EVIDENCE FOR THE EXISTENCE OF A COMMON TRANSPORT SYSTEM OF β-LACTAM ANTIBIOTICS IN ISOLATED RAT HEPATOCYTES

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The inhibition effect of several β-lactam antibiotics on the uptake of [^{14}C]benzylpenicillin (PCG) into isolated rat hepatocytes was studied.

Monobasic β-lactam antibiotics such as apalcillin, cloxacillin, nafcillin, piperacillin, cefmetazole, cefoperazone, cefpiramide and cephalothin significantly inhibited the uptake of PCG, while amphoteric β-lactam antibiotics such as amoxicillin, ciclacillin, cephradine, cephalexin and cephaloridine had a slight inhibitory effect on the uptake of PCG.

Five monobasic compounds of these antibiotics used (apalcillin, nafcillin, piperacillin, cefmetazole and cefoperazone) which have a tendency to be excreted into bile to a large extent, inhibited the initial uptake rate of PCG in a fully competitive fashion according to the Lineweaver-Burk plots and the corresponding modified Inui-Christensen plots.

Thus, it was concluded that almost all β-lactam antibiotics have a common carrier system responsible for their uptake into isolated rat hepatocytes, but it is still uncertain whether or not amphoteric β-lactam antibiotics have another specific transport system.

In our previous paper, it was reported that benzylpenicillin (PCG), cefazolin and cefpiramide are taken up by isolated rat hepatocytes, following the combination kinetics of an unsaturable first-order rate process and a saturable carrier-mediated process.[1,2] Though the saturable process is not influenced by amino acids and peptides, PCG has a common carrier system with probenecid and exhibits some interaction with the transport system of taurocholic acid.[1,3]

To date, many kinds of derivatives of β-lactam antibiotics have been developed. Their pharmacokinetic characteristics differ widely from each other, especially, those of several derivatives which are exclusively eliminated from the liver into bile. However, it is uncertain whether or not these biliary excretion-type derivatives are transported by a common carrier system with PCG.

On the other hand, organic compounds are generally said to be excreted into bile *via* transport routes depending on their molecular electric charge.[4] Since there are two types of β-lactam antibiotics, one of which has only an anionic charge and the other both anionic and cationic charges, it is important to clarify whether all kinds of β-lactam antibiotics are taken up by isolated rat hepatocytes *via* the carrier-mediated system common to PCG.

The purpose of the present study was to confirm the presence of a transport system common to PCG and also to such derivatives as monobasic-type including biliary excretion-type and amphoteric-type β-lactam antibiotics.

Materials and Methods

Materials

[^{14}C]Benzylpenicillin (54 mCi/mmol) and [^{14}C]inulin (5 mCi/mmol) were purchased from Amersham International Ltd., Amersham, U.K. Collagenase (Clostridiopeptidase A) was obtained from

Boehringer-Mannheim Gmbh, Mannheim, F.R.G. β-Lactam antibiotics used in this work were kindly supplied as follows: Amoxicillin (AMPC, 852 μg/mg) from Fujisawa Pharmaceutical Co., Ltd., Osaka; ampicillin anhydrate (ABPC, 1,004 μg/mg) and ciclacillin (ACPC, 1,011 μg/mg) from Takeda Chemical Industries, Ltd., Osaka; apalcillin (APPC, 860 μg/mg) from Sumitomo Chemical Co., Ltd., Osaka; benzylpenicillin potassium (PCG, 1,595 U/mg) and cloxacillin sodium (MCIPC, 904 μg/mg) from Meiji Seika Kaisha, Ltd., Tokyo; cefmetazole sodium (CMZ, 960 μg/mg), piperacillin (PIPC, 921 μg/mg) and cephradine (CED, 970 μg/mg) from Sankyo Co., Ltd., Tokyo; cefoperazone sodium (CPZ, 1,073 μg/mg) from Toyama Chemical Co., Ltd., Tokyo; cefpiramide sodium (CPM, 899 μg/mg) from Sumitomo Chemical Co., Ltd., Osaka and Yamanouchi Pharmaceutical Co., Ltd., Tokyo; cephaloridine (CER, 970 μg/mg), cephalexin (CEX, 940 μg/mg) and cephalothin (CET, 959 μg/mg) from Shionogi & Co., Ltd., Osaka; nafcillin monohydrate sodium (NFPC, 911 μg/mg) from Wyeth Japan Co., Tokyo. All other chemicals were of reagent grade and were used without further purification.

Animals

Male Wistar rats (Sankyo Laboratory Co., Toyama, Japan) weighing 280~320 g, which had free access to food and water, were used.

Uptake Experiments

Liver cells were isolated according to the procedure of MOLDEUS *et al.*[5]. The detailed procedures concerning the cell isolation and viability test for each cell preparation have been described previously.[2] Uptake experiments employed were also essentially the same as described previously.[2] The washed cells were incubated in modified Krebs-Henseleit buffer solution (pH 7.4) containing 2% bovine serum albumin (Fraction V, Sigma Chemicals Co., St. Louis, U.S.A.). After preincubation of hepatocytes for 5 minutes at 37°C, substrates including [^{14}C]PCG were added and uptake was started. The initial uptake rate was determined from the slope constructed from the measuring points at 15, 30, 45 and 60 seconds after addition of [^{14}C]PCG. The transport reaction was stopped by a centrifugal filtration technique through a layer of silicone oil.[6] The amount of PCG in the extracellular fluid of cells was corrected from the separate experiments by incubating the cells with [^{14}C]inulin. All studies were performed within 4 hours after cell isolation. Cellular protein was measured by the method of LOWRY *et al.*[7] using bovine serum albumin as a standard.

Data Analysis

The kinetic parameters in Table 1 were obtained by solving the following simultaneous equations by a nonlinear least-squares method, using the NONLIN computer program.[8]

$$v_{c,app}=V_{max}C/(K_t+C)+K_dC \quad (1)$$

$$v_{i,app}=V_{max}C/[K_t(1+I/K_i)+C]+K_dC \quad (2)$$

where, $v_{c,app}$ and $v_{i,app}$ represent the apparent uptake rate of PCG in the absence and the presence of inhibitor, respectively; C and I represent the respective concentrations of PCG and inhibitor; K_t and K_i represent respectively the Michaelis constant of PCG uptake and the inhibition constant of the inhibitor; V_{max} is the maximum uptake rate of PCG in the saturable uptake component; K_d is the apparent first-order rate constant.

Results

Effect of Monobasic β-Lactam Antibiotics on PCG Uptake into Isolated Rat Hepatocytes

The kinetics of inhibition by several monobasic β-lactam antibiotics on the uptake of PCG into isolated rat hepatocytes was examined.

Figs. 1 and 2 show the Lineweaver-Burk plots of PCG uptake in the absence and the presence of NFPC or CPZ at the respective concentrations of 1.5 and 4 mM. At both inhibitor concentrations, the maximum uptake rate (V_{max}) of PCG was not affected, but the Michaelis constant (K_t) was affected by the inhibitors. This result indicates that NFPC and CPZ competitively inhibit the PCG

Fig. 1. Lineweaver-Burk plot of PCG uptake rate showing inhibition by NFPC.

NFPC was simultaneously added at the initiation of PCG uptake. The concentration of NFPC was 0 mM (●), 1.5 mM (▲) and 4 mM (■). Solid lines were obtained by linear regression analysis of the plots.

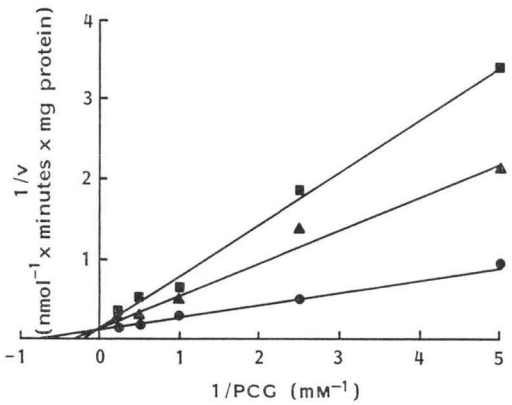


Fig. 2. Lineweaver-Burk plot of PCG uptake rate showing inhibition by CPZ.

CPZ was simultaneously added at the initiation of PCG uptake. The concentration of CPZ was 0 mM (●), 1.5 mM (▲) and 4 mM (■). Solid lines were obtained by linear regression analysis of the plots.

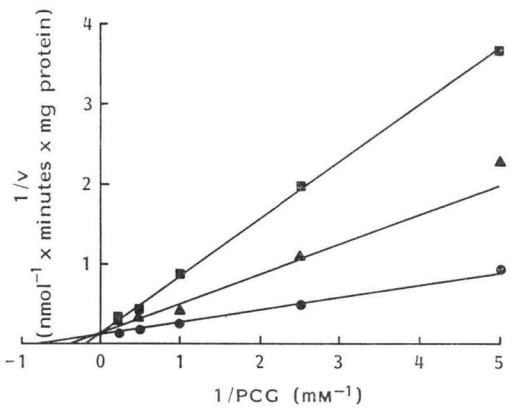


Fig. 3. Lineweaver-Burk plot of PCG uptake rate showing inhibition of APPC (○), PIPC (▲) and CMZ (■).

Each β-lactam antibiotic was simultaneously added at the initiation of PCG uptake. The concentration of β-lactam antibiotics was 0 mM (●) and 5 mM (○, ▲, ■). Solid lines were obtained by linear regression analysis of the plots.

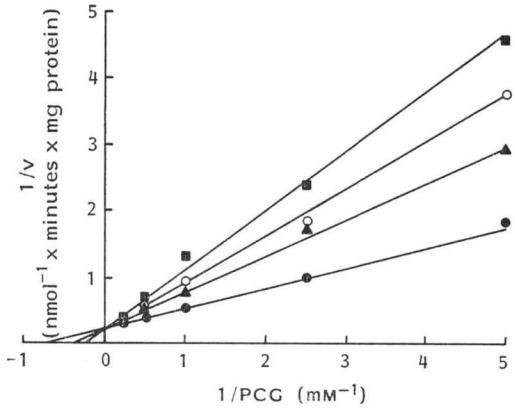


Table 1. Michaelis constant of PCG uptake and the inhibition constants of five β-lactam antibiotics on the uptake of PCG.

β-Lactam antibiotics	Michaelis or inhibition constants[a] (mM)
PCG	1.24±0.42[b]
NFPC	1.12±0.10[c]
CPZ	1.31±0.24[c]
CMZ	2.45±0.22[c]
APPC	3.86±0.23[c]
PIPC	6.63±1.25[c]

[a] The results represent the mean value ± S.D. calculated by nonlinear regression analysis according to the equation described in Materials and Methods.

[b] The value represents the Michaelis constant, K_t.

[c] The value represents the inhibition constant, K_i.

uptake. Fig. 3 shows the Lineweaver-Burk plots of PCG uptake in the absence and the presence of APPC, PIPC and CMZ at a concentration of 5 mM. These three β-lactam antibiotics also inhibit PCG uptake competitively in a similar manner to NFPC and CPZ. The inhibition constants (K_i) for these five β-lactam antibiotics and the Michaelis constant for PCG uptake (K_t) are summarized in Table 1. The reason why the K_t value of PCG uptake differ from that reported in the previous paper (0.473± 0.158 mM)[1] is probably due to inter-experimental variation.

In order to evaluate the above inhibition effects of these β-lactam antibiotics on PCG uptake, their kinetics were represented by modified Inui-Christensen plots[9] of $1/(1-v_i/v_c)$ vs. the reciprocal of the inhibitor concentration, where v_i and v_c represent the uptake rates of the saturable component

Fig. 4. Modified Inui-Christensen plot of PCG uptake rate showing inhibition of NFPC.

NFPC was simultaneously added at the initiation of PCG uptake. The concentration of PCG was 0.2 mM (●), 1 mM (▲) and 2 mM (■). Solid lines were obtained by linear regression analysis of the plots.

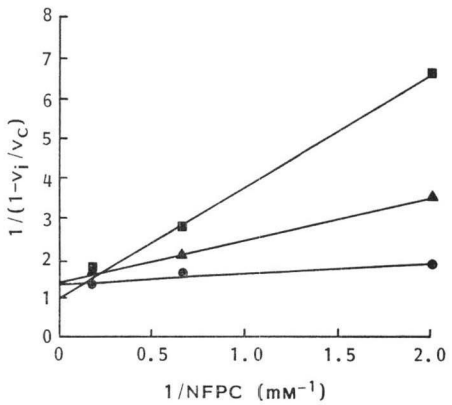
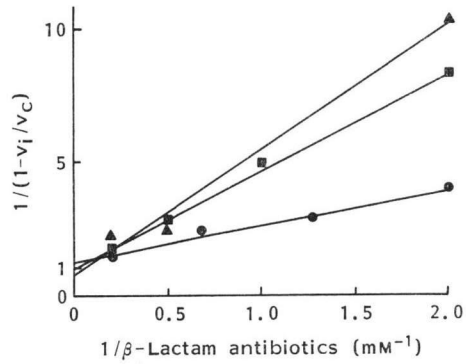


Fig. 5. Modified Inui-Christensen plot of PCG uptake rate showing inhibition by APPC (■), CMZ (▲) and CPZ (●).

Each β-lactam antibiotic was simultaneously added at the initiation of PCG uptake. The concentration of PCG was 1 mM. Solid lines were obtained by linear regression analysis of the plots.



of PCG uptake which was calculated by subtracting the first-order uptake rate from the apparent uptake rate in the presence and the absence of inhibitor, respectively. Fig. 4 shows such modified Inui-Christensen plots of PCG uptake at PCG concentrations of 0.2, 1.0 and 2.0 mM in the presence of NFPC. When extrapolated to infinite concentration of NFPC, the mean values on the y-axis obtained from each line were 1.23 ± 0.16 (with S.E.M.), giving no significant difference from unity ($P<0.05$). Fig. 5 shows the modified Inui-Christensen plots of PCG uptake at a concentration of 1 mM in the presence of APPC, CMZ and CPZ. The extrapolated values on the y-axis were 1.38, 0.95 and 0.75, respectively. These values are also close to unity. In the case of PIPC, the extent of inhibitory effect was so low that analysis by this method was not possible.

Effect of Amphoteric β-Lactam Antibiotics on PCG Uptake into Isolated Rat Hepatocytes

In Fig. 6, the inhibition effects of several amphoteric β-lactam antibiotics which have both an electrically positive and negative charge at physiological pH, are represented as percentage of control PCG uptake rate at a concentration of 200 μM. ACPC, AMPC, CED, CER and CEX, but not ABPC, did not show significant inhibition at a concentration of 1 mM, while they inhibited significantly at a higher concentration of 5 mM to 57.2 ± 1.6, 72.6 ± 2.8, 81.2 ± 3.6 and 75.3 ± 5.2 percent of the control, respectively. On the other hand, ABPC significantly inhibited uptake at a concentration of 1 mM to 63.9 ± 6.7 percent of the control. For the sake of comparison, similar inhibition effects were examined using monobasic β-lactam antibiotics such as PCG as inhibitors. The results are shown in Fig. 6. In the presence of 1 mM MCIPC, CET and CPM, the initial uptake rate of PCG was inhibited significantly to 60.6 ± 7.0, 48.4 ± 8.0 and 31.0 ± 4.6 percent of the control, respectively.

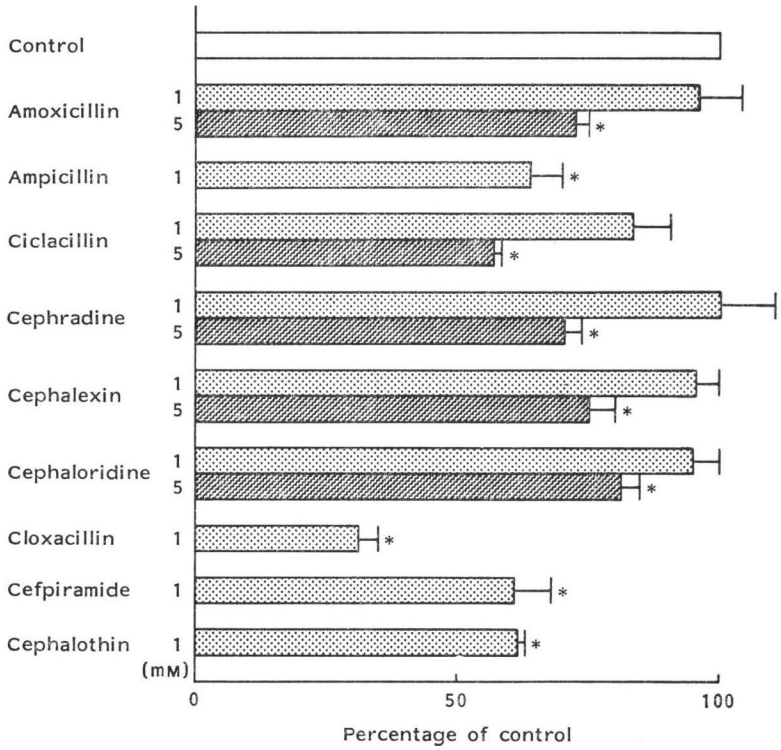
Discussion

Studies on the mechanism of β-lactam antibiotic uptake into isolated hepatocytes has centered on the classification of these antibiotics into urinary excretion-type and biliary excretion-type based on

Fig. 6. Effect of β-lactam antibiotics on PCG uptake.

Each bar represents the mean and S.E.M. of three independent experiments. The concentration of PCG was 200 μM.

* Level of significance was set at $P < 0.05$.



the respective characteristics of their elimination pathways. From a previous study carried out at this laboratory, the transport system of PCG into isolated rat hepatocytes has been proved to be composed of carrier-mediated and first-order processes.[1,2] The carrier system of PCG has some interaction with those for taurocholic acid and probenecid but no interaction with those for amino acids and peptides.[1,3] However, it has not yet been determined whether this carrier system is common to all kinds of β-lactam antibiotics. Accordingly, the present investigation was carried out to examine the inhibitory effect of various monobasic β-lactam antibiotics, including biliary excretion-types and amphoteric types, on the uptake of PCG into isolated rat hepatocytes.

The competitive inhibitory effects of five monobasic β-lactam antibiotics, APPC, NFPC, PIPC, CMZ and CPZ, on PCG uptake suggest that there is a common affinity site between the carrier system of these derivatives and that of PCG (Figs. 1~3). The results demonstrating a y-axis value of almost unity in their modified Inui-Christensen plots (Figs. 4 and 5) indicate that at an infinite concentration of these inhibitors PCG uptake is fully inhibited. Consequently, it is supposed that the carrier systems responsible for PCG and the above five β-lactam antibiotic derivatives are entirely identical, or that the carrier system of PCG at least partially shares the carrier systems of these derivatives. Since these five β-lactam antibiotics used as inhibitors have a tendency to be excreted extensively into bile as compared with PCG,[10~14] a specific transport system for these biliary excretion-type derivatives may exist.

On the other hand, amphoteric β-lactam antibiotics which have an amino group (ACPC, AMPC, CED and CEX) or have a pyridinium cation (CER) in addition to a carboxylic acid group, had a tendency to require a higher concentration to show significant inhibition on PCG uptake than that for monobasic β-lactam antibiotics. This result suggests that they have lower affinity for the carrier system of PCG uptake than monobasic derivatives, probably due to the electrostatic difference between

them. However, it is noteworthy that only ABPC had a significant inhibitory effect at a concentration of 1 mM. It is anticipated that the carrier system of anionic compounds is different from that of cationic compounds in the liver.[4] In the kidney, transport systems differentiated by the electric charge of respective β-lactam antibiotics have also been proved.[15] Accordingly, there is a possibility that amphoteric β-lactam antibiotics share in part the transport system sensitive to monobasic-type derivatives and also have another specific, high-affinity transport system.

In the case of fully competitive inhibition, the K_i values obtained from the kinetic analysis (Table 1) can be regarded as the Michaelis constant (K_t) of uptake into hepatocytes, which means the affinity for the carrier system. Although, the K_t value of PCG is the same as those of NFPC and CPZ and smaller than those of APPC, PIPC and CMZ, as shown in Table 1, the extent of biliary excretion of PCG is the lowest among those for the other monobasic derivatives[16] examined in this study. Accordingly, there seems to be no correlation between the extent of biliary excretion and the affinity for the carrier system of uptake into hepatocytes. Considering these findings and a report that conjugation with glutathione and/or interaction with a hepatic ligandin plays an important role in the hepatic excretion of an organic anion[17], it may be said that the rate-limiting step in the biliary excretion of β-lactam antibiotics cannot exist in the step of uptake from blood to hepatocytes but in other steps of intracellular interaction with protein and/or bile canalicular membrane transport.

In conclusion, there exists a carrier-mediated transport system common to all kinds of β-lactam antibiotics for the first process of elimination from the liver. However, it is still uncertain whether or not amphoteric β-lactam antibiotics have another specific transport system. In order to elucidate the mechanism of biliary excretion of these antibiotics, it is necessary to investigate their intracellular metabolism, intracellular binding and their bile canalicular membrane transport. Further experiments are now in progress along these lines.

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