EFFECT OF THE HYDROXYL GROUP OF THE *p*-HYDROXYPHENYL MOIETY OF ASPOXICILLIN, A SEMISYNTHETIC PENICILLIN, ON ITS PHARMACOKINETIC PROPERTY

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The serum concentrations, urinary and biliary excretions of six penicillin derivatives including aspoxicillin (ASPC) were studied in rats and the correlation between the values of pharmacokinetic parameters thus obtained and the Rm values measured by means of reversed phase TLC were analyzed.

Among the penicillins studied, the hydrophilicity of amoxicillin was the highest (the lowest Rm value), which was followed by those of ASPC, ampicillin, *p*-hydroxypiperacillin, dehydroxyaspoxicillin and piperacillin in descending order. These Rm values were then correlated with the AUC values at 20 mg/kg of dosing, giving the results that more hydrophilic penicillins having a hydroxyl group show higher serum concentrations as well as greater AUC values. The studies of correlation between the Rm values and the urinary or biliary excretion revealed that hydrophilic penicillins were almost excreted into urine, but more hydrophobic ones were mainly eliminated into bile.

From the above results, a hydroxyl group introduced to the phenyl group of ASPC was considered to have a role that increases the hydrophilicity of ASPC, giving higher and longer persistency of the serum levels and increasing the excretion of drugs into urine.

In recent years, ampicillin (ABPC) derivatives such as piperacillin (PIPC) and mezlocillin have been widely used in clinic as parenteral penicillins with broad antibacterial spectrum.

Aspoxicillin (ASPC), a newly developed semisynthetic penicillin is a derivative of amoxicillin (AMPC) in which the amino group was substituted by N^4-methyl-D-asparagine moiety.[1] In order to analyze the correlation between chemical structure and some pharmacokinetic properties in rats, the Rm values and the pharmacokinetic properties of structurally related penicillin derivatives with ASPC were examined in this study. In addition to four reference penicillins, dehydroxyaspoxicillin (AB-ASPC)[1] and *p*-hydroxypiperacillin (AM-PIPC) in which the amino group of ABPC and the phenyl group of PIPC were substituted with N^4-methyl-D-asparagine and a hydroxyl group, respectively, were newly synthesized in our Organic Chemistry Research Laboratory.

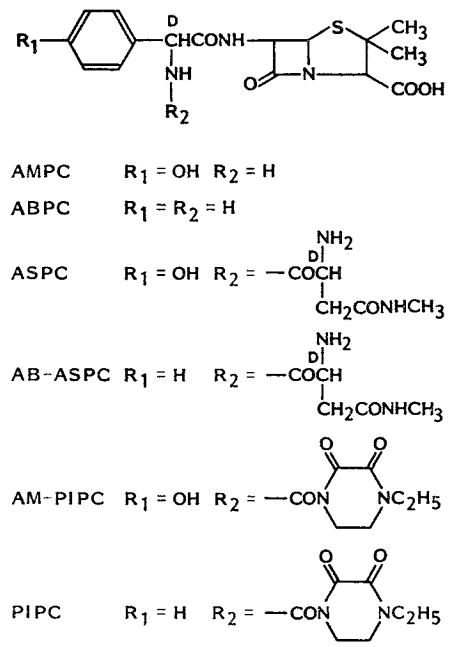
The role of a hydroxyl group introduced to the phenyl group of ASPC was analyzed with special emphasis in this study.

Materials and Methods

Penicillins

The structures of these penicillins were shown in Fig. 1. Penicillins used in this study were as follows: ASPC (Tanabe Seiyaku Co., Ltd., Osaka, Japan), ABPC (Meiji Seika Kaisha, Ltd., Tokyo, Japan), PIPC (Toyama Chemical Co., Ltd., Tokyo, Japan) and AMPC (Antibioticos S.A., León, Spain). AB-ASPC and AM-PIPC were synthesized in our Organic Chemistry Research Laboratory.

Fig. 1. Structures of ASPC and other penicillins.



Measurement of Antibacterial Activity

Standard strains stocked in our laboratory were used in determining MICs by serial 2-fold dilutions of each penicillin in Sensitivity Disk Agar-N (Nissui, Tokyo, Japan). After incubation for about 18 hours at 37°C with an inoculum size of 10^6 cfu/ml, the lowest concentration causing complete inhibition of visible growth was considered to be the MIC.

Experimental Animal

The male SLC-SD rats, weighing from 220 to 300 g (7 to 8 weeks old), were used for these experiments.

Drug Administration

Each penicillin was dissolved in a sterile distilled water and administered *via* the tail vein of rats.

Sample Preparation

Serum: Group of four rats were used for each penicillin. After administration of each penicillin at a dose of 20 mg/kg, the blood samples were collected by amputation of the femoral artery or vein at appropriate intervals. After the blood was allowed to stand for 1 to 2 hours at room temp, the serum were separated by centrifugation (1,500×*g*, 10 minutes) for analysis.

Urine: Group of four to five rats were used for each penicillin. After administration of each penicillin at a dose of 40 mg/kg, the rats were kept in metabolic cages and the urine samples were continuously collected during 0~2, 2~4 and 4~6 hours intervals. The urines were being frozen with dry-ice during the collection and kept for analysis.

Bile: Group of four to five rats were used for each penicillin. The rats were anesthetized with urethane (ip, 1 g/kg) and the common bile duct was cannulated with polyethylene tube. After administration of each penicillin at a dose of 40 mg/kg, the bile samples were continuously collected at 1 or 2 hours-intervals for 6 hours in dry-iced vessels.

Determination of Drug Concentration

The concentrations of each penicillin in the samples of serum, urine and bile of rats were determined by a paper-disc agar plate diffusion method. The concentrations of ASPC, AB-ASPC, PIPC and AM-PIPC were assayed by using *Escherichia coli* ATCC 27166 as a test strain, and those of ABPC and AMPC were assayed by using *Bacillus subtilis* ATCC 6633 as a test strain. Standard solutions for the assay of serum samples were prepared by diluting each penicillin with rat serum and standard solutions for urine and bile assays were diluted with phosphate buffer solution (1/15 M, pH 7.0). The lower limits of assay for ASPC, AB-ASPC and AM-PIPC, PIPC, ABPC and AMPC were 0.78 μg/ml and 0.39 μg/ml, respectively. Serum concentrations are reported as the mean value±SE.

Determination of Hydrophilicity

The hydrophilicity of each penicillin was determined by means of a reverse-phase TLC.[2,3] The stationary phase used was Silica gel 60 F_{254} (Merck Institute for Therapeutic Research, Rahway, N.J., U.S.A.) impregnated with silicone oil TSF451 (Toshiba Silicone Co., Tokyo, Japan) which had been prepared by developing the plate with ether containing 5% silicone oil and successive drying. The mobile phase was prepared by saturation of phosphate buffer solution (1/20 M, pH 7.0) with the above silicone oil and addition of 0~6% acetone. The phosphate buffer solution (1/20 M, pH 7.0) contain-

ing each penicillin (6 mg/ml) was prepared and 1 μl of the solution was applied on thin-layer plate. When the front of solvent migrated about 10 cm, the developed plates were dried and the spots of penicillins were detected with iodine vapor. The Rf values of each penicillin in various mobile phases containing 0~6% acetone concentration were measured on 4~5 plates and the mean value was adapted as Rf value.

The Rm value was calculated from the following equation:

$$Rm = \log (1/Rf - 1)$$

and these values were plotted against the concentration of acetone in the mobile phase. The regression lines were calculated by means of the least squares method and from which Rm values at acetone concentration of 0% were also estimated. The regression line of AMPC was unable to calculate because this compound migrated to the front of solvents by developing the plate with mobile phases containing 2~6% acetone concentration. Consequently only the Rm value of AMPC for 0% acetone concentration was calculated by using above equation.

Results

Antibacterial Activity of ASPC and Other Penicillins

The antibacterial activity of each penicillin against strains tested was shown in Table 1. No significant differences among the MIC of ASPC and AB-ASPC, PIPC and AM-PIPC, and AMPC and ABPC against strains tested were found.

Hydrophilicity of ASPC and Other Penicillins

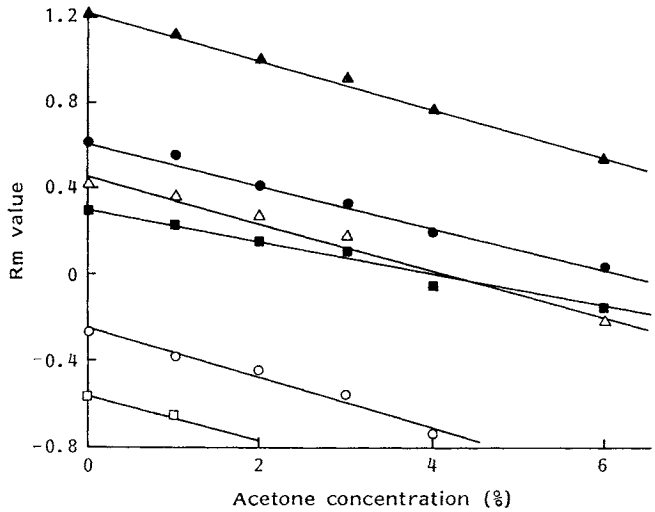
As shown in Fig. 2, the Rm values of each penicillin gave good correlation with the concentrations of acetone in the mobile phase. Moreover, the calculated Rm values corresponding to the acetone concentration of 0% were in good accordance with the estimated Rm values from the regression lines. Among the penicillins tested, AMPC was the lowest in Rm value, which was followed by ASPC, ABPC, AM-PIPC, AB-ASPC and PIPC in ascending order, indicating that AMPC is the most hydrophilic penicillin among penicillins tested. Comparison of the Rm value of ASPC (−0.26) and AB-ASPC (0.62) demonstrated that a hydroxyl group introduced in *para* position of the phenyl group of ASPC increased the hydrophilicity of the compound. Similar results were obtained in a pair of PIPC and AM-PIPC as well as ABPC and AMPC.

Table 1. Antibacterial activity of ASPC and other penicillins.

Strain	AMPC	ABPC	ASPC	AB-ASPC	AM-PIPC	PIPC
Staphylococcus aureus 209P JC-1	0.78[a]	0.2	1.56	1.56	0.78	0.78
S. aureus 252R	>100	50	100	>100	>100	100
Escherichia coli NIHJ JC-2	12.5	12.5	3.13	12.5	1.56	3.13
E. coli ML-1410 RGN-823	>100	>100	>100	>100	>100	>100
Klebsiella pneumoniae PCI-602	>100	100	50	50	6.25	3.13
Morganella morganii Kono	50	50	>100	100	3.13	1.56
Proteus mirabilis TU-1698	0.78	0.2	≦0.2	≦0.2	≦0.2	≦0.2
P. vulgaris IID-874	>100	>100	50	12.5	0.78	0.78
P. vulgaris GN76/C-1	>100	>100	>100	100	6.25	3.13
Citrobacter freundii TL-12	50	25	3.13	3.13	0.78	3.13
C. freundii GN-346	>100	>100	>100	>100	100	25
Pseudomonas aeruginosa PI-67	>100	>100	12.5	12.5	0.39	0.78
P. aeruginosa TU-408	>100	>100	6.25	12.5	0.39	0.78
P. aeruginosa 35R	>100	>100	100	100	1.56	1.56

[a] MIC (μg/ml): 10^6 cfu/ml.

Fig. 2. Rm values of ASPC and other penicillins.
▲ PIPC, ● AB-ASPC, △ AM-PIPC, ■ ABPC, ○ ASPC, □ AMPC.



Rm values of penicillins (acetone, 0%): PIPC; 1.22, AB-ASPC; 0.62, AM-PIPC; 0.47, ABPC; 0.30, ASPC; −0.26, AMPC; (−0.58)*.

* The Rm value of AMPC at 0% acetone concentration was calculated from the following equation: Rm= log (1/Rf−1).

TLC plate: Silica gel 60 F_{254} (Merck Institute for Therapeutic Research) treated with 5% silicone oil TSF451 (Toshiba Silicone Co.) in ether.

Solvent: 0.05 M phosphate buffer (pH 7.0) containing acetone (0 to 6%).

Serum Concentrations of ASPC and Other Penicillins

In Table 2, the serum concentrations, the area under the serum concentration-time curve ($AUC_{0\sim1}$) and the half-life ($T_{1/2}$) of each penicillin tested were shown. All penicillins gave the maximum serum concentrations at 7.5 minutes after administration and the maximum values were as follows: ASPC; 45.8±2.3, AB-ASPC; 29.1±3.5, AM-PIPC; 36.0±1.0, PIPC; 15.0±1.4, AMPC; 40.5±5.0, and ABPC; 20.4±1.0 μg/ml. After administration, the levels of ASPC, AMPC and ABPC gradually decreased and maintained the following levels at 1 hour after administration: ASPC; 3.3±0.4, AMPC; 1.9±0.1 and ABPC; 0.9±0.1 μg/ml. On the other hand, the levels of AB-ASPC, PIPC and AM-PIPC quickly decreased and diminished to the level under the limit of detection at 1 hour after administration. The following order of the AUC values was obtained: AMPC; 25.3, ASPC; 20.2, AM-PIPC; 12.0, AB-ASPC; 10.7, ABPC; 10.3 and PIPC; 4.9 μg·hours/ml. The $T_{1/2}$ values of ABPC, AMPC, ASPC, AB-ASPC, AM-PIPC and PIPC were 15.2 (β), 14.5 (β), 14.1, 7.1, 6.2 and 4.6 minutes, respectively. Thus, the maximum serum concentration values at 7.5 minutes after administration and the AUC values of ASPC and AM-PIPC, both being AMPC derivatives, were found to be higher than those of AB-ASPC and PIPC, the derivatives of ABPC. From the values of $T_{1/2}$, it was also revealed that ASPC and AM-PIPC could persist higher and longer serum levels than AB-ASPC and PIPC, the derivatives of ABPC. The $T_{1/2}$ values of ABPC and AMPC were similar, but the maximum serum concentration and the AUC value of AMPC were higher than those of ABPC.

Relationship between the Serum Concentrations at 7.5 Minutes after Administration and the Rm Values of ASPC and Other Penicillins

As shown in Fig. 3 the serum concentrations of ASPC, AMPC and AM-PIPC at 7.5 minutes after

Table 2. Serum concentrations of ASPC and other penicillins after an intravenous bolus injection of 20 mg/kg in rats.

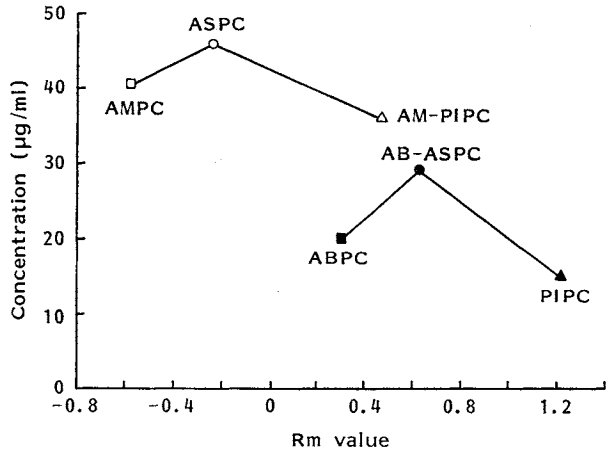
	Serum concentration[a]				$T_{1/2}$ (minutes)	AUC (μg·hours/ml)
	7.5 minutes	15 minutes	30 minutes	60 minutes		
ASPC	45.8±2.3	29.3±1.6	14.1±1.8	3.3±0.4	14.1	20.2
AB-ASPC	29.1±3.5	15.0±2.3	3.4±0.2	ND	7.1	10.7
AM-PIPC	36.0±1.0	14.7±0.6	2.8±0.6	ND	6.2	12.0
PIPC	15.0±1.4	4.6±0.3	0.5±0.06	ND	4.6	4.9
AMPC	40.5±5.0	16.1±1.3	8.2±0.9	1.9±0.1	14.5 (β)	25.3
ABPC	20.4±1.0	7.6±0.4	3.7±0.4	0.9±0.1	15.2 (β)	10.3

[a] Each value represents the mean (μg/ml)±SE of 4 animals.

Bioassay: ASPC, AB-ASPC, AM-PIPC, PIPC; test strain *Escherichia coli* ATCC 27166, test medium 1.5% peptone, 1.5% agar. AMPC, ABPC; test strain *Bacillus subtilis* ATCC 6633, test medium Trypto soy agar.

ND: Not detected.

Fig. 3. Relationship between the serum concentrations at 7.5 minutes after an intravenous bolus injection of 20 mg/kg in rats and the Rm values of ASPC and other penicillins.



administration were higher than those of structurally corresponding penicillins which have no hydroxyl group. Among the AMPC and AMPC derivatives, the serum concentration of ASPC was the highest, AMPC came second, and next in order was AM-PIPC. When compared among ABPC and its derivatives, the following order was observed: AB-ASPC>ABPC>PIPC. Consequently, ASPC and AB-ASPC containing a side chain of N^4-methyl-D-asparagine gave higher serum concentration than AMPC and AM-PIPC, ABPC and PIPC, respectively.

Correlation between the AUC Values and the Rm Values of ASPC and Other Penicillins

As shown in Fig. 4 when the AUC values of penicillins tested were plotted against the Rm values, a linear regression line expressed as $Y=-11.21X+17.20$ ($r=-0.97$) was obtained, which gave a good correlation between the both parameters. From these data, it was shown that the higher hydrophilicity gave the greater AUC values for the penicillins tested.

Fig. 4. Correlation between the AUC values and the Rm values of ASPC and other penicillins.
$Y=-11.21X+17.20$ ($r=-0.97$).

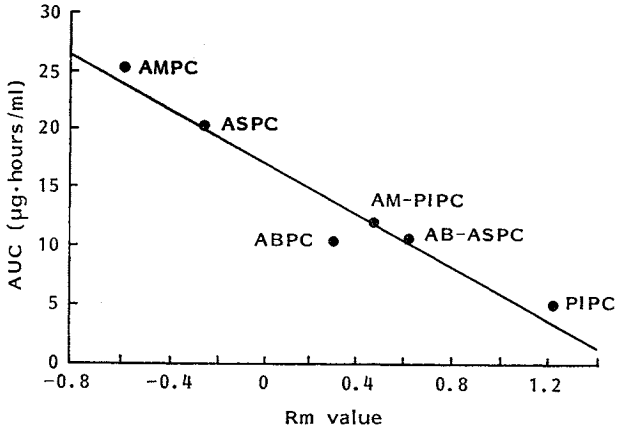


Table 3. Urinary and biliary excretions of ASPC and other penicillins after an intravenous bolus injection of 40 mg/kg in rats.

	Urine: Mean (%)±SE (n=4~5)			Bile: Mean (%)±SE (n=4~5)			
	0~2 hours	0~4 hours	0~6 hours	0~1 hour	0~2 hours	0~4 hours	0~6 hours
ASPC	54.2±1.0	57.3±0.7	58.0±0.8	12.2±1.7	13.9±1.6	14.7±1.5	14.8±1.5
AB-ASPC	25.1±1.4	26.2±1.5	26.4±1.5	32.8±7.1	39.2±7.2	43.0±6.5	44.1±6.2
AM-PIPC	20.9±0.9	21.7±0.9	22.3±0.9	46.6±2.4	51.6±1.8	53.8±2.1	54.6±2.4
PIPC	18.0±2.0	19.2±1.8	19.6±1.9	48.4±4.0	54.5±1.8	56.1±1.1	56.9±0.7
AMPC	41.9±3.2	48.3±2.7	50.9±2.6	5.5±0.9	8.5±0.7	10.6±0.5	11.3±0.6
ABPC	43.9±4.0	49.5±4.4	50.6±4.4	4.8±0.9	12.6±1.3	19.6±2.2	20.7±2.2

Bioassay method: ASPC, AB-ASPC, AM-PIPC, PIPC; test strain *Escherichia coli* ATCC 27166, test medium 1.5% peptone, 1.5% agar. AMPC, ABPC; test strain *Bacillus subtilis* ATCC 6633, test medium Trypto soy agar.

Urinary and Biliary Excretions of ASPC and Other Penicillins

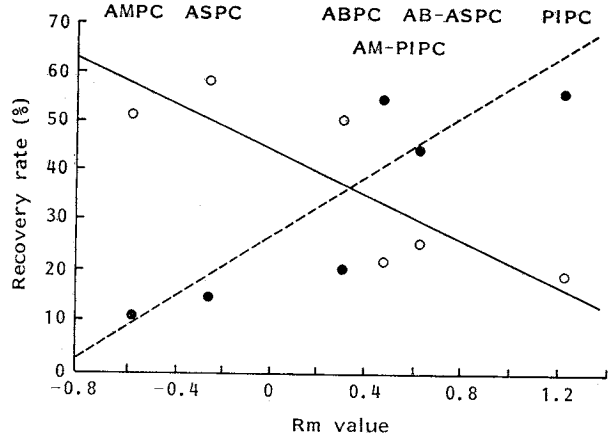
The urinary and biliary excretions after the administration of each penicillin into rats were shown in Table 3. On comparison of the urinary recovery rates within 0~6 hours after administration, the mean urinary recovery rate of ASPC (58.0%) was the highest and was followed by those of AMPC (50.9%), ABPC (50.6%), AB-ASPC (26.4%), AM-PIPC (22.3%) and PIPC (19.6%) in descending order. These urinary excretion were almost complete within the first 2 hours. On the other hand, PIPC (56.9%) gave the highest biliary recovery rate (0~6 hours), which was followed by those of AM-PIPC (54.6%), AB-ASPC (44.1%), ABPC (20.7%), ASPC (14.8%) and AMPC (11.3%) in descending order. From the above results, ASPC, a derivative of AMPC mainly excreted into urine and a great part of AB-ASPC derived from ABPC was eliminated into bile, indicating that the presence of a hydroxyl group affects the route of excretion. Similar results has been reported on a pair of cephalosporin derivatives, LY88011 and LY89439.[4]

Correlation between the Urinary or Biliary Recovery Rates and the Rm Values of ASPC and Other Penicillins

As shown in Fig. 5 the correlation between the urinary recovery rates of penicillins and the Rm values was expressed as $Y=-22.20X+44.52$ ($r=-0.84$). Similarly the equation for biliary elimina-

Fig. 5. Correlation between the urinary or biliary recovery rates and the Rm values of ASPC and other penicillins.

Urinary recovery rate (○): $Y=-22.20X+44.52$ ($r=-0.84$), biliary recovery rate (●): $Y=28.33X+25.38$ ($r=0.89$).



tion was calculated as $Y=28.33X+25.38$ ($r=0.89$). From these results, the excretion rates into the both body fluids and the Rm values of penicillins tested showed a good correlation and it was suggested that the more hydrophilic penicillins mainly excrete into urine and the more hydrophobic penicillins were mostly eliminated into bile.

Discussion

TOBIKI *et al.*[5] synthesized an AMPC derivative which has the same *N*-acyl side chain of PC-904 (apalcillin), an ABPC derivative, and showed that this derivative exhibits approximately 2-fold higher serum concentration and much higher urinary excretion than that of parent PC-904 after intramuscular adminstration in rats.

In general, more hydrophilic β-lactam antibiotics have been suggested to pass more easily through the outer-membrane of Gram-negative bacteria.[6,7] Therefore, more hydrophilic β-lactams may be expected to be more active against Gram-negative bacteria. The results obtained in this study was somewhat contradictory to the above expectation: A hydroxyl group on phenyl group of penicillins seems to affect their pharmacokinetic properties rather than their antibacterial activity. NEU and WINSHELL[8] also reported that AMPC has antibacterial activities similar to those of ABPC.

Further study on the relationship between the structure and the pharmacokinetics of ASPC having other amino acid side chains would be of interest, since ASPC and AB-ASPC containig a side chain of N^4-methyl-D-asparagine exhibited higher serum concentrations than AMPC and AM-PIPC, ABPC and PIPC, respectively.

Very little is known about the relationship between the structure and the pharmacokinetic properties of β-lactam antibiotics, though much has been published on the structure-activity relationship of these antibiotics.

WRIGHT and LINE[4] reported that the excretion rates of cephalosporins into the bile of rats were related to their molecular weight. Our present study demonstrated that the lipophilic character, a physico-chemical property also greatly affects the pharmacokinetic properties of penicillins. Thus, we think that not only molecular weight but also physico-chemical properties should be taken into consideration when the pharmacokinetic properties of chemotherapeutic agents in the body is investigated.

This correlation between chemical structure and pharmacological property will be useful to decide on drug design for new penicillin derivatives.

References

1) WAGATSUMA, M.; M. SETO, T. MIYAGISHIMA, M. KAWAZU, T. YAMAGUCHI & S. OHSHIMA: Synthesis and antibacterial activity of asparagine derivatives of aminobenzylpenicillin. J. Antibiotics 36: 147~154, 1983
2) BIAGI, G. L.; A. M. BARBARO, M. F. GAMBA & M. C. GUERRA: Partition data of penicillins determined by means of reversed-phase thin-layer chromatography. J. Chromatogr. 41: 371~379, 1969
3) MURAKAMI, K. & T. YOSHIDA: Penetration of cephalosporins and corresponding 1-oxacephalosporins through the outer layer of gram-negative bacteria and its contribution to antibacterial activity. Antimicrob. Agents Chemother. 21: 254~258, 1982
4) WRIGHT, W. E. & V. D. LINE: Biliary excretion of cephalosporins in rats: influence of molecular weight. Antimicrob. Agents Chemother. 17: 842~846, 1980
5) TOBIKI, H.; H. YAMADA, N. TANNO, K. SHIMAGO, Y. EDA, H. NOGUCHI, T. KOMATSU & T. NAKAGOME: Studies on β-lactam antibiotics. V. Synthesis of 6-[D(−)-α-(acylamino)-4-hydroxyphenylacetamido]penicillanic acids and antibacterial activity. Yakugaku Zasshi (Japan) 100: 133~139, 1980
6) BIAGI, G. L.; M. C. GUERRA, A. M. BARBARO & M. F. GAMBA: Influence of lipophilic character on the antibacterial activity of cephalosporins and penicillins. J. Med. Chem. 13: 511~516, 1970
7) SAWAI, T.; K. MATSUBA, A. TAMURA & S. YAMAGISHI: The bacterial outer-membrane permeability of β-lactam antibiotics. J. Antibiotics 32: 59~65, 1979
8) NEU, H. C. & E. B. WINSHELL: In vitro antimicrobial activity of 6[D(−)α-amino-*p*-hydroxyphenylacetamido]penicillanic acid, a new semisynthetic penicillin. Antimicrob. Agents Chemother.-1970: 407~410, 1971