Characteristics of Ceftibuten Uptake into Caco-2 Cells

Noriyuki Muranushi,[1] Kazutoshi Horie,[1] Kazuyoshi Masuda,[1] and Koichiro Hirano[1]

Received April 6, 1994; accepted July 4, 1994

The characteristics of ceftibuten uptake into Caco-2 cells grown in a collagen-coated dish were examined. Ceftibuten showed stereoselective and pH-dependent uptake. The pH-dependency of ceftibuten was more marked than that of cefaclor or cephalexin, but all three antibiotics showed maximal uptake at pH 5.5. Ceftibuten uptake was linear for the initial 1 hr and then reached a plateau. The initial uptake (15 min) was markedly reduced by the addition of 2,4-dinitrophenol or FCCP (a protonophore), or by lowering the incubation temperature. The uptake of ceftibuten into the brush-border membrane vesicles prepared from cultured Caco-2 cells showed an overshoot in the presence of an H^+-gradient. These findings indicated that the uptake of ceftibuten was energy-dependent, especially H^+-gradient-dependent. Uptake inhibition by various compounds was compared using Caco-2 cells. Amino acids and a tetrapeptide did not inhibit uptake, whereas di- or tri-peptides were effective inhibitors. These observations suggest that ceftibuten is taken up by a carrier-mediated transport system(s) for dipeptides. Various antibiotics differed in their ability to inhibit uptake, with cyclacillin showing maximum inhibition. Differences in the inhibitory effect may be accounted for by the heterogeneity (multiplicity) of the transport systems.

KEY WORDS: ceftibuten; Caco-2; uptake; brush-border membrane vesicles; oligopeptide; oral cephem.

INTRODUCTION

Caco-2 cells have been used to evaluate the oral absorbability of drugs or to study their absorption mechanisms. Caco-2 cells differentiate in culture to epithelial cells and possess intestinal enterocyte-like properties [1]. Caco-2 cells express the transport systems of nutrients such as sugars [2, 3], amino acids [4, 5], dipeptides [6] and bile acids [7].

Uptake of the cephalosporins, cephalexin and cefaclor, into Caco-2 cells was mediated by the proton-dependent dipeptide transporter [8, 9]. Inui et al. also demonstrated cephradine transport via a H^+/dipeptide cotransport system in Caco-2 cells [10]. Ceftibuten does not possess an α-amino group in the side chain at the 7 position of the cephem skeleton (Fig. 1), which distinguishes it from amino β-lactams. Previously, we characterized the uptake of ceftibuten into rat intestinal brush-border membrane vesicles [11, 12]. Ceftibuten uptake into rat, rabbit, and human brush-border membrane vesicles showed a typical overshoot phenomenon [11, 13, 14]. The uptake characteristics of ceftibuten were different from those of cefaclor [12] and cephalexin [14], although the uptake of both drugs might occur via dipeptide transport systems.

[1] Shionogi Research Laboratories, Shionogi & Co., Ltd., Sagisu, Fukushima-ku, Osaka, 553 Japan.

Experiments using isolated brush-border membrane suffer from several artifacts. In this study, we investigated the characteristics of ceftibuten uptake into Caco-2 cells which maintain morphology and physiology as living cells.

MATERIALS AND METHODS

Materials. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, Md.). Ceftibuten and latamoxef were used as obtained from Shionogi Research Laboratories. Cefaclor and cephalexin were supplied from Eli Lilly Co. (Indianapolis, Ind.). Cephradine, cefadroxil and ampicillin were purchased from Sigma Chemical Co. (St. Louis, Mo.), cyclacillin from Takeda Chemical Industries (Osaka, Japan), and cefazolin from Fujisawa Pharmaceutical Co. (Osaka, Japan). γ-L-Glutamyl-L-alanyl-L-alanine was obtained from Bachem (Bubendorf, Switzerland). All other peptides used were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Cell Culture. Caco-2 cells were cultured in Dulbecco's Modified Eagle medium (Life Technologies, Inc., N.Y.) containing 10% fetal calf serum (Bioproduct, Inc., Md.) and 1% non-essential amino acids (Life Technologies Inc., N.Y.) without addition of antibiotics. For uptake study, 1×10^6 cells were seeded in collagen-coated dishes (9 cm^2, Costar, Cambridge, Mass.) and the medium was replaced every two or three days.

Uptake Study into Cells. Hank's balanced salt solution (HBSS, GIBCO) containing 25 mM Mes was used. To assess the extracellular pH effect, citric acid and Hepes were used at a concentration of 25 mM for pH 4.5 and 7.0, respectively. The Caco-2 cells cultured in a dish were washed three times with 1 ml of HBSS and incubated at 37°C for 10 min with 1 ml of HBSS. Then 1 ml of 1 mM drug solution was added to start an uptake study at 37°C. After a certain incubation time, the dish was washed three times with 1 ml of ice-cold HBSS, then 1 ml of 50% acetonitrile solution was added. The cells were stripped off from the dish and transferred into a 10 ml-test tube and 2 ml of dichloromethane was added. After shaking and centrifugation, the drug concentration in the supernatant was measured. Non-specific binding of the drug to the cells was determined by the same procedure as above at 0°C. All uptake amounts were corrected by subtraction of this binding although this was very small. In the case of the inhibition study, various compounds were added to the incubation medium in a concentration of 10 mM.

Uptake Study into Brush-Border Membrane Vesicles. Brush-border membrane vesicles were isolated from Caco-2 cells, which were grown in 8 incubation flasks (175 cm^2) for 14 days, by the Ca^{2+}-precipitation method of Kessler et al. [15] as described previously [11]. Uptake studies were carried out at 37°C by the rapid filtration technique as reported previously [11]. The enrichment factor of specific activity of alkaline phosphatase, a marker enzyme for brush-border membrane, was 16.1, compared to that in the starting homogenate.

Analytical Method. Antibiotics were measured with a high-performance liquid chromatograph LC-6A (Shimadzu Co., Kyoto, Japan). The conditions were as follows: column, Nucleosil $_5C_{18}$ 4.6 mm × 15 cm (Macherey-Nagel, Ger-

0724-8741/94/1200-1761$07.00/0

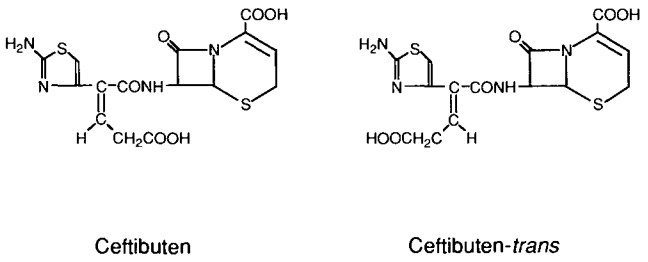

Fig. 1. Chemical structure of ceftibuten and ceftibuten-trans.

many) for all drugs; mobile phase, 0.1 M ammonium acetate/MeOH = 93/7 for ceftibuten, ceftibuten-trans, latamoxef and cefadroxil, 0.1 M ammonium acetate/acetonitrile = 89/11 for cefaclor, cephalexin, cefazolin, and cephradine, 0.05% phosphoric acid/MeOH = 80/20 for cyclacillin; flow rate, 1.0 ml/min; wavelength, 210 nm for cyclacillin, 262 nm for the other drugs. Protein was assayed by a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, Cal.) with bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

Time Course of Ceftibuten Uptake

Caco-2 cells differentiate to epithelial cells gradually in culture. The cells reach a monolayer and steady state density 6–7 days after seeding [1]. Maximal uptake was observed in cells during 10–14 days of culture, which was therefore used in subsequent experiments. Figure 2 shows the time course of ceftibuten uptake at pH 5.5. An almost linear uptake was observed for the initial 60 min, and then the uptake reached a plateau. Ceftibuten was concentrated 8.9 times in the cells against the extracellular drug concentration at 1 hr when considering the cellular volume to be 3.66 μl/mg protein [2]. Since the uptake of ceftibuten was linear for the initial 15 min, uptake over 15 min was subsequently used to represent the initial uptake.

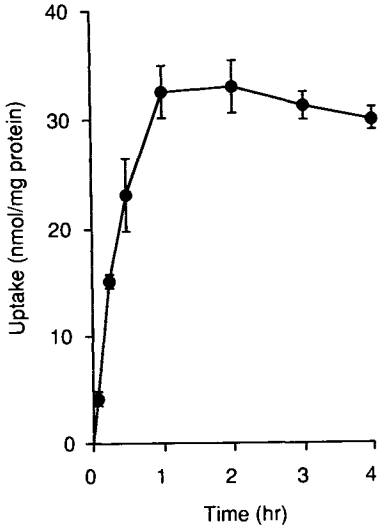

Fig. 2. Time course of ceftibuten uptake into Caco-2 cells. Cells were incubated with 1mM ceftibuten at pH 5.5. Each value is the mean ± S.D. (n = 4).

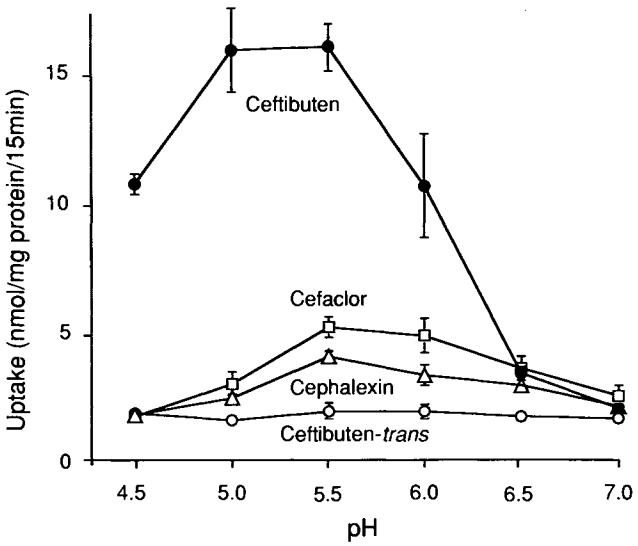

Fig. 3. pH dependence of uptake of cephems into Caco-2 cells. Cells were incubated with 1 mM drug solution for 15 min. Data represent the mean ± S.D. (n = 5).

pH Dependence of Oral Cephems Uptake

Figure 3 shows the initial uptake of oral cephems at various pHs. Ceftibuten possesses a double bond in its side chain is cis isomer (Fig. 1). The trans isomer is not readily absorbed from rat intestine [16]. While the extracellular pH had no effect on the uptake of ceftibuten-trans, it affected those of ceftibuten, cefaclor and cephalexin. Although the maximal uptake was observed at pH 5.5 in these three oral cephems, ceftibuten was affected most by pH, and the uptake of ceftibuten was much larger than those of cefaclor and cephalexin. Similar differences between ceftibuten and cefaclor were also observed in the uptake into rat intestinal brush-border membrane vesicles [11, 12].

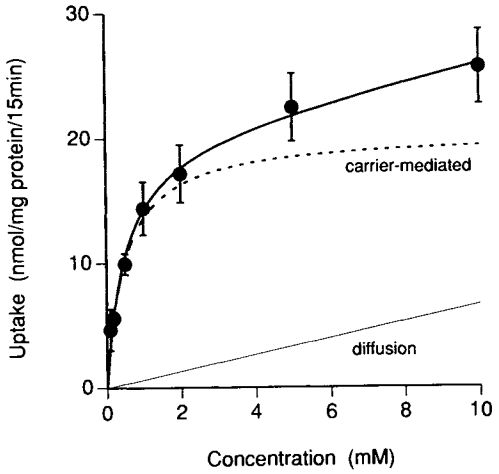

Fig. 4. Concentration-dependent uptake of ceftibuten into Caco-2 cells. Uptake of ceftibuten for the initial 15 min was measured at various concentrations (0.1–10.0 mM) and pH 5.5. The curve was generated from Eq. (1) using kinetic parameters estimated by NONLIN. The dotted curve and the linear line indicate the contribution of carrier-mediated and diffusion process in the equation, respectively.

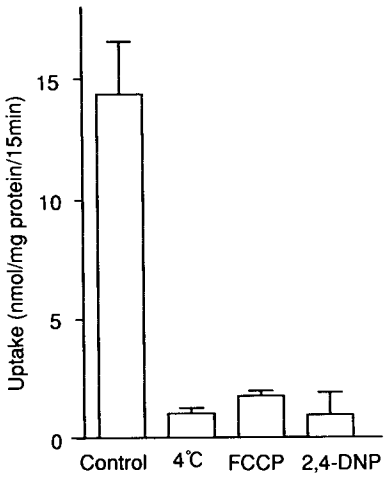

Fig. 5. Effect of energy poisons on the uptake of ceftibuten into Caco-2 cells FCCP and 2,4-dinitrophenol were added to the incubation medium at a concentration of 50 and 500 μM, respectively. Data represent the mean ± S.D. (n = 4).

Kinetic Analysis of Ceftibuten Uptake

To ensure a carrier-mediated transport, the concentration dependence of the uptake was examined (Fig. 4). Uptake was indeed concentration dependent, and the kinetic parameters of ceftibuten estimated by NONLIN [17] by fitting the data to equation (1) are as follows: Km = 0.50 mM; Vmax = 1.36 nmole/mg protein/min; Kdif = 0.044 nmole/mg protein/min/mM.

$$V = Vmax \cdot C/(Km + C) + Kdif \cdot C \quad (1)$$

where V is the initial uptake rate, C is the initial concentration, Vmax is the maximum uptake rate by the carrier-mediated process, Km is the Michaelis constant, and Kdif is the coefficient of simple diffusion.

The dotted line in Fig. 4 indicates the portion of Michaelis-Menten equation and the linear line is the contribution of diffusion. This result shows that ceftibuten mainly is taken up by a carrier-mediated transport system.

Effect of Energy Poisons

The initial uptake of ceftibuten was markedly reduced by the addition of 2,4-dinitrophenol or FCCP (a protonophore), or by lowering the incubation temperature (Fig. 5). Therefore, the concentrative uptake of ceftibuten is not due to the binding to cell components. Ceftibuten uptake is considered to be energy-dependent, especially, H^+-gradient dependent because FCCP exerts a strong inhibitory effect.

Ceftibuten Uptake into Caco-2 Brush Border Membrane Vesicles

To ascertain whether ceftibuten uptake was H^+-gradient dependent, the uptake of ceftibuten into the brush-border membrane vesicles prepared from Caco-2 cells was examined. As Fig. 6 shows, a typical overshoot uptake of ceftibuten was observed in the presence of an H^+-gradient, as seen in rat intestinal brush-border membrane vesicles [11]. H^+-gradient had no effect on the uptake of ceftibuten-trans. These findings demonstrated that the driving force of the ceftibuten uptake in Caco-2 cell is H^+-gradient. In the brush-border membrane vesicles system, only a transient concentrative (overshoot) uptake was observed while a continuous concentrative uptake was seen in the Caco-2 cell system (Fig. 2). In viable Caco-2 cells the intracellular pH is 7.3, and the H^+-gradient across the brush border membrane is maintained. On the other hand, in the brush-border membrane vesicles system the H^+-gradient declines during uptake to an equilibrium state.

Inhibitory Effect of Oligopeptides and Amino Acids in Caco-2 Cell

To determine the transport route of ceftibuten, inhibition of amino acids and oligopeptides on the uptake of ceftibuten in the Caco-2 cell system were examined (Fig. 7).

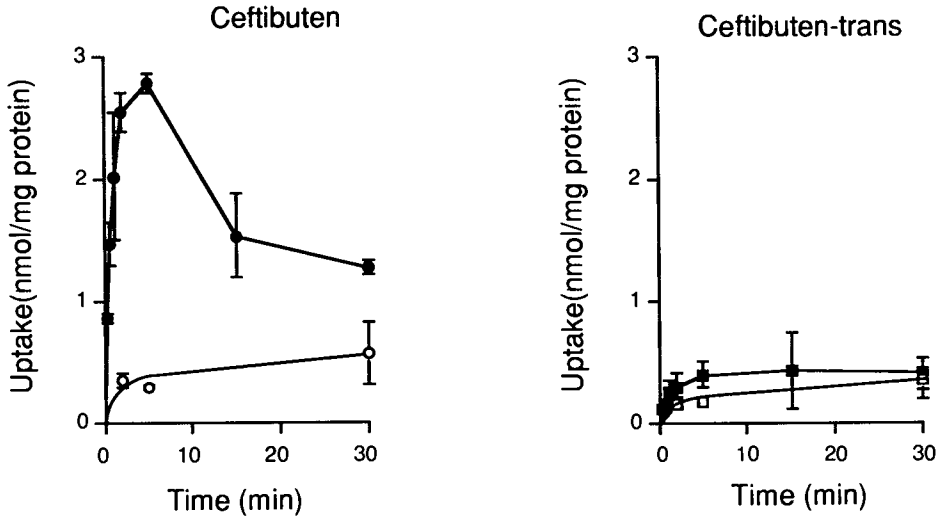

Fig. 6. Effect of an inwardly directed H^+-gradient on ceftibuten and ceftibuten-trans uptake into the brush border membrane vesicles prepared from Caco-2 cells. The intravesicular pH is 7.5. The pH of incubation medium is 5.5 (●, ■) or 7.5 (○, □). Data represent the mean ± S.D. (n = 3).

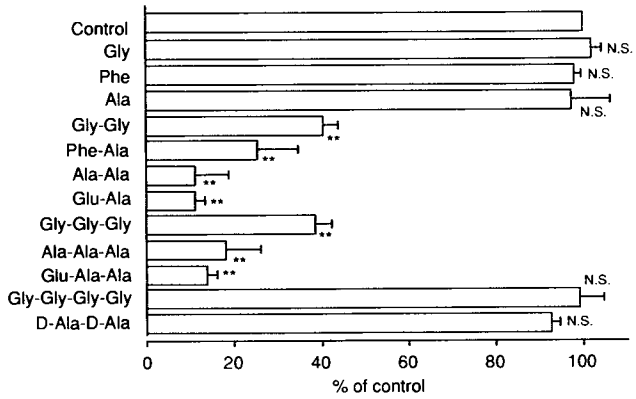

Fig. 7. Inhibitory effects of amino acids and oligopeptides on ceftibuten uptake into Caco-2 cells. Amino acids and peptides were added in a concentration of 10 mM to the incubation medium. Each value is the mean ± S.D. (n = 4–5). (significance: N.S.; not significant, **;$p < 0.01$).

Although amino acids (Gly, L-Ala, L-Phe) and a tetrapeptide (Gly-Gly-Gly-Gly) had no effect, dipeptides and tripeptides strongly inhibited the ceftibuten uptake. Since D-Ala-D-Ala had no inhibitory effect, the inhibition by L-Ala-L-Ala is considered to be stereospecific. These findings strongly suggest that ceftibuten is taken up via a dipeptide transport system(s) in Caco-2 cells.

Inhibitory Effect of Antibiotics

Since the carrier-mediated transport mechanism has been reported to participate in the uptake of cefaclor, cephalexin and cephradine by Caco-2 [8–10], the effect of other antibiotics involving those for parenteral use on ceftibuten uptake was studied (Fig. 8). The inhibitory effects varied among antibiotics, cyclacillin exhibiting the strongest inhibition. Therefore, the uptake of these drugs into Caco-2 cells was also examined (Fig. 9). There seems to be a positive correlation between the extent of the inhibitory effects and the extent of uptake of the antibiotic itself into Caco-2 cells except for latamoxef. Abe et al. also suggested multiple transport systems for dipeptides [18]. They proposed a different transport system for lipophilic dipeptides (such as Phe-Phe) from that for hydrophilic dipeptides (such as Gly-Gly) in toad small intestine. These findings along with those obtained previously on rat intestinal brush-border membrane vesicles in our laboratory [12] suggest the existence of multiple transport systems in Caco-2 cells; one is H^+-gradient-sensitive (active transport) and the other is H^+-gradient-insensitive (facilitated diffusion). The contribution ratio of these two systems may vary among oral antibiotics. Because ceftibuten uptake into Caco-2 cells is most affected by the extracellular pH and is markedly higher than that of any other oral antibiotic examined (Fig. 3), it is considered to be predominantly taken up by the active transport system. Therefore, the more the contribution of the active transport on the antibiotic, the stronger is its inhibitory effect on ceftibuten. Multiple transport systems for the uptake of oral cephems (originally for dipeptides) in Caco-2 cells can account for the above phenomena. These findings were similar to those obtained with rat intestinal brush border membrane vesicles.

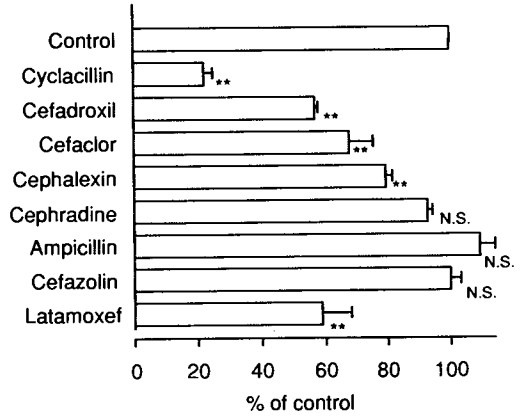

Fig. 8. Effects of various antibiotics on ceftibuten uptake into Caco-2 cells. Antibiotics were added in a concentration of 10 mM to the incubation medium. Data represent the mean ± S.D. (n = 4–5). (significance: N.S.; not significant, **;$p < 0.01$).

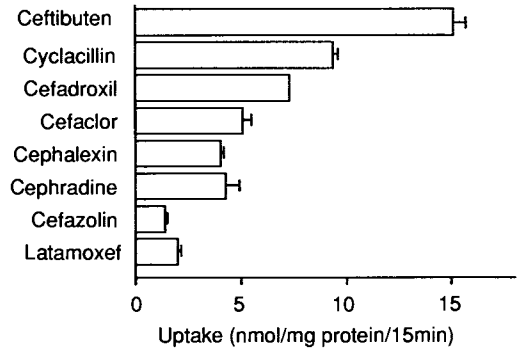

Fig. 9. Uptakes of various antibiotics into Caco-2 cells. Data represent the mean ± S.D. (n = 3–5) of at pH 5.5 for 15 min.

We conclude that in Caco-2 cells, ceftibuten is taken up dominantly via an oligopeptide transporter whose driving force is the transmembrane H^+-gradient.

REFERENCES

1. I. J. Hidalgo, T. J. Raub, and R. T. Borchart. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology, 96:736–749(1989)
2. A. Blais, P. Bissonnette, and A. Berteloot. Common characteristics for Na^+-dependent sugar transport in Caco-2 cells and human fetal colon. J. Membr. Biol. 99:113–125(1987)
3. L. Mahraoui, M. Rousset, E. Dussaulx, D. Darmoul, A. Zweibaum, and E. Brot-Laroche. Expression and localization of GLUT-5 in Caco-2 cells, human small intestine. Am. J. Physiol. 263:G312–G318(1992)
4. I. J. Hidalgo and R. T. Borchart. Transport of a large neutral amino acid(phenylalanine) in a human intestinal epithelial cell line: Caco-2. Biochim. Biophys. Acta 1028:25–30(1990)
5. P. L. Nicklin, W. J. Irwin, I. F. Hassan, and M. Mackay. Proline uptake by monolayers of human intestinal absorptive (Caco-2) cells. Biochim. Biophys. Acta 1104:283–292(1992)
6. D. T. Thwaites, C. D. A. Brown, B. H. Hirst, and N. L. Simmons. H^+-coupled dipeptide(glycylsarcosine) transport across apical and basal borders of human intestinal Caco-2 cell monolayers display distinctive characteristics. Biochim. Biophys. Acta 1151:237–245(1993)
7. I. J. Hidalgo and R. T. Borchart. Transport of bile acids in a human intestinal epithelial cell line, Caco-2. Biochim. Biophys. Acta 1035:97–103(1990)

8. A. H. Dantzig and L. Bergin. Uptake of the cephalosporin, cephalexin, by a dipeptide transport carrier in the human intestinal cell line, Caco-2. Biochim. Biophys. Acta 1027:211–217(1990)
9. A. H. Dantzig, L. B. Tabas, and L. Bergin. Cefaclor uptake by the proton-dependent dipeptide transport carrier of human intestinal Caco-2 cells and comparison to cephalexin. Biochim. Biophys. Acta 1112:167–173(1992)
10. K. Inui, M. Yamamoto, and H. Saito. Transepithelial transport of oral cephalosporins by monolayers of intestinal epithelial cell line Caco-2: Specific transport systems in apical and basolateral membranes. J. Pharmacol. Exp. Ther. 261:195–201(1992)
11. T. Yoshikawa, N. Muranushi, M. Yoshida, T. Oguma, K. Hirano and H. Yamada. Transport characteristics of ceftibuten (7432-S), a new oral cephem, in rat intestinal brush-border membrane vesicles: Proton-coupled and stereoselective transport of ceftibuten. Pharm. Res. 6:302–307(1989)
12. N. Muranushi, T. Yoshikawa, M. Yoshida, T. Oguma, K. Hirano and H. Yamada. Transport characteristics of ceftibuten, a new oral cephem, in rat intestinal brush-border membrane vesicles: Relation to oligopeptide and amino β-lactam transport. Pharm. Res. 6:308–312(1989)
13. M. Sugawara, T. Toda, K. Iseki, K. Miyazaki, H. Shiroto, Y. Kondo and J. Uchino. Transport characteristics of cephalosporin antibiotics across intestinal brush-border membrane in man, rat and rabbit. J. Pharm. Pharmacol. 44:968–972(1992)
14. M. Sugawara, K. Iseki, K. Miyazaki, H. Shiroto, Y. Kondo and J. Uchino. Transport characteristics of ceftibuten, cefixime and cephalexin across human jejunal brush-border membrane. J. Pharm. Pharmacol. 43:882–884 (1991)
15. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Müller and G. Semenza. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Biochim. Biophys. Acta, 506, 136–154(1978)
16. N. Muranushi, T. Yoshikawa, M. Nishiuchi, T. Oguma, K. Hirano and H. Yamada, Characteristics of the intestinal absorption of 7432-S, a new orally active cephem, cephalosporin. J. Pharmacobio-Dyn. 10:s–72(1987)
17. C. M. Metzler "NONLIN, A Users manual for NONLIN and associated programs. The Upjohn Co., Kalamazoo, Mich., 1974
18. M. Abe, T. Hoshi, and A. Tajima, Characteristics of transmural potential changes associated with the proton-peptide cotransport in toad small intestine. J. Physiol. (London) 394:481–499(1987)