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Quantitative prediction of oral absorption of PEPT1 substrates based on *in vitro* uptake into Caco-2 cells

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Abstract

The method for predicting the fraction absorbed (F_a) of the PEPT1 substrates was established based on the *in vitro* uptake into Caco-2 cells. Uptake of a drug into Caco-2 cells was measured, and the carrier-mediated initial uptake clearance (ΔCL_{uptake}) was calculated as the difference between the uptake clearance in the absence of glycyl-sarcosine (Gly-Sar) and that in the presence of 30 mM Gly-Sar. The ΔCL_{uptake} of each drug was then divided by that of cephradine to obtain ΔCL^*_{uptake}, which was a normalized parameter to correct for inter-day and/or inter-cell variability. Then, cephradine (CED), cefixime (CFIX), and cefotiam (CTM) were selected as marker compounds having excellent, medium and poor absorption, respectively. The ΔCL^*_{uptake} and F_a values for CED, CFIX and CTM were fitted to the equation derived from the complete radial mixing (CRM) model, and the scaling factor (A') was obtained. Using the A' value, F_a was predicted from the ΔCL^*_{uptake} value of each drug. Good correlation was observed between the predicted and reported F_a values, which demonstrated that F_a of PEPT1 substrates can be predicted based on the *in vitro* uptake in Caco-2 cells.

Keywords: β-Lactam antibiotics; PEPT1; Oral absorption; Prediction; Caco-2 cells

1. Introduction

Drug transporters play important roles in the processes of absorption, distribution and excretion of various drugs. Among these transporters, the peptide transporter expressed in the small intestine (PEPT1, SLC15A1) is responsible for the oral absorption of not only di/tripeptides but also peptide mimetic drugs such as β-lactam antibiotics (Herrea-Ruiz and Knipp, 2003; Bai and Amidon, 1992). Recently, it has been shown that PEPT1 transports a wide variety of structurally unrelated compounds even without a peptide bond, which makes this transporter a target for drug delivery for intestinal absorption (Rubio-Aliaga and Daniel, 2003).

Oral absorption of passively absorbed drugs can be predicted to some extent *in silico* and/or *in vitro*. It is reported that the fraction absorbed (F_a) of passively absorbed drugs can be predicted from the polar molecular surface area of the drug molecule (Palm et al., 1998) or the permeability through an artificial membrane, e.g., PAMPA, as well as a Caco-2 cell monolayer (Chong et al., 1996; Gres et al., 1998; Corti et al., 2006). Moreover, the prediction of F_a from the *in situ* perfusion study has been successful for drugs not only with passive absorption but also with transporter-mediated absorption (Amidon et al., 1980, 1988). The *in situ* perfusion method has the advantage of providing a means of predicting F_a irrespective of the mechanism of absorption, although it is time-consuming and not suitable for screening a number of drug candidates.

To screen a large number of drug candidates, an *in silico* or *in vitro* system which can predict transporter-mediated absorption is desirable. However, the prediction of F_a *in silico* or *in vitro*

Abbreviations: CRM model, complete radial mixing model; CLuptake, uptake clearance; F_a, fraction absorbed; ABPC, ampicillin; ACPC, cyclacillin; CCL, cefaclor; CED, cephradine; CEX, cephalexin; CETB, ceftibuten; CEZ, cefazolin; CFIX, cefixime; CTM, cefotiam; Gly-Sar, glycyl-sarcosine; Glu-Ala, glutamyl-alanine; Gly-Pro, glycyl-proline; HEPES, 2-[4-(2-hydroxy ethyl)-1-piperazinyl] ethane sulfonic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; MES, 2-morpholinoethanesulfonic acid, monohydrate.

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has been unsuccessful for drugs that are absorbed by a carrier-mediated system (Chong et al., 1996; Gres et al., 1998; Gochoco et al., 1994). In our previous study using rat intestinal brush border membrane vesicles (BBMV), F_a values of cephalosporins were successfully predicted based *in vitro* uptake into BBMV (Kohda-Shimizu et al., 2001). However, the lot-to-lot variability among BBMV preparations as well as the time and labor required for BBMV preparation make it extremely difficult to use the method for screening purposes. In the present study, Caco-2 cells were used to establish an *in vitro* method to predict F_a of PEPT1 substrates.

2. Materials and methods

2.1. Materials

The β-lactam antibiotics used in the present study are listed in Table 1. Cefaclor (CCL) and ceftibuten (CETB) were kindly donated by Shionogi & Co. (Osaka, Japan). Cefixime (CFIX), cefotiam (CTM) and cyclacillin (ACPC) were kindly donated by Fujisawa Pharmaceutical Co. (Osaka, Japan), Takeda Chemical Industries (Tokyo, Japan) and Takata Seiyaku Co. (Tokyo, Japan), respectively. Ampicillin (ABPC), cefazolin (CEZ), cephalexin (CEX), cephradine (CED), glycyl-sarcosine (Gly-Sar), glutamyl-alanine (Glu-Ala) and glycyl-proline (Gly-Pro) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid), Tris (2-amino-2-hydroxymethyl-1,3-propanediol) and MES (2-morpholinoethanesulfonic acid, monohydrate) were purchased from Wako Pure Chemicals (Osaka, Japan). [^{3}H]-Gly-Sar was purchased from Moravek Biochemicals (Brea, CA, USA). All other chemicals were of the highest grade available.

2.2. Cell culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD, USA) at passage 18. The cells were cultured in six well plates (9.6 cm^2/well; Falcon) and used for uptake studies between 21 and 28 days after seeding. The culture conditions were the same as those reported previously (Li et al., 1999). The cells between the 30th and 40th passages were used in the present study.

2.3. Uptake study

Hank's balanced salt solution (HBSS: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$) containing 5 mM D-glucose and 5 mM MES (for pH 6.0) was used as the uptake medium. HBSS containing 5 mM D-glucose and 5 mM HEPES (for pH 7.4) was used as the rinse medium.

Table 1
Fraction absorbed (F_a) in humans, the reported K_m or K_i values, and the estimated maximum concentration in the gastrointestinal (GI) tract

	F_a	K_m or K_i (mM)	Estimated maximum concentration (mM) in the GI tract[a]
Cyclacillin (ACPC)	0.95[1]	1.15[c,10]	3.7
Cephradine (CED)	0.94[2]	8.3[11]	3.6
Cefaclor (CCL)	0.90[3]	7.6[12]	3.4
Cephalexin (CEX)	0.90[4]	2.9[13], 7.5[14]	3.6
Ceftibuten (CETB)	0.70[5]	0.5[15], 1.0[11]	2.4
Ampicillin (ABPC)	0.57[6]	14.5[c,10]	6.2
Cefixime (CFIX)	0.47[7]	1.4[11], 17[16]	1.1
Cefazolin (CEZ)	0.063[b,8]	–[d]	–[e]
Cefotiam (CTM)	0.031[b,9]	–[d]	–[e]

[1] Tanaka et al. (1992).
[2] Schwinghammer et al. (1990).
[3] Sinko et al. (1993).
[4] Gilman et al. (2001).
[5] Unpublished observation.
[6] Ehrnebo et al. (1979).
[7] Faulkner et al. (1988).
[8] Yoshimura et al. (1985).
[9] Nishimura et al. (1986).
[10] Bretschneider et al. (1999).
[11] Matsumoto et al. (1995).
[12] Dantzig et al. (1992).
[13] Dantzig and Bergin (1990).
[14] Gochoco et al. (1994).
[15] Muranushi et al. (1994).
[16] Dantzig et al. (1994).
[a] The maximum concentration when the drug is completely dissolved 200 mL of water.
[b] F_a in mice.
[c] K_i value.
[d] Not reported.
[e] Not applicable.

For the uptake study, plated Caco-2 cells were rinsed twice and pre-incubated with the rinse medium (pH 7.4) for 10 min at 37 °C. Uptake was initiated by adding 1 mL of the pre-incubated drug solution (pH 6.0). The drug solution was aspirated at the appropriate time to terminate the uptake, and then the cells were rinsed twice with ice-cold rinse medium (pH 7.4). To determine the uptake amount of [^{3}H]-Gly-Sar, the cells were lysed with 1 mL of 0.1% Triton X-100®, and a 0.8 mL aliquot of the cell lysate was transferred to a scintillation vial. Then, 10 mL of Aquasol™-2 (Packard Instrument, Meriden, CT, USA) was added, and the radioactivity was measured with a liquid scintillation count. To determine the uptake amount of other drugs, 1 mL of the mobile phase (see below) was added. The cells were then detached and collected with a cell scraper. The mixture was vortexed, centrifuged at 3500 × *g* for 15 min, and a 100-μL aliquot of the supernatant was injected onto HPLC.

Adsorption of the drug to the cells was measured in the same manner as described above, but the drug solution was removed immediately after being added to the plate. The adsorbed amount was subtracted to obtain the net uptake. In the inhibition study, each inhibitor was added to the drug solution at an appropriate concentration, and uptake of the drug was measured in the same manner as described above. For protein determination, Caco-2 cells were dissolved in 1 mL of 0.1% Triton X-100® and the protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Labs, Richmond, CA) using bovine serum albumin as a standard.

2.4. HPLC conditions

High-performance liquid chromatograph (HPLC) was used to determine amount of the drug. Mightysil-18® (4.6 mm (i.d.) × 150 mm; KANTO CHEMICAL, Tokyo, Japan) was used as an analytical column. The mobile phase for the determination of ABPC, ACPC, CCL, CED, CEX, CEZ and CTM was 20 mM CH_3COONH_4–CH_3OH (80:20 (vol/vol)). The mobile phase for CFIX was a mixture (27.5:72.5 (vol/vol)) of CH_3CN and the aqueous solution containing 10 mM tetra-*n*-butylammonium bromide and 20 mM CH_3COONH_4. The mobile phase for CETB was 50 mM phosphate buffer (pH 3.0)-CH_3OH (87.5:12.5 (vol/vol)). CFIX, CETB and FRPM were detected at 290, 262 and 313 nm, respectively. Other drugs were detected at 220 nm. The flow rate was 0.8 mL/min for all drugs.

2.5. Data analysis

To calculate kinetic parameters for Gly-Sar uptake, the following equation (Eq. (1)) was used and the parameter values were obtained by a non-linear least-squares regression analysis (Yamaoka et al., 1981).

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + k_d \cdot [S] \quad (1)$$

where v is the initial uptake rate, V_{max} is the maximum uptake rate, K_m is the Michaelis constant, k_d is the uptake clearance by passive diffusion, and $[S]$ is the initial concentration of [^{3}H]-Gly-Sar.

Uptake clearance (CL_{uptake}) was calculated according to Eq. (2).

$$CL_{uptake} = \frac{v}{[S]} \quad (2)$$

where v is the initial uptake rate.

The carrier-mediated uptake clearance (ΔCL_{uptake}) was obtained by subtracting the uptake clearance in the presence of 30 mM Gly-Sar from that in the absence of Gly-Sar (Eq. (3)).

$$\Delta CL_{uptake} = CL_{uptake}(\text{without Gly-Sar}) - CL_{uptake}(\text{with 30 mM unlabeled Gly-Sar}) \quad (3)$$

According to the CRM model, the fraction absorbed (F_a) following oral administration of a drug can be described by Eq. (4) (Amidon et al., 1980, 1988).

$$F_a = 1 - \exp(-A \cdot CL_{abs}) \quad (4)$$

where A is the scaling factor and CL_{abs} is the absorption clearance. For the drugs that are absorbed by a carrier-mediated process, CL_{abs} in Eq. (4) can be replaced by the carrier-mediated uptake clearance as shown in Eq. (5) (Kohda-Shimizu et al., 2001).

$$F_a = 1 - \exp(-A \cdot \Delta CL_{uptake}) \quad (5)$$

where ΔCL_{uptake} is the carrier-mediated uptake clearance.

Moreover, cephradine (CED) was selected as the standard compound to correct for (or normalize) the inter-day and inter-cell variability. The value of ΔCL_{uptake} of each drug was divided by that of CED, and the normalized ΔCL_{uptake} was defined as ΔCL^*_{uptake}. Then, ΔCL_{uptake} in Eq. (5) was replaced by ΔCL^*_{uptake} to obtain Eq. (6).

$$F_a = 1 - \exp(-A' \cdot \Delta CL^*_{uptake}) \quad (6)$$

where $A' = A \cdot \Delta CL_{uptake}(\text{CED})$ and $\Delta CL^*_{uptake} = \Delta CL_{uptake} / \Delta CL_{uptake}(\text{CED})$. The A' value was obtained by fitting F_a and ΔCL^*_{uptake} of the marker compounds (CTM, CFIX and CED) to Eq. (6) by a non-linear least-squares method (Yamaoka et al., 1981).

2.6. Statistical analysis

Student's *t*-test was used for statistical analysis with a p value of less than 0.05 being considered statistically significant.

3. Results

3.1. Time course of uptake

The time course of the uptake of [^{3}H]-Gly-Sar, ABPC, ACPC, CED and CEX in the presence of an H^+ gradient (pH 6.0) is shown in Fig. 1. Because the amount of each drug taken up in the cells increased linearly at least up to 10 min, the initial uptake rate was calculated from the uptake amount at 5 min in the following studies.

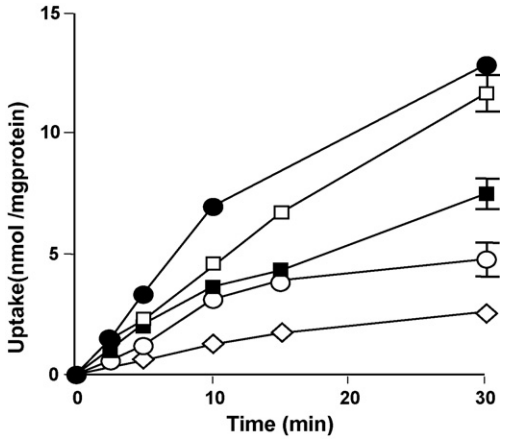

Fig. 1. Time course of the uptake 1 mM ABPC (◯), ACPC (●), CED (□), CEX (■) and 5 μM ^{3}H-Gly-Sar (◊) into Caco-2 cells. Each point represents the mean ± S.D. of three determinations.

3.2. Concentration dependence of Gly-Sar uptake

The initial uptake of [^{3}H]-Gly-Sar at various concentrations (0–32 mM) was measured in the presence of an H^+ gradient (pH 6.0), and the results are shown in Fig. 2. The uptake parameters calculated according to Eq. (1) were as follows: $K_m = 0.97$ mM, $V_{max} = 2.41$ nmol/mg protein/ min, and $k_d = 1.10$ μL/mg protein/min.

3.3. Inhibition studies

The inhibitory effects of dipeptides and β-lactam antibiotics on the initial uptake of 36 μM [^{3}H]-Gly-Sar were studied. The results are summarized in Table 2. Gly-Sar uptake decreased significantly in the presence of the dipeptides (Gly-Sar, Gly-Pro and Glu-Ala), ABPC, ACPC, AMPC, CCL, CED, CEX and CFIX. On the other hand, the uptake of Gly-Sar was not influenced by 10 mM CEZ or 20 mM CTM. Unexpectedly, 20 mM CEZ inhibited Gly-Sar uptake (see Section 4).

Table 2
Inhibitory effects of various compounds on 36 μM [^{3}H]-Gly-Sar uptake into Caco-2 cells

Compound	Uptake (% of control) mean ± S.D.	n
Control (36 μM Gly-Sar)	100.0 ± 7.1	12
+20 mM Gly-Sar	13.5 ± 1.8**	
+20 mM Gly-Pro	13.7 ± 3.5**	3
+20 mM Glu-Ala	5.7 ± 1.1**	3
+5 mM ACPC	6.3 ± 2.1**	3
+20 mM ABPC	73.7 ± 4.7**	3
+5 mM CCL	52.7 ± 4.8**	3
+10 mM CCL	16.7 ± 1.9**	3
+20 mM CED	26.0 ± 3.4**	6
+20 mM CEX	28.8 ± 2.7**	3
+5 mM CFIX	62.1 ± 3.5**	3
+10 mM CEZ	83.0 ± 10.5	3
+20 mM CEZ	57.5 ± 3.9**	12
+20 mMCTM	89.5 ± 10.8	3

** Significantly different from the control, $p < 0.01$.

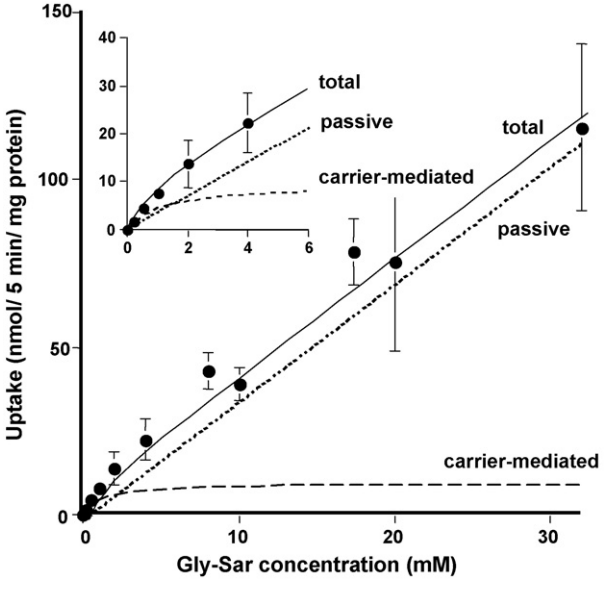

Fig. 2. Concentration dependence of the initial uptake rate (v) of Gly-Sar into Caco-2 cells at pH 6.0. Initial uptake rate was calculated from the uptake of Gly-Sar in 5 min. Each point represents the mean ± S.D. of six determinations. The solid lines are the results of nonlinear regression analysis. The dashed lines represent the passive and carrier-mediated components simulated with the obtained parameter values. Inset: magnified for the lower concentrations (0–4 mM).

3.4. Prediction of F_a based on the uptake into Caco-2 cells

The initial uptake of ABPC, ACPC, AMPC, CCL, CED, CEX, CETB, CFIX, CEZ and CTM were measured at pH 6.0 in the absence or presence of 30 mM unlabeled Gly-Sar. The concentration in the uptake medium was 0.5 mM for CETB and 1 mM for other drugs. The uptake clearance was calculated according to Eq. (2) and the results are shown in Fig. 3. The uptake clearances of ABPC, ACPC, AMPC, CCL, CED, CETB, CEX and CFIX in the absence of Gly-Sar were significantly greater than those in the presence of 30 mM Gly-Sar. In contrast, the uptake clearances of CEZ and CTM in the presence of 30 mM Gly-Sar did not differ from those in the absence of Gly-Sar.

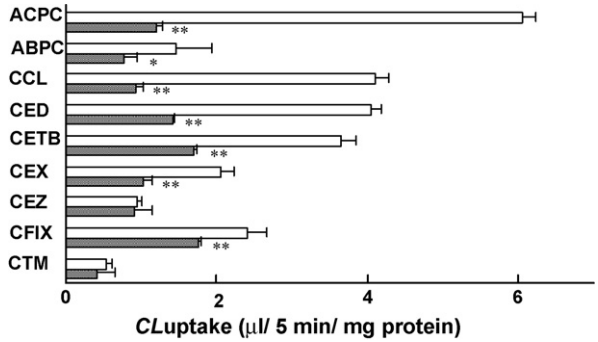

Fig. 3. Uptake clearance of various compounds into Ccao-2 cells in the absence (open column) or presence (shaded column) of 30 mM unlabeled Gly-Sar. Uptake was measured at pH 6.0 for 5 min. Each column represents the mean ± S.D. of at least three determinations. The uptake clearance was significantly different from that in the absence of Gly-Sar ($^*p < 0.05$, $^{**}p < 0.01$).

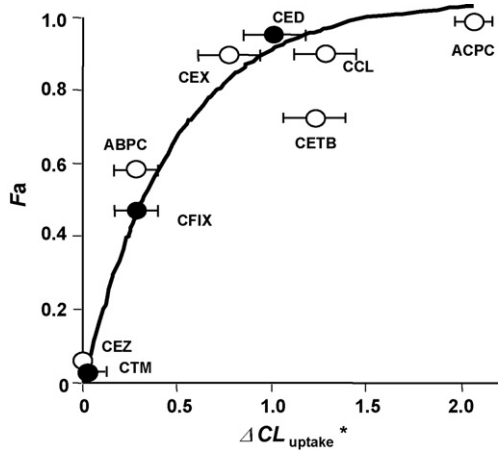

Fig. 4. The ΔCL^*_{uptake} versus reported F_a. The solid line shows the fitting curve for CTM, CFIX and CED. Each point represents the mean ± S.E. of at least three determinations.

The uptake clearance by PEPT1 (ΔCL_{uptake}) was calculated according to Eq. (3). In every uptake experiment, the uptake of CED was measured together with several other drugs, and the ΔCL_{uptake} of each drug was divided by that of CED to obtain ΔCL^*_{uptake}. In Fig. 4, the ΔCL^*_{uptake} was plotted against the reported F_a (Table 1).

CED, CFIX and CTM were selected as the marker compounds with excellent, medium and poor absorption, respectively. The values of F_a and ΔCL^*_{uptake} for these marker compounds were fitted to Eq. (6) (Fig. 4), and the A' value of 2.40 was obtained. Using this A' value, the F_a value of each drug was calculated from ΔCL^*_{uptake} (Eq. (6)). The calculated (predicted) and reported F_a values were plotted in Fig. 5. Good correlation was observed between the predicted and reported F_a values, demonstrating that the F_a values of PEPT1 substrates can be predicted according to the present method.

4. Discussion

In the present study, Caco-2 cells were used to predict the F_a values of β-lactam antibiotics which are absorbed by PEPT1

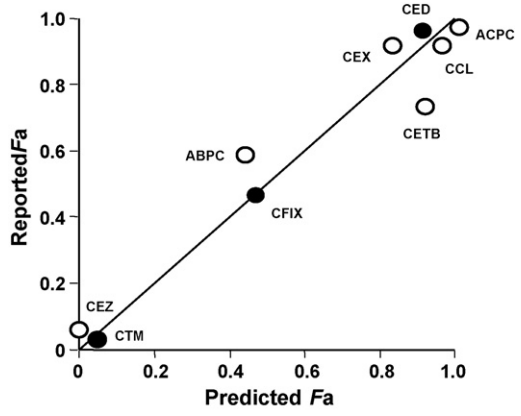

Fig. 5. Correlation between the reported and predicted F_a. Predicted F_a values of test drugs (compounds indicated with ○) were calculated according to Eq. (6) using the A' value of 2.40. The A' value was obtained from the data of the marker compounds (compounds indicated with ●) as shown in Fig. 4.

(Table 1). Since the F_a values of CEZ and CTM in humans were unavailable, the reported F_a values in mice were assumed to be equal to those in humans for these drugs. The microclimate pH at the apical surface of intestinal epithelial cells is 5.8–6.2 (Lucas, 1983; Said et al., 1986; Shimada and Hoshi, 1988) and PEPT1 uses the H^+ gradient as a driving force. Therefore, the uptake of a drug into Caco-2 cells was measured at the outside pH of 6.0 in this study. Reported K_m and/or K_i values in Caco-2 cells at pH of 6.0 are also summarized in Table 1.

Since the drugs in Table 1 are highly hydrophilic, the contribution of passive diffusion in the intestinal absorption *in vivo* can be negligible. Indeed, it has been shown that the contribution of passive diffusion is insignificant in the intestinal absorption of cephalosporins in rat *in situ* perfusion studies (Sinko and Amidon, 1988; Tamai et al., 1997). Moreover, the intestinal absorption of cephalexin and cefadroxil in rats is correlated with the PepT1 expression level (Chu et al., 2001; Naruhashi et al., 2002). Furthermore, the polar molecular surface areas (PMSA) of β-lactam antibiotics are so large that the intestinal absorption of these drugs by passive diffusion is expected to be negligible (Clark, 1999). All these observations strongly indicate that the carrier-mediated pathway is predominant in the intestinal absorption of the drugs in Table 1. Therefore, the carrier-mediated uptake was estimated and used as the parameter to predict F_a in the present study.

The expression of PEPT1 in Caco-2 cells is much lower than that in the human enterocytes (Chong et al., 1996; Seithel et al., 2006), which indicates that the contribution of passive diffusion in the total uptake of drugs into Caco-2 cells is much greater than that into enterocytes *in vivo*. Therefore, the PEPT1-mediated uptake clearance was estimated by using an excess concentration of an inhibitor (Gly-Sar) and used for the prediction.

Since all drugs in Table 1, except CEZ and CTM, are PEPT1 substrates, it is reasonable to assume that Gly-Sar inhibits the uptake of the drugs competitively. Then, the inhibitory effect of 30 mM Gly-Sar on the PEPT1-mediated uptake of the drugs can be estimated according to the following equation (Eq. (7)).

$$\frac{v'}{v} = \frac{K_m + [S]}{K_m \cdot (1 + [I]/K_i) + [S]} \quad (7)$$

where v' and v are the PEPT1-mediated initial uptake rate of a drug in the presence and absence of Gly-Sar, respectively, K_m is the Michaelis constant of the uptake of a drug, $[S]$ is the drug concentration (0.5 mM for CETB and 1 mM for other drugs), $[I]$ is the inhibitor (i.e., Gly-Sar) concentration (30 mM), and K_i is the inhibition constant of Gly-Sar. The K_i value of Gly-Sar can be assumed to be equal to the K_m value (0.97 mM). The v'/v ratios were calculated by substituting the K_m (or K_i) values (Table 1) to Eq. (7), and the obtained v'/v ratios were approximately 0.03 or less. The calculations indicate that the PEPT1-mediated initial uptake rate of each drug was inhibited by at least 97% in the presence of 30 mM Gly-Sar. Therefore, the value of ΔCL_{uptake} in the present study genuinely represents the PEPT1-mediated uptake.

Moreover, the value of ΔCL_{uptake} of the drug was further divided by that of CED for normalization in order to correct for

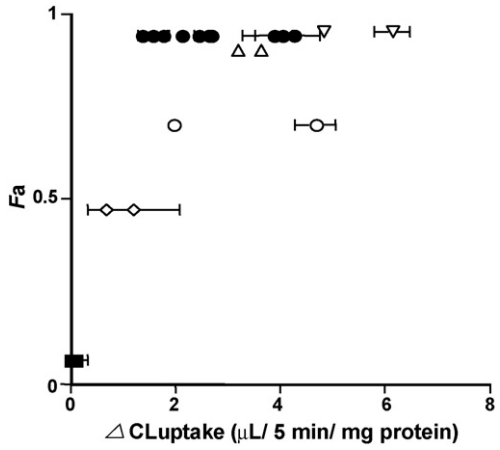

Fig. 6. The ΔCL_{uptake} versus reported F_a on different days with various passages of Caco-2 cells. (●) CED, (△) CCL, (▽) ACPC, (◯) CETB, (◇) CFIX, and (■) CEZ. Each point represents the mean ± S.E. of at least three determinations.

inter-day and/or inter-cell variability. In fact, when the uptake of 1 mM CED was measured on ten different occasions (different days with various passages of Caco-2 cells), the uptake clearance varied by a factor of greater than four. Significant inter-day and/or inter-cell variability was also observed in the uptake of other drugs. Without the normalization by the CED uptake, the present prediction was not possible due to large inter-day and/or inter-cell variability (Figs. 4 and 6).

The estimated maximum concentrations (EMCs) of the drugs in the gastrointestinal (GI) tract are also listed in Table 1. These values were obtained based on the assumption that the drugs are administered with 200 mL of water and are completely dissolved in the same volume of liquid. For CED, CCL, CEX, ABPC, and CFIX, K_m (or K_i) values are greater than the EMCs in the GI tract. The EMCs in the GI tract are, in turn, similar to or greater than the concentration used in the uptake study ($[S] = 1$ mM). Since $[S] \leq$ EMCs $< K_m$ (or K_i), no saturation should be expected for CED, CCL, CEX, ABPC, and CFIX in the absorption *in vivo* as well as in the present uptake study.

In contrast, the concentrations of CETB and ACPC in the uptake study ($[S] = 0.5$ and 1 mM for CETB and ACPC, respectively) are close to the corresponding K_m (or K_i) values (Table 1). The EMCs in the GI tract are greater than the $[S]$ and K_m (or K_i), i.e., $[S] \cong K_m$ (or K_i) < EMCs. Therefore, saturation may take place both in the present uptake study and in the absorption *in vivo*, and the effect of saturation may be greater in the absorption process *in vivo*. This may be reflected in the prediction of F_a (Fig. 5), where the predicted F_a value is greater than the observed value for CETB. Saturation in PEPT1-mediated absorption will have to be taken into account for better prediction in future studies.

In Table 2, lack of inhibition by 10 mM CEZ and 20 mM CTM also agrees with the fact that the two drugs are not substrates of PEPT1. It was totally unexpected that 20 mM CEZ decreased Gly-Sar uptake. In our recent study using PEPT1 cDNA transfected cells, CEZ inhibited the uptake of Gly-Sar competitively (data not shown). Since the uptake of CEZ was not inhibited by Gly-Sar (Fig. 3), CEZ is likely to be an inhibitor, not a substrate, of PEPT1. These observations strongly indicate that the affinity with the transporter (e.g., K_i or IC50) is not a good predictor of F_a.

Corticosterone is passively and completely absorbed in the small intestine following oral administration. The uptake of corticosterone into Caco-2 cells was measured, and the ΔCL^*_{uptake} was calculated. The obtained ΔCL^*_{uptake} of corticosterone was null, which resulted in the predicted F_a value being zero (data not shown). Therefore, the present method can be applied only to PEPT1 substrates, and it is unlikely the F_a of a passively absorbed drug is erroneously predicted as a PEPT1 substrate.

Only the PEPT1-mediated uptake was taken into account in the present prediction, although the contribution of passive diffusion may not be negligible for the PEPT1 substrates with high lipophilicity. In addition, it has been reported that ABPC and CEZ may be transported in the secretory direction with an energy dependent process in the rat intestinal segments (Saitoh et al., 1996a,b). In contrast, no such secretory transport has been observed for CTM in Caco-2 cells (Inui et al., 1992). In our transport study with Caco-2 cells, the permeability of ABPC from the basal to apical side was almost equal to that in the opposite direction. However, the permeability of CEZ from the basal to apical side was approximately double that in the opposite direction (data not shown). These results indicate that CEZ, not ABPC or CTM, may be transported in the secretory direction in Caco-2 cells. It is also reported that other peptide transporters, such as HPT1, may be involved in the intestinal absorption of PEPT1 substrates (Landowski et al., 2003). Incorporation of the contribution of passive diffusion into the present prediction may be necessary for the better prediction. Contribution of passive diffusion, other uptake and efflux transporters will have to be taken into account for the better prediction.

In conclusion, the present study has demonstrated that the F_a of PEPT1 substrates can be predicted based on the *in vitro* uptake into Caco-2 cells. The present method should be tested with a variety of PEPT1 substrates in future. Considering the broad substrate specificity of PEPT1, the present method is expected to contribute to the screening of a drug candidate that is targeted for PEPT1-mediated absorption.

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