined the effect of food intake on the diurnal rhythm of intestinal PEPT1 using fed and fasted rats, and also determined whether such variation affected the pharmacokinetics of peptide-like drugs. In fed rats, PEPT1 protein level was significantly higher at 20:00 h than at 8:00 h. However, during fasting for 2-4 days, the differences of PEPT1 protein levels between 8:00 h and 20:00 h gradually disappeared. Intestinal absorption of an oral antibiotic ceftibuten (CETB), a pharmacological substrate for PEPT1, was also greater at 20:00 h than at 8:00 h in fed rats, but not different in 4 days-fasted rats. In contrast to PEPT1 protein levels, PEPT1 mRNA levels retained a diurnal rhythm after 4 days fasting. Pharmacokinetic analyses of CETB after intraintestinal administration demonstrated that both $C_{\text{max}}$ and $AUC_{0-3\ h}$ were greater at 20:00 h than at 8:00 h in fed rats. In contrast, pharmacokinetic parameters showed no significant difference between 8:00 h and 20:00 h for intraintestinal administration in 4 days-fasted rats, and for intravenous administration in fed and 4-days-fasted rats. These findings suggested that the diurnal rhythm of intestinal PEPT1 transport activity was disrupted by fasting, and that diurnal variation of intestinal PEPT1 functionality could influence the pharmacokinetics of peptide-like drugs such as CETB.
Di- and tripeptides and various peptide-like drugs such as β-lactam antibiotics are taken up into the intestinal and renal epithelial cells by H⁺-coupled peptide cotransporters. Cloning studies have identified two peptide transporters, PEPT1 and PEPT2, and many functional studies using heterologous expression systems have demonstrated molecular natures in their transport characteristics (Daniel and Herget, 1997; Inui and Terada, 1999; Leibach and Ganapathy, 1996). Furthermore, molecular identification of PEPT1 and PEPT2 provided a novel opportunity to determine the mechanisms of their regulation. For example, it was reported that the intestinal PEPT1 is regulated by various factors including dietary conditions (Naruhashi et al., 2002; Ogihara et al., 1999; Shiraga et al., 1999), hormones such as insulin, leptin and thyroid hormone (Ashida et al., 2002; Buyse et al., 2001; Gangopadhyay et al., 2002; Thamotharan et al., 1999), epidermal growth factor (Nielsen et al., 2001), development (Shen et al., 2001) and some pharmacological agents (Berlioz et al., 2000; Fujita et al., 1999).

In addition to the above described regulations, we recently found that PEPT1 in the rat small intestine is under the regulation of diurnal rhythm (Pan et al., 2002). Briefly, the transport of [¹⁴C]glycylsarcosine (Gly-Sar), a typical substrate for PEPT1, by in situ intestinal loop and everted intestine was greater in the dark phase rather than the light phase, and PEPT1 protein and mRNA levels varied significantly, with a maximum at 20:00 h and minimum at 8:00 h (Pan et al., 2002).
In contrast to the intestine, renal PEPT1 and PEPT2 showed little diurnal rhythmicity. Since rodents show nocturnal feeding behavior, the diurnal rhythm of intestinal PEPT1 is reasonable for the preparation of anticipated dietary load.

In our previous study, we used rats kept with free access to water and laboratory chow to clarify the diurnal rhythmicity under standard environmental conditions. However, as feeding conditions greatly affected the expression and function of intestinal PEPT1 (Naruhashi et al., 2002; Ogihara et al., 1999; Shiraga et al., 1999), there is a possibility that feeding also influence the diurnal rhythm of this transporter. Based on these hypotheses, in the present study, we examined the effect of food intake on the diurnal rhythm of intestinal PEPT1 using fed and fasted rats. In addition, using an oral β-lactam antibiotic ceftibuten (CETB), a good pharmacological substrate of PEPT1, we examined whether the regulation of intestinal PEPT1 in the diurnal rhythm and fasting could affect the intestinal absorption and pharmacokinetics of this drug. The expressional changes of renal PEPT1 and PEPT2 were also examined to clarify the tissue specificity of the diurnal rhythm and fasting effect.
Materials and Methods

**Materials.** Ceftibuten (CETB) was supplied by Shionogi Co. (Osaka, Japan). All other chemicals used were of the highest purity available.

**Animals.** Male Wistar rats (160–180 g) were housed in an air-conditioned room at 22 ± 0.5°C with a 12-hour lighting schedule (8:00 h-20:00 h). Animals were fed *ad libitum* on the light/dark schedule for 1 week before they were divided into fed and fasted groups. Two different groups of rats were used in this study: (1) control group fed normal chow *ad libitum* (F0); (2) fasted for 1-4 days (F1, F2, F3 and F4). Water was available *ad libitum* to all groups throughout the experiments. Five or six rats were used in each group. Daily changes in body and mucosal weights were examined over the 4 days of fasting (27% and 35% decreased in body and mucosal weights after 4 days of fasting, respectively, compared with those in fed rats). The animal experiments were performed in accordance with the *Guidelines for Animal Experiments of Kyoto University.*

**Western blot analysis.** Under anesthesia, the duodenum and kidney were removed at 8:00 h and 20:00 h of one day. The duodenum was flushed with ice cold phosphate-buffered saline, and the mucosa was scraped. The kidney was decapsulated, and slices of the renal cortex were prepared with a Stadie-Riggs microtome. A portion of the mucosa and renal slices were rapidly frozen in liquid nitrogen for later preparation of brush-border membranes and total RNA.
border membranes from rat small intestine and kidney cortex were prepared as described previously (Inui et al., 1984; Okano et al., 1986). The membrane fractions (small intestine, 10 µg/lane; kidney cortex, 50 µg/lane) were separated by 8.5% SDS-PAGE, and analyzed by Western blot analyses as reported (Saito et al., 1995 and 1996). The relative densities of the bands in each reaction were determined using NIH Image 1.61 (National Institutes of Health, Bethesda, MD).

**Northern blot analysis.** Total RNA was isolated from small intestinal mucosa using TRIzol reagents (1 ml/100 mg tissue) (Invitrogen Japan KK, Tokyo, Japan) per the manufacturer’s protocols. Total RNA (20 µg/lane) was electrophoresed in 1% denaturing agarose gel containing formaldehyde and Northern blot analyses were performed as reported previously (Pan et al., 2002; Saito et al., 1995 and 1996).

**In situ loop technique.** CETB absorption was examined by the in situ loop technique at 8:00 h and at 20:00 h in fed or 4 days-fasted rats as described previously (Pan et al., 2002). The CETB (3 mg/3 ml/kg body wt) was introduced into a duodenum 10 cm-loop with a microsyringe, and blood was withdrawn from the portal vein at 0, 3, 6, 9, 12, 15, 18 and 30 min after CETB injection. The blood samples were centrifuged for 2 min at 14,000 ×g, and 100 µl of plasma samples were analysed by high-performance liquid chromatography (HPLC).
In vivo experiments. Rats were anesthetized with sodium pentobarbital (40 mg/kg). For intraintestinal infusion of CETB, a catheter with a 26G needle was carefully fixed with cyanoacrylate glue into the middle part of the duodenum (Yamaguchi et al., 2002). For intravenous infusion of CETB, the jugular vein was cannulated with a polyethylene tube (PE-10, Becton Dickinson, Parsippany, NJ). A single 3 mg/kg body weight dose of CETB dissolved in an isotonic phosphate-buffer (pH 6.0) was administered by the intraintestinal or the intravenous to fed and 4 days-fasted rats at 8:00 h or 20:00 h. Blood samples were collected from the contralateral jugular vein at 0, 8, 15, 30, 45, 60 min, and 1.5, 2.0, 2.5, 3.0 h after CETB injection. The blood samples were centrifuged for 2 min at 14,000 ×g, and 100 µl of plasma samples were analysed by HPLC.

Pharmacokinetic analysis. A conventional one-compartment model was employed to analyze the plasma concentration-time profiles of CETB after intravenous administration in rats. Estimated pharmacokinetic parameters were total area under the plasma concentration-time curve from 0 h to 3 h (linear trapezoidal method), maximum plasma concentration (C max), time after administration needed to obtain C max (T max), elimination rate constant (K e), half-life (T 1/2), volume of distribution (V d), total body clearance (CL tot) for CETB.
**HPLC analysis.** Plasma concentration of CETB was measured with a HPLC LC-6A (Shimadzu, Kyoto, Japan) equipped with an UV spectrophotometric detector SPD-10A (Shimadzu). The condition was as follows: column, Zorbax ODS 4.6-mm inside diameter ×150 mm (Agilent, Palo Alto, CA); the mobile phase, 50 mM ammonium acetate /methanol = 80:20; flow rate, 1.0 ml/min; wavelength, 262 nm; the injection volume, 50 µl, and column temperature, 45°C. Peak area size was measured with a Chromatopac C-R6A (Shimadzu).

**Data analysis.** Values are expressed as means ± SE. Analysis of variance (ANOVA) and Fisher’s test were used for the statistical significance of CETB concentration and CETB pharmacokinetic parameters. The statistical significance of differences between mean values of other data was analyzed using the non-paired \( t \) test or one-way analysis of variance followed by Fisher’s test when multiple comparisons were needed. Differences were considered significant at \( p < 0.05 \).
Results

In our previous study, we found that PEPT1 protein and mRNA expressions were minimum at 8:00 h (beginning of the light phase) and maximum at 20:00 h (beginning of the dark phase), respectively (Pan et al., 2002), and thus, we typically examined PEPT1 expression levels at these times. Furthermore, in preliminary experiments, the body weight and small intestinal mucosal mass of rats fasted for 1-4 days were significantly lower than those of fed rats. There was no significant difference in the loss of body weight or intestinal mucosal mass between at 8:00 h and at 20:00 h of each day. This result served to confirm the change of the intestinal mucosa during the fasting process.

Effect of fasting on diurnal variation of intestinal PEPT1 protein and mRNA levels in fasted rats. It has been demonstrated that starvation markedly increased the amount of intestinal PEPT1 mRNA (Naruhashi et al., 2002) and protein (Ogihara et al., 1999). In addition, in the present experiments, PEPT1 mRNA and protein levels in 4 days-fasted rats were significantly increased compared with those in fed rats, and this effect was observed at both 8:00 h and 20:00 h (Fig. 1).

Next, we assessed diurnal regulation of intestinal PEPT1 protein using 1-4 days-fasted rats. As shown in Fig. 2, in fed (F0) and the 1 day-fasted rats (F1), PEPT1 protein level was significantly higher at 20:00 h than at 8:00 h. However, after fasting for 2-4 days (F2-F4), the differences of PEPT1 protein levels between 8:00 h and 20:00 h...
gradually disappeared. To exclude the possibility of the phase shift for intestinal PEPT1 biorhythm, we examined PEPT1 protein levels at 4-hour intervals in 4 days-fasted rats. As shown in Fig. 3, the intestinal PEPT1 protein of 4 days-fasted rats showed no significant diurnal rhythm throughout one day.

To assess whether abolished diurnal variation of intestinal PEPT1 protein in 4 days-fasted rats were transcriptionally regulated or not, Northern blot analysis was performed. As shown in Fig. 4, PEPT1 mRNA levels at 20:00 h were significantly higher in 4 days-fasted rats as well as in fed rats. These findings suggested that diurnal regulation of PEPT1 mRNA expression was maintained in the fasting state.

**Effect of fasting on diurnal variation of renal PEPT1 and PEPT2 protein levels in fasted rats.** We next examined the effect of fasting on renal PEPT1 and PEPT2 protein levels at 8:00 h and 20:00 h in fed and 4 days-fasted rats. As shown in Fig. 5A, PEPT1 protein levels remained mostly constant between fed and 4 days-fasted rats both at 8:00 h and 20:00 h. PEPT2 protein levels showed a similar pattern as PEPT1, but a modest decrease was observed at 8:00 h (Fig. 5B).

Renal PEPT1 and PEPT2 did not show diurnal variation in the fed rats (Pan et al., 2002). This result was confirmed in the present study, as shown in F0 of Figs. 6A and 6B. However, as the fasting period was prolonged, renal PEPT1 and PEPT2 protein levels showed a slight diurnal variation, with higher expression...
levels at 20:00 h than 8:00 h in 4 days-fasted rats (Figs. 6A and 6B), suggesting distinct regulation of renal peptide transporters from that of the intestine.

*CETB pharmacokinetics at 8:00 h and 20:00 h in fed and 4 days-fasted rats.*

It has been demonstrated that several drugs vary in potency and/or toxicity based on the rhythmicity of biochemical, physiological and behavioral processes (Labrecque and Belanger, 1991; Lemmer, 1999; Lemmer and Labrecque, 1987). There is a possibility that the diurnal regulation of intestinal PEPT1 may affect the intestinal absorption of peptide-like drugs, which in turn may influence the pharmacokinetic parameters of such drugs. Thus, we assessed this hypothesis using a representative pharmacological PEPT1 substrate CETB. Fasting effects on the intestinal absorption and the pharmacokinetic parameters of CETB were also investigated. As shown in Fig. 7, in the fed state, the initial absorption rate of CETB was significantly higher at 20:00 h than at 8:00 h. On the other hand, in the 4 days-fasted state, the initial absorption rate of CETB was not significantly different between 8:00 h and 20:00 h. Comparing the initial absorption rate of CETB between fed and fasted rats, the latter showed higher absorption rate.

We next measured plasma concentrations of CETB after intraintestinal and intravenous administration at 8:00 h and 20:00 h in fed and 4 days-fasted rats. Pharmacokinetic parameters of CETB after intraintestinal and intravenous administration are summarized in Tables 1 and 2, respectively. In the fed condition, rats
administered at 20:00 h showed greater $C_{\text{max}}$ and $\text{AUC}_{0-3\ h}$, and faster $T_{\text{max}}$ rather than those administered at 8:00 h (Table 1 and Fig. 8A). However, there were no significant differences of pharmacokinetic parameters of CETB between 8:00 h and 20:00 h in 4 days-fasted rats (Table 1 and Fig. 8B). Comparison of pharmacokinetic parameters between fed and 4 days-fasted rats, all parameters exhibited higher absorption of CETB in 4 days-fasted rats compared with fed rats irrespective of administration time (Table 1). In the intravenous administration, there were no differences in $K_e$, $CL_{\text{tot}}$ and $T_{1/2}$ between 8:00 h and 20:00 h in the fed and 4 days-fasted rats (Table 2, Figs. 8C and 8D), although there were significant differences between fed and 4 days-fasted rats at both administration times (Table 2).
Discussion

A daily periodicity in the intestinal transport activity and several other digestive proteins were documented before the responsible genes were identified. Nevertheless, the observed activity changes were clearly ascribed to the feeding pattern (*ad libitum* and scheduled) rather than to an inherent circadian signal (Fisher and Gardner, 1976; Stevenson and Fierstein, 1976). In our previous study, we demonstrated that the function and expression of intestinal PEPT1 showed a diurnal rhythm in rat feeding *ad libitum* (Pan et al., 2002). As the intestinal PEPT1 diurnal rhythm was also synchronized with food intake, which is one of the major factors regulating of the intestinal PEPT1, the present study was performed to examine the effect of food intake on diurnal variation of intestinal PEPT1 using fed and fasted rats.

The intestinal PEPT1 protein showed significant diurnal rhythm in feeding and 1 day-fasted conditions. However, this diurnal rhythm disappeared after 2 days fasting. In accordance with the diurnal rhythm of PEPT1 protein expression in feeding and fasting conditions, the CETB absorption rate assessed by the *in situ* loop technique was higher at 20:00 h rather than at 8:00 h in the feeding state, but not in the 4 days-fasted condition. Interestingly, the diurnal regulation of the PEPT1 mRNA level was maintained even in the 4 days-fasted state. This finding suggested that the transcription of intestinal PEPT1 gene in the diurnal rhythm was
mediated by factors other than food intake. Currently, there is no information regarding transcription factors involved in the constitutive and regulatory expression of intestinal PEPT1 mRNA. Further studies about promoter analysis of rat PEPT1 may clarify the molecular mechanisms for the diurnal rhythm of intestinal PEPT1 mRNA expression. For Na\(^+\)/glucose cotransporter 1 (SGLT1), it was demonstrated that periodicity in transcription factor hepatocyte nuclear factor-1 (HNF-1) contributes to circadian changes in glucose transport activity in the intestine (Rhoads et al., 1998). Since there is a potential site for HNF-1 in the rat PEPT1 promoter region (Shiraga et al., 1999), this factor can be a candidate for diurnal transcriptional regulation of the intestinal PEPT1 expression.

The diurnal variation of intestinal PEPT1 protein expression was abolished in 4 days-fasted rats, although the diurnal variation in the mRNA expression was retained. There are some possible explanations for this finding. First, the rates of the translation process and degradation of PEPT1 protein at 8:00 h and 20:00 h may be different between the fed and fasted conditions. Second, the alteration in the cellular distribution of PEPT1 protein (cytoplasmic pool and apical membranes) appears to be involved in the disappearance of diurnal variation. For examples, the increased translocation of the cytoplasmic pool of PEPT1 to the apical membrane was demonstrated by hormone treatment such as insulin (Gangopadhyay et al., 2002; Thamotharan et al., 1999) and leptin (Buyse et al., 2001). Insulin and leptin
showed a diurnal rhythm with a nocturnal peak, but this rhythm of both hormones was completely abolished by the fasting state (Ahrén, 2000), which appeared to correspond to the alteration of PEPT1 membrane expression between the fed and fasted conditions.

Several clinical studies, performed in a cross-over design, have provided evidence that the pharmacokinetics of many lipophilic drugs can be circadian phase-dependent in $C_{\text{max}}$ and $T_{\text{max}}$ (Lemmer, 1999). Since most drugs tested are absorbed by passive diffusion, the circadian variation in gastric emptying time (Goo et al., 1987) and the perfusion of the gastrointestinal tract (Lemmer and Nold, 1991) has been considered for this reason. In the present study, we found that pharmacokinetic parameters of CETB such as $C_{\text{max}}$ and $T_{\text{max}}$ showed a significant diurnal variation after intraintestinal administration in fed rats. The initial absorption rate of CETB assessed by the *in situ* loop technique was significantly higher at 20:00 h than at 8:00 h, and pharmacokinetic parameters of intravenous administration of CETB did not show diurnal variation. These findings suggested that pharmacokinetic variation of CETB between 8:00 h and 20:00 h was caused by the intestinal absorption process, and that the diurnal rhythm of the intestinal PEPT1 expression plays a pivotal role in this variation. The diminished diurnal variation of the pharmacokinetic parameters for CETB administered intraintestinally in 4 days-fasted rats corresponding to the disappearance of the
diurnal rhythm of intestinal PEPT1 also supported this idea. As far as we know, this is the first demonstration that the intestinal transporter is involved in the diurnal variation of pharmacokinetics of drugs.

In the intravenous administration, there were no significant differences in pharmacokinetic parameters of CETB between 8:00 h and 20:00 h both in fed and 4 days-fasted rats, although the expressions of renal PEPT1 and PEPT2 were increased in 4 days-fasted rats. Although we did not assess the renal handling of CETB in detail, it is plausible that renal PEPT1 and PEPT2 may little affect the CETB pharmacokinetics.

CETB pharmacokinetic parameters such as $C_{\text{max}}$ and $AUC_{0-3\,h}$ obtained by the intestinal administration were significantly larger in 4 days-fasted rats compared with the fed rats. In addition, the CETB absorption rates assessed by the in situ loop technique in 4 days-fasted rats were significantly increased compared with the fed rats both at 8:00 h and at 20:00 h. All these findings appeared to be accountable by increased intestinal PEPT1 protein expression. Recently, Naruhashi et al. (2002) reported that PEPT1 mRNA expression was induced by starvation and its level correlated with absorptive transport of cefadroxil, an oral $\beta$-lactam antibiotic, in the rat intestine. Furthermore, it was reported that the expression level of PEPT1 assessed by Western blot analysis in the rat jejunum was significantly correlated with intestinal loop single perfusion of an another oral $\beta$-
lactam antibiotic cephalexin (Berlioz et al., 1999). All these findings suggested that the expression level of intestinal PEPT1 is one of the key factors determining the absorption of oral β-lactam antibiotics.

In conclusion, we have demonstrated that the diurnal rhythm of intestinal PEPT1 transport activity is altered by fasting. The diurnal rhythm of intestinal PEPT1 would be responsible for the diurnal variation for pharmacokinetics of peptide-like drugs such as CETB, which was also abolished by fasting.
References


Footnotes

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Address reprint requests to: Professor Ken-ichi Inui, Ph.D., Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan
FIGURE LEGENDS

**Fig. 1.** Effect of fasting on PEPT1 protein (A and B) and mRNA (C and D) expression in the rat duodenum. A and B. Duodenal brush-border membranes were prepared from fed (❑) and 4 days-fasted rats (■) killed at 8:00 h and at 20:00 h, and Western blot analyses were carried out. Representative data are shown in A. C and D. Total RNA prepared from fed (❑) and 4 days-fasted rats (■) killed at 8:00 h and at 20:00 h, and Northern blot analyses were performed. Representative data are shown in C. Signal intensities on the film were subjected to scanning densitometry, and protein and mRNA abundances were expressed as a ratio of the value of fed rats. Each column represents the mean ± SE of five to six rats. *p < 0.05, significantly different from fed rats.

**Fig. 2.** Effect of fasting on diurnal variation of duodenal PEPT1 protein expression in the fed and fasted rats. A. Duodenal brush-border membranes were prepared from fed and each fasted condition rats killed at 8:00 h and at 20:00 h, and Western blot analyses were carried out. Representative data are shown. B. Signal intensities on the film were subjected to scanning densitometry, and protein abundances were expressed as a ratio of the value at 8:00 h (❑, 8:00 h; ■, 20:00 h). Each column represents the mean ± SE of five to six rats. *p < 0.05, significantly different from 8:00 h.
Fig. 3. Effect of fasting on duodenal PEPT1 protein expression in the 4 days-fasted rats through one day. A. Duodenal brush-border membranes were prepared from rats killed at the indicated time through one day, and Western blot analyses were carried out. Representative data are shown. B. Signal intensities on the film were subjected to scanning densitometry, and protein abundances were expressed as a ratio of the value at 4:00 h. □ and ■ show light and dark phases, respectively. Each point represents the mean ± SE of 4 rats.

Fig. 4. Diurnal variation of duodenal PEPT1 mRNA expression in the fed and 4 days-fasted rats. A. Total RNA prepared from duodenum of fed and 4 days-fasted rats killed at 8:00 h and at 20:00 h, and Northern blot analyses were performed. Representative data are shown. B. PEPT1 message levels were quantified by scanning densitometry, and expressed as a ratio of the value at 8:00 h (□, 8:00 h; ■, 20:00 h). Each column represents the mean ± SE of five rats. *p < 0.05, significantly different from at 8:00 h.

Fig. 5. Effect of fasting on renal PEPT1 (A) and PEPT2 (B) protein expression in fed and fasted rats. Brush-border membranes of rat kidney cortex were prepared from fed (□) and fasted (■) rats killed at 8:00 h and 20:00 h, and Western blot analyses were carried out. Signal intensities on the film were subjected to scanning densitometry, and
protein abundances were expressed as a ratio of the value of fed rats. Each column represents the mean ± SE of six rats. *p < 0.05, significantly different from at 8:00 h.

**Fig. 6.** Diurnal variation of renal PEPT1 (A) and PEPT2 (B) protein expression in the fed and fasted rats. Renal brush-border membranes were prepared from fed and each fasted condition rats killed at 8:00 h and at 20:00 h of one day, and Western blot analyses were carried out. Signal intensities on the film were subjected to scanning densitometry, and protein abundances were expressed as a ratio of the value at 8:00 h (▴, 8:00 h; ■, 20:00 h). Each column represents the mean ± SE of six rats. *p < 0.05, significantly different from at 8:00 h.

**Fig. 7.** Time course of portal vein CETB concentration at 8:00 h and 20:00 h assessed by *in situ* duodenum loop techniques in the fed and 4 days-fasted rats. ○, 8:00 h in fed rats; ●, 20:00 h in fed rats; △, 8:00 h in 4 days-fasted rats; ▲, 20:00 h in 4 days-fasted rats. Each point represents the mean ± SE of five to six rats. *, F0: 8:00 h versus F0: 20:00 h; #, F0: 8:00 h versus F4: 8:00 h; ##, F0: 20:00 h versus F4: 20:00 h. p < 0.05, significantly different between each group.

**Fig. 8.** Effect of fasting on plasma concentration of CETB after intraintestinal (A, B) and intravenous (C, D) administration in fed and 4 days-fasted rats at 8:00 h and 20:00 h
of one day. CETB was injected at single dose of 3 mg/kg at 8:00 h or 20:00 h. Blood samples were collected at the specified times after the injection. ○, dosing at 8:00 h; ●, dosing at 20:00 h. Each point represents the mean ± SE of six (A, B) or three (C, D) rats. *p < 0.05, significantly difference between each group.
Table 1. Pharmacokinetic parameters of CETB after intraintestinal administration in fed and 4 days-fasted rats at 8:00 h and 20:00 h of one day.

<table>
<thead>
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<th>fed rats</th>
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<th>4 days-fasted rats</th>
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<tr>
<td></td>
<td>8:00 h</td>
<td>20:00 h</td>
<td>8:00 h</td>
<td>20:00 h</td>
</tr>
<tr>
<td>(T_{\text{max}}) (min)</td>
<td>42.4 ± 4.6</td>
<td>32.5 ± 2.5(^{a})</td>
<td>60.0 ± 10.3(^{b})</td>
<td>54.0 ± 10.2(^{c})</td>
</tr>
<tr>
<td>(C_{\text{max}}) (ml/min/kg)</td>
<td>2.0 ± 0.4</td>
<td>2.7 ± 0.3(^{a})</td>
<td>4.8 ± 0.7(^{b})</td>
<td>4.6 ± 0.4(^{c})</td>
</tr>
<tr>
<td>(\text{AUC}_{0-3\text{ h}}) ((\mu\text{g} \times \text{min/ml}))</td>
<td>208.6 ± 20.9</td>
<td>275.4 ± 27.5(^{a})</td>
<td>599.9 ± 160.9(^{b})</td>
<td>521.1 ± 99.1(^{c})</td>
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</table>

CETB was intraintestinally injected as a dose of 3 mg/kg. Each value represents the mean ± SE of four to five rats. \(a\), F0: 8:00 h versus F0: 20:00 h; \(b\), F0: 8:00 h versus F4: 8:00 h; \(c\), F0: 20:00 h versus F4: 20:00 h. \(p < 0.05\), significant difference between each group.
Table 2. Pharmacokinetic parameters of CETB after intravenous administration in fed and 4 days-fasted rats at 8:00 h and 20:00 h of one day.

<table>
<thead>
<tr>
<th></th>
<th>fed rats</th>
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<tr>
<td></td>
<td>8:00 h</td>
<td>20:00 h</td>
<td>8:00 h</td>
<td>20:00 h</td>
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<tr>
<td>$K_e$ (1/min)</td>
<td>0.029 ± 0.004</td>
<td>0.025 ± 0.000</td>
<td>0.011 ± 0.001$^a$</td>
<td>0.014 ± 0.002$^b$</td>
</tr>
<tr>
<td>$T_{1/2}$ (min)</td>
<td>26.9 ± 2.7</td>
<td>28.1 ± 0.4</td>
<td>67.3 ± 10.0$^a$</td>
<td>58.1 ± 19.5$^b$</td>
</tr>
<tr>
<td>$V_d$ (liter/kg)</td>
<td>0.43 ± 0.09</td>
<td>0.40 ± 0.06</td>
<td>0.37 ± 0.06</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>$CL_{tot}$ (ml/min/kg)</td>
<td>10.5 ± 0.6</td>
<td>9.9 ± 1.5</td>
<td>3.8 ± 0.1$^a$</td>
<td>4.5 ± 0.7$^b$</td>
</tr>
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</table>

CETB was intravenously injected as a dose of 3 mg/kg. Each value represents the mean ± SE of three rats. $^a$, F0: 8:00 h versus F4: 8:00 h; $^b$, F0 20:00 h versus F4: 20:00 h. $p$ < 0.05, significant difference between each group.
Fig. 1

A

F0  F4
8:00
20:00

B

PEPT1 Protein Levels
(ratio to F0)

0
0.5
1.0
1.5
2.0
2.5

8:00  20:00

Time of Day

* *

C

F0  F4
8:00
20:00

D

PEPT1 mRNA Levels
(ratio to F0)

0
0.5
1.0
1.5
2.0
2.5

8:00  20:00

Time of Day

* *
Fig. 2

A

B

PEPT1 Protein Levels (ratio to 8:00 h)

Days of Fasting

F0 F1 F2 F3 F4

* *
Fig. 3

[Image of a graph showing the variation of PEPT1 protein levels over time, with peaks at certain times of the day.]
Fig. 4

A

B

PEPT1 mRNA Levels
(ratio to 8:00 h)

Days of Fasting

F0

F4

8:00

20:00

*
Fig. 5

A

PEPT1 Protein Levels (ratio to F0)

8:00 20:00

B

PEPT2 Protein Levels (ratio to F0)

8:00 20:00

Time of Day
Fig. 6

A

PEPT1 Protein Levels
(ratio to 8:00 h)

F0  F1  F2  F3  F4

B

PEPT2 Protein Levels
(ratio to 8:00 h)

F0  F1  F2  F3  F4

Days of Fasting
Fig. 7

Portal Vein CETB Concentration (μg/ml) vs Time after Injection (min)

- # indicates a significant difference at the P < 0.05 level.
- ## indicates a significant difference at the P < 0.01 level.
Fig. 8

A

B

C

D

Plasma CETB Concentration (µg/ml)

Time after Injection (min)

F0

F4

Plasma CETB Concentration (µg/ml)

Time after Injection (min)