Characteristics of Uptake of Cefroxadine by Rabbit Small Intestinal Brush Border Membrane Vesicles

Shuji Kitagawa* and Yoshio Sugaya

Niigata College of Pharmacy, Kamishin’ei-cho 5-13-2, Niigata 950-21, Japan.

Received August 10, 1995; accepted October 20, 1995

Characteristics of transport of an oral aminoccephalosporin, cefroxadine, in rabbit small intestinal brush border membrane vesicles were examined. Uptake rate of cefroxadine was saturable in the presence of an inward H⁺ gradient, and kinetic parameters were similar to those of cephradine. However, the uptake rate was almost linear with the concentration in the absence of an inward H⁺ gradient up to 5 μM. Overshoot phenomenon was observed in the presence of an inward H⁺ gradient at 37°C, but it disappeared with decrease of temperature. The Arrhenius plot of uptake rate constant showed a break point at approximately 30°C. Cefroxadine uptake was optimum in the vicinity of pH 5.5 at 37°C, but the dependence on extravesicular pH disappeared at 15°C. The uptake of cefroxadine in the presence of an inward H⁺ gradient was markedly inhibited by other aminoccephalosporins such as cephalixin, but the inhibition was only slight in the absence of an inward H⁺ gradient. Alkyl alcohols such as n-hexyl alcohol also inhibited H⁺-coupled uptake of cefroxadine at the concentration range at which the alcohols increased the membrane fluidity, and overshoot phenomenon diminished, suggesting that H⁺-coupled transport of cefroxadine is sensitive to the alcohol-induced increase in membrane fluidity. On the other hand, the alcohols rather stimulated its uptake in the absence of an H⁺ gradient.

Key words cefroxadine; small intestine; membrane transport; brush border membrane vesicle; membrane fluidity; alcohol

Oral cephalosporins such as cephradine and cephalixin are effectively absorbed from the small intestine, although they are present as ions at a physiological pH and have very low lipid solubility. Studies with brush border membrane vesicles of the rabbit small intestine have revealed that aminoccephalosporin antibiotics such as cephadine are actively absorbed via an H⁺-coupled dipeptide transport system. However, it is controversial whether the transport mechanism of aminoccephalosporins is common in other mammalian species or not. It is also not clear whether absorption via the H⁺-coupled dipeptide transport system by itself can explain the transport characteristics of aminoccephalosporins. The existence of a common carrier transport system for aminoccephalosporins and dianionic cephalosporins such as cefixime is also controversial.

Herein we examined the characteristics of uptake of an oral aminoccephalosporin, cefroxadine, the transport behavior in vitro of which has not been reported in detail. The chemical structure of cefroxadine shown in Fig. 1 resembles that of cephadine: 3-OCH₃ group in cefroxadine is replaced with CH₃ group in cephadine. We used the small intestinal brush border membrane vesicle, a good system with which we can examine the transport characteristics and mechanisms of hydrophilic drugs, the adsorption to the membrane surface of which can be easily distinguished from the uptake into the inside of the vesicles. These vesicles have been used to examine the transport of β-lactam and cephalosporin antibiotics in the small intestine. We compared our findings obtained in the rabbit with those obtained with other aminoccephalosporins such as cephadine and cephalixin, which have been revealed to be taken up via an H⁺-coupled dipeptide transport system in the rabbit small intestine. We studied the uptake of cefroxadine in the presence and absence of an inward H⁺ gradient at different concentrations and at different temperatures and the effect of alcohols on its uptake to clarify the characteristics of the transport system, because alcohols as well as temperature differ in their effects on carrier transport and simple diffusion.

MATERIALS AND METHODS

Reagents Cefroxadine (Ciba Geigy Japan Co., Tokyo, Japan) was generously donated. Carbonyl cyanide p-trifluoromethoxyphenyl hydradione (FCCP) and diphenyl-hexatriene (DPH) were obtained from Sigma (St. Louis, MO). All other reagents were purchased from Sigma or Wako Pure Chemical Industries (Osaka, Japan).

Preparation of Brush Border Membrane Vesicles Brush border membrane vesicles were isolated from the rabbit small intestine by the CaCl₂ precipitation method of Kessler et al. The membrane vesicles obtained were suspended in experimental buffer (100 mM mannitol, 100 mM KCl and 10 mM HEPES–KOH (pH 7.5)).

Measurement of Uptake of Cefroxadine The uptake of cefroxadine was measured by a rapid filtration technique following Okano et al. Membrane vesicles (10 μl) were incubated at a certain temperature with nine-fold volume of the substrate mixture comprising 100 mM mannitol, 100 mM KCl buffered with either MES–KOH (pH 4.5–6.0) or HEPES–KOH (pH 6.5–7.5). From aliquots of the samples drawn cefroxadine trapped on a nitrocellulose membrane filter (0.45 μm, 2.5 cm diameter) was extracted with 300 μl of distilled water and was used for the determination by high performance liquid chro-

Fig. 1. Chemical Structure of Cefroxadine

© 1996 Pharmaceutical Society of Japan
matography (HPLC). Deviation of data from preparation to preparation of the vesicles was within 25%.

**Analytical Method** The concentration of cefroxadine was determined by HPLC (L-6000, Hitachi Ltd., Tokyo, Japan) equipped with an L-4000 UV detector (Hitachi, Ltd.) at 269.5 nm. Separation was achieved on a reversed phase column (ODS, Shodex C18-5A, 4.6 mm i.d. 150 mm) using a mobile phase consisting of acetonitrile and 30 mM phosphate buffer, pH 7.0 (1 : 7, v/v) at a flow rate of 1.0 ml/min. Protein was measured by the method of Lowry et al.\textsuperscript{15} with bovine serum albumin as the standard.

**Measurement of Fluorescence Anisotropy** The fluorescence anisotropy of DPH was measured as described previously.\textsuperscript{16} A suspension of the membrane vesicles described above was mixed with the experimental buffer in which various concentrations of alcohols were dissolved by vigorous vortex mixing at 37 °C. The suspension was then incubated at a final concentration of 0.5 μM DPH at 37 °C for 4 min before measurement. The fluorescence intensity was measured at 37 °C in an F-4010 spectrofluorometer (Hitachi, Ltd.) equipped with an excitation and emission polarizer and the steady-state fluorescence anisotropy was calculated. The excitation and emission wavelengths used for DPH were 363 and 428 nm, respectively.

**RESULTS**

**Concentration-Dependence of Uptake of Cefroxadine** We examined the uptake of cefroxadine by brush border membrane vesicles, at intravesicular pH 7.5, in incubation medium adjusted to pH 5.0 or 7.5. As shown in Fig. 2a, the initial uptake rate was saturable at pH 5.0, at which an inward H\(^{+}\) gradient was present. On the other hand, as shown in Fig. 2b, it was almost linear with the concentration up to 5 mM at pH 7.5, at which the H\(^{+}\) gradient was absent. The concentration dependence of cefroxadine uptake was analyzed by assuming that the transport process consists of a carrier mediated process and simple diffusion using nonlinear least-squares regression analysis.\textsuperscript{17} Kinetic parameters were calculated from the following equation:

\[ V = \frac{V_{\text{max}}[S]}{K_m + [S]} + K_d[S] \] (1)

where \( V \) is the initial uptake rate, \([S]\) is the initial concentration, \( V_{\text{max}} \) is the maximum uptake rate by carrier-mediated process, \( K_m \) is the Michaelis constant, and \( K_d \) is the coefficient of simple diffusion.\textsuperscript{2} At pH 5.0, the values of apparent \( K_m \) and \( V_{\text{max}} \) for carrier-mediated transport were 1.71 ± 0.44 mM and 2.66 ± 0.63 nmol/mg protein per 15s, respectively. The Lineweaver-Burk plot after correction for the nonsaturable component showed a linear relationship (Fig. 2a, inset). The \( K_m \) and \( V_{\text{max}} \) values revealed here were only slightly smaller than the values of cephradine in rabbit intestinal brush border membrane vesicles at the same pH reported by Inui et al.\textsuperscript{3} Since the uptake at pH 7.5 was almost linear at the concentration tested, parameter values with statistical significance could not be obtained by this analysis. The present findings suggest that in the presence of an inward H\(^{+}\) gradient, cefroxadine is taken up by a carrier mediated process in addition to simple diffusion, and that the uptake of cefroxadine in the absence of an H\(^{+}\) gradient occurs mainly by simple diffusion.

**Temperature-Dependence of Uptake of Cefroxadine in the Presence of H\(^{+}\)-Gradient** We examined the temperature dependence of uptake of cefroxadine at the concentr-
The plot was characterized by two slopes and by a break point at approximately 30°C. Activation energy below the break point was 98 kJ/mol and that above the break point was 67 kJ/mol. The break point temperature resembled that reported for α-glucose transport in rat intestinal brush border membrane vesicles, but the values of activation both above and below the break point shown here were much larger than those for α-glucose transport. The break point temperature was slightly higher than that for cefixime uptake in rat intestinal brush border membrane vesicles (22°C), whose activation energy below the break point was similar to that revealed here for cefoxadine but above the break point was lower.

**pH-Dependence of Uptake of Cefoxadine** There exists an optimum extravesicular pH for the uptake of aminoccephalosporins: that is, uptake rates of cefoxadine at 37°C in rabbit small intestinal brush border membrane vesicles have been reported to be fastest at approximately pH 6.0, 7.0. Therefore, we examined the dependence of uptake rate of cefoxadine on the extravesicular pH at 37°C. Since the overshoot phenomenon in the presence of an inward H⁺ gradient depended on temperature, we also examined it at 15°C. The concentration of cefoxadine was 0.5 mM at an extravesicular pH of 5.0. As reported for other aminoccephalosporins like cephradine, the transport was stimulated by an inward H⁺ gradient, and a prominent overshoot phenomenon was observed at 37°C (Fig. 3). The uptake of cefoxadine depending on temperature and overshooting was hardly observable at 25°C. At 15°C, at which the overshoot phenomenon completely disappeared, permeation of cefoxadine occurred very slowly and it took more than 60 min to reach the equilibrium concentration. The overshoot phenomenon and acceleration of uptake of cefoxadine by an inward H⁺ gradient was also inhibited by addition of a protonophore, FCCP (Fig. 3) as reported for other aminoccephalosporins such as cephradine.

Figure 4 shows the Arrhenius plot of an apparent cefoxadine uptake rate constant kₚ by assuming the pseudo first order transport equation shown in Eq. 2 at a relatively low concentration of solute in Eq. 1.

\[ V = k_c[S] \]  
\[ k_p = \frac{V_{\text{max}}}{K_m} + K_d \]

The uptake for 15 s was determined. Each value represents the mean ± S.D. of three determinations.
Cefradine was 1 mm and intravesicular pH was fixed at 7.5. At 37°C the optimum extravesicular pH for cefradine uptake was about 5.5 as shown in Fig. 5, which was similar to that for cephradine. We also confirmed that the optimum pH of uptake of cefaclor and cefadroxil was also about 5.5 at 37°C. However, the pH-dependence almost disappeared at 15°C.

**Effects of Other Aminoccephalosporins on Cefradine Uptake**

The transport of aminoccephalosporins in rabbit small intestinal brush border membrane vesicles in the presence of an inward H⁺ gradient is inhibited by substrates of H⁺/dipeptide transporter including other aminoccephalosporins. The effects of 20 mm cephalixin shown in Fig. 6a and the effects of other aminoccephalosporins and glycylglycine shown in Table 1 indicated that the dipeptide and other aminoccephalosporins significantly inhibited the uptake of cefradine at extravesicular pH 5.0, at which an inward H⁺ gradient was absent, as shown for the effect of cephalixin in Fig. 6b. These findings are also consistent with the speculation that uptake of cefradine was conducted primarily by the H⁺/dipeptide transporter in the presence of an inward H⁺ gradient, but mainly by simple diffusion in its absence.

**Effects of Alcohols on H⁺-Coupled Transport of Cefradine**

The transport of solutes is often reported to be influenced by the fluidity of the membrane lipid bilayer. Alcohols increase the fluidity of this bilayer and modify solute transport mediated by membrane transport proteins. The modification has been suggested to be related to alcohol-induced perturbation of the membrane lipid bilayer. Although alcohols also modify simple diffusion of solutes through a membrane, their effects are quite different from those on carrier transport. Since there have been few reports on the effects of alcohols on transport of amino β-lactam antibiotics, we examined such effects on the transport of cefradine and their relation with the change in membrane fluidity.

As shown in Fig. 7 for the effects of n-hexyl alcohol, n-alkyl alcohols inhibited the overshoot uptake of the antibiotic in the presence of an inward H⁺ gradient. As shown in Fig. 8 for the effects of n-hexyl alcohol, at extravesicular pH 5.0, at which an inward H⁺ gradient
was present, uptake of cefroxadine was inhibited at the concentration at which it increased fluidity of lipid bilayer of the membrane as monitored by the decrease of fluorescence anisotropy of DPH. Changes in the volume and integrity of the vesicles were not induced by the alcohols at the concentrations tested. At extravascular pH 7.5 at which the H⁺ gradient was absent, the uptake was, on the contrary, stimulated by the same concentration of alcohols as shown for n-hexyl alcohol. Similar inhibitory effects on cefroxadine uptake in the presence of an inward H⁺ gradient and stimulatory effects on the uptake in its absence were also observed for other alkyl alcohols such as n-butyl and n-amyl alcohols (data not shown). Enhancement of uptake in the absence of an inward H⁺ gradient was consistent with the effects of chlorpromazine on the permeability of amino β-lactam antibiotics like cephradine in rat intestinal brush border membrane reported by Iseki et al., who related the inhibition to increase in membrane surface fluidity induced by chlorpromazine, which differs in its effect on membrane fluidity in different depths of membrane. The present findings suggest that H⁺-coupled transport of aminopenicillins such as cefroxadine is selectively inhibited by the alcohol-induced increase in fluidity of the membrane lipid bilayer. The findings also suggest that the major mechanism of uptake of cefroxadine in the absence of an H⁺ gradient is different from that in the presence of an H⁺ gradient: possibly simple diffusion.

**DISCUSSION**

Transport features of cefroxadine in rabbit small intestinal brush border membrane resembled those of other aminopenicillins, cephradine and cephalaxin in rabbit vesicles: the overshoot phenomenon in the presence of an inward H⁺ gradient, its inhibition by FCCP and optimum pH all resembled those of cephradine and cephalaxin. The saturation phenomenon was also observed in the presence of an H⁺ gradient. The K_m value (1.71 ± 0.44 mm) resembles that of cephradine (2.1 mm). The V_max value was about three fourths of that of cephradine. Similarity of these values is probably due only to the difference of 3-OCH₃ group in cefroxadine which is replaced with CH₃ in cephradine. Significant inhibition of cefroxadine uptake in the presence of an inward H⁺ gradient by other aminopenicillins is also consistent with those of other aminopenicillins such as cephradine. Therefore, its transport pathway seems to be common with other aminopenicillins and uptake of cefroxadine in the presence of an inward H⁺ gradient seems to be done mainly by a H⁺/dipeptide transporter at least in rabbit. However, simple diffusion seems to have a major role for the uptake in the absence of an H⁺ gradient.

Temperature dependent pH-dependence of cefroxadine transport and relatively high activation energy for the uptake of cefroxadine indicate that H⁺-coupled transport of this substance is highly temperature-dependent, which is often found in carrier transport. The loss of pH-dependence of the permeation rate of this aminopenicillcin at a low temperature is just like the result on the modification of histidyl residues of membrane proteins which led to a complete loss of pH dependence of transport of cephalaxin and cefxin. Likewise, pH-dependence of the uptake of cephradine and bestatin, which seem to be taken up by the H⁺/dipeptide transport system in the rabbit small intestine, markedly diminishes in the presence of other transport substrates. Therefore, pH dependence of the uptake of aminopenicillins seems to be mainly due to pH dependence of the uptake via H⁺/dipeptide transport system. Uptake of the aminopenicillins in the absence of H⁺ gradient also depends on pH and is slightly higher at a neutral pH than at an acidic pH as revealed for cephradine and cefroxadine (data not shown). This may be due to the difference in dissociation of cefroxadine at pH 5.0 and 7.5, because pK_a values of cefroxadine are 3.2 and 7.5.

Alcohols have been found to increase membrane fluidity and inhibit various membrane protein-mediated transport processes. For example, alcohols inhibit glucose transport both in erythrocytes and in intestinal brush border membrane vesicles. They also inhibit transport of anions and choline in erythrocytes. However, alcohols enhance drug penetration by simple diffusion via lipid bilayer and have been applied as drug penetration enhancers. In relation to their enhancing effects, Orme et al. reported that n-alkyl alcohols stimulate the transmembrane fluxes of a hydrophobic cation and anion in the erythrocyte membrane. Kutchai et al. also revealed that the alcohols stimulate membrane permeation of erythritol in human erythrocytes.

As shown in this study, alcohols inhibited H⁺-coupled uptake of cefroxadine into brush border membrane vesicles. Furthermore, alcohol addition to brush border membrane vesicles almost caused elimination of the overshooting of the aminopenicillcin at the concentra-
tion range at which alcohols increased the fluidity of the lipid bilayer of the brush border membrane. The present findings suggest that the H⁺-coupled aminoccephalosporin transport system is also sensitive to alcohol-induced increase in membrane fluidity as in other carrier protein-mediated transport systems. Alcohols affect the fluidity of a membrane lipid bilayer differentially depending on the depth.  

This may be one reason why they differ in their effect on cefuroxime uptake from temperature rise which also induces an increase in membrane fluidity. However, alcohols such as n-hexyl alcohol may affect H⁺-coupled uptake of cefuroxime by another mechanism such as direct perturbation of the structure of membrane protein or removal of lipids from the lipid-protein interface. However, the possibility also exists that alcohol-induced enhancement of depletion of H⁺ gradient inhibits H⁺-coupled transport of cefuroxime, because H⁺ transport is reportedly increased by the alcohols. 

In the absence of H⁺ gradient, uptake of cefuroxime was, in contrast, stimulated by alcohols. These findings are also in agreement with the assumption that uptake in the absence of H⁺ gradient is mainly due to simple diffusion. Contribution of simple diffusion to the uptake of aminoccephalosporins cannot be neglected, although these drugs are completely ionized and therefore have very small partition coefficients. The transport mechanism of this simple diffusion should also be examined in the future.

Acknowledgments This work was supported by grants from the Japanese Ministry of Education, Science and Culture (06304044), the Takeda Science Foundation, and the Research Foundation for Pharmaceutical Sciences.

REFERENCES