IN VITRO AND *IN VIVO* LABORATORY STUDIES ON THREE HYDROXYIMINOPHENYLACETYL CEPHALOSPORINS WITH PARTICULAR REFERENCE TO SK&F 80303, AN UNUSUALLY LONG-ACTING CEPHALOSPORIN

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(Received for publication September 4, 1979)

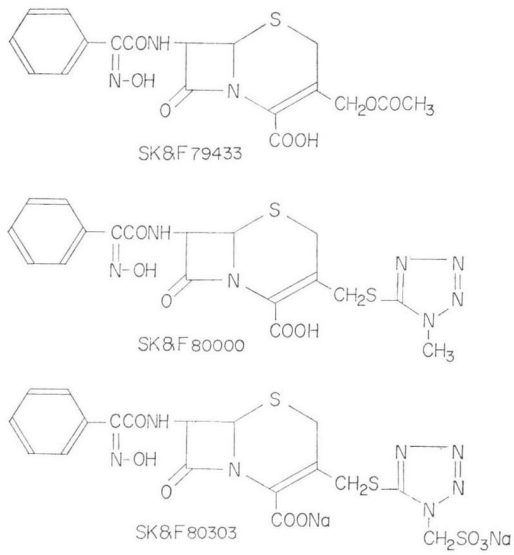
Three cephalosporins with 7-(2-hydroxyiminophenylacetamido) side chains (SK&F 79433, 80000 and 80303), differing in their 3-substituents, exhibited similar broad-spectrum antibacterial activity *in vitro* against strains of *Staphylococcus aureus, Streptococcus faecalis* and various Gram-negative bacilli. All three were active *in vivo* (s.c., mouse) against *S. aureus, Escherichia coli* or *Klebsiella pneumoniae,* but they differed significantly in serum pharmacokinetic profiles. SK&F 80303 produced high and extremely prolonged serum levels and protected mice when administered up to 24 hours prior to challenge with β-lactamase-producing *S. aureus* or *K. pneumoniae.* It was resistant to hydrolysis by β-lactamases from *S. aureus,* and variably so to β-lactamases from *E. coli* strains. SK&F 80303 was bacteriolytic to logarithmically growing *S. aureus, E. coli, Proteus mirabilis, K. pneumoniae* and *Enterobacter cloacae* (partially).

SK&F 80303 illustrates further the effect of the 3-sulfoalkyltetrazole substituent on the pharmacokinetic properties of cephalosporins. Its combined biological properties make it a possible candidate for therapeutic and long-term prophylactic use.

A number of 7-(α-oxyiminoarylacetamido)-cephalosporins have been reported to be potent, broad-spectrum antibacterial agents with a high degree of resistance to most β-lactamases[10,13,14] and several are now undergoing laboratory and clinical trials[4,6,8,12]. The chemical structures of three additional cephalosporins of this type, which are the subject of this report, are indicated in Fig. 1. All have the syn-configuration since it has been demonstrated that these isomers are more potent than the corresponding anti-isomers[5,10,14].

All three analogs exhibited broad-spectrum antibacterial activities, and their pharmacokinetic properties in laboratory animals were equal to or better than those of cefazolin. But while they protected mice from acute bacterial infec-

Fig. 1. Structures of three 7-(2-hydroxyiminophenylacetamido)cephalosporins.



tion when administered subcutaneously, the ED_{50} values observed were higher than those obtained with cefazolin using the same regimen. However, the high and prolonged serum concentrations produced by SK&F 80303 suggest that this cephalosporin should be effective when used prophylactically, and the ED_{50} values in acute infections do not preclude therapeutic efficacy for this analog in most situations. Consequently, additional studies were carried out on SK&F 80303 to delineate more broadly its activity *in vitro* and to test its efficacy potential for prophylactic use.

Materials and Methods

Antibiotics

SK&F 79433, 80000, 80303 and cefazolin were synthesized at Smith Kline and French Laboratories.

Bacterial Cultures

The bacterial strains used for the MIC and ED_{50} determinations are those regularly employed in our laboratory for the primary and secondary testing of cephalosporins and penicillins[18]. They are for the most part clinical isolates obtained from various geographical locations in the United States.

β-Lactamase Activity Determinations

The β-lactamase activities of the bacterial strains used in this study were determined on the basis of color development with nitrocefin (Glaxo 87/312)[11] using whole cell suspensions in buffered peptone-glucose broth as previously described[11,17,18]. In addition, the resistance of these strains to various β-lactam antibiotics was verified by biological assay.

In Vitro Assays

The minimum inhibitory concentrations (MIC's) were determined either by agar dilution or by microdilution techniques as previously reported[1,2,3,18]. The media for *S. aureus* and Gram-negative bacilli were Trypticase soy agar or MUELLER-HINTON broth, buffered to pH 7.0 with 10% MCILVAINE's citric acid-phosphate buffer. The *Neisseria* spp. were grown on MUELLER-HINTON agar fortified with HEPES buffer (0.05 M), IsoVitaleX (0.5%), Fildes enrichment (2.5%), dextrose (1%) and laked sheep erythrocytes (2.5%). For *Haemophilus influenzae,* MUELLER-HINTON agar containing HEPES buffer (0.05 M), yeast extract (0.5%), nicotinamide adenine dinucleotide, IsoVitaleX (1%), dextrose (1%) and hemin (10 mg/ml) was used. The *Streptococcus* spp. were tested on TODD-HEWITT agar supplemented with HEPES buffer (0.05 M) and laked sheep erythrocytes (2.5%). In the agar-dilution assays, bacterial cultures containing approximately $10^4 \sim 10^6$ cells were used to inoculate the surface with the aid of a STEERS' inocula replicating device[16]. Plates were incubated overnight at 37°C. The MIC's determined are those concentrations which completely inhibited colony-formation. For broth dilution assays, a semiautomated microdilution-technique (Microtiter, Cooke Engineering Co.) was used. Inocula used in the microtiter assay were approximately 5×10^5 colony-forming units/ml.

Microbiological Assay for Antibiotic Concentration

All serum and urine samples were assayed for antibiotic concentrations by the disc agar-diffusion method, using *Bacillus subtilis* ATCC 6633. Standards and test samples were diluted using appropriate pooled animal sera. For urine assays, the diluent was 0.01 N phosphate buffer, pH 6.0. Assay plates were incubated overnight at 37°C. Zone diameters were measured with a Fisher-Lilly zone reader. Samples were also examined for biologically active metabolites using thin-layer chromatography.

In Vitro Binding to Serum Proteins

Serum protein binding (mouse, squirrel monkey, rabbit, human) was estimated by comparing microbiologically assayed standard dose-response curves in phosphate buffer (pH 6.0) with those obtained in appropriate serum[15].

Mode of Action Studies

The comparative bacteriolytic activity of SK&F 80303 was studied in peptone-glucose broth (pH 7.3, 37°C) using stationary growing cultures[17,18] of *S. aureus, E. coli, P. mirabilis, K. pneumoniae* and *Enterobacter cloacae.* When the cultures reached optical density (Spectronic 70, 500 nm) of about

0.4 (rapid exponential growth phase) the antibiotic was added to final concentrations of 50, 25, 12, 6, 3 and 1.5 μg/ml. Optical density readings were taken stat, at hourly intervals for 8 hours and at 24 hours and were plotted versus time.

Animals

Male, white, Swiss-Webster mice with average weight of 18~21 g were used for efficacy and pharmacokinetic experiments. Rabbits (2.25~3.5 kg) and squirrel monkeys (0.8~1.1 kg) were also used for serum level studies[8].

Pharmacokinetics

Serum and urinary antibiotic levels during the first 4 hours after dosing were determined in duplicate pooled groups of ten mice after subcutaneous injection of 20 mg/kg of the cephalosporins[1,2,3,19]. Serum levels were also determined in rabbits and squirrel monkeys injected intramuscularly with 20 mg/kg of drug. The serum samples were kept frozen (−20°C) until assayed.

Chemotherapeutic Efficacy Studies

The *in vivo* protective efficacy studies followed procedures previously reported[1,2,3,18]. Acute infections were produced by injection (i.p.) into mice (groups of ten) of a predetermined number of organsims diluted in 5% gastric mucin to produce uniform lethality in non-treated animals. In the therapeutic studies the test cephalosporins were administered subcutaneously at 1 and 5 hours after infection; in the prophylactic studies, the mice were injected preinfection as indicated in the table. ED_{50} and LD_{50} values were calculated using the method of LITCHFIELD and WILCOXON[9].

Results and Discussion

Comparative *In Vitro* Studies

Table 1 compares the *in vitro* antibacterial activities (MIC values) of the three cephalosporin analogs under study with those of cefazolin against 16 representative bacterial strains. The three test compounds exhibited broad-spectrum activity similar to cefazolin. They were slightly more active than cefazolin against *Proteus morganii* and they inhibited a strain of *Serratia marcescens* (6.3~12.5 μg/ml) resistant to this standard. *Pseudomonas aeruginosa* was not inhibited by any of these cephalosporins.

The range of MICs and median MIC values for SK&F 80303 and cefazolin against multiple strains of *S. aureus* and *E. coli*, including β-lactamase-producing strains of both species, are listed in Table 2. The activity of SK&F 80303 against *Neisseria, Haemophilus* and *Strepto-*

Table 1. *In vitro* activity of three 7-(2-hydroxyiminophenylacetamido)cephalosporins and cefazolin against sixteen bacterial strains.

Organism	Minimal inhibitory concentration (μg/ml)			
	SK&F 79433	SK&F 80000	SK&F 80303	Cefazolin
Staph. aureus	0.2	0.2	0.8	0.2
*Staph. aureus**	0.2	0.2	0.8	0.4
*Staph. aureus***	25.0	12.5	100.0	100.0
Strep. faecalis	3.1	3.1	6.3	12.5
E. coli	6.3	3.1	1.6	1.6
*E. coli**	6.3	3.1	3.1	3.1
K. pneumoniae	1.6	1.6	1.6	1.6
*K. pneumoniae**	3.1	1.6	0.8	0.8
S. paratyphi	1.6	1.6	0.8	0.8
S. paradysenteriae	1.6	0.8	0.4	0.4
*P. aeruginosa**	>200.0	>200.0	>200.0	>200.0
*Serratia marcescens**	12.5	6.3	12.5	>200.0
P. mirabilis	NT	NT	3.1	3.1
*P. morganii**	50.0	3.1	25.0	200.0
*E. aerogenes**	12.5	6.3	6.3	1.6
*E. cloacae**	6.3	3.1	1.6	0.8

* β-Lactamase producer; **Methicillin resistant and β-lactamase producer; NT: Not tested.

Table 2. *In vitro* activities of SK&F 80303 and cefazolin (CEZ) against non-β-lactamase and β-lactamase producing strains of *Staphylococcus aureus* and *Escherichia coli*.

Organism	Compound	No. of strains tested	Number of strains inhibited at concentration (μg/ml)										Median
			≤0.2	0.4	0.8	1.6	3.2	6.3	12.5	25	50	100	
S. aureus[a]	80303	12	3	4	5								0.4
Non-β-lactamase producer	CEZ		6	6									0.3
S. aureus[b]	80303	13		2	10	1							0.8
β-lactamase producer	CEZ		4	3	4	2							0.4
E. coli[c]	80303	8			1	4	3						1.6
Non-β-lactamase producer	CEZ				1	5	2						1.6
E. coli[d]	80303	17					4	4	4	3	2		12.5
β-lactamase producer	CEZ					1	3	4	4	2	1	2	12.5

[a] Penicillin G sensitive; [b] Penicillin G resistant; [c] Ampicillin sensitive; [d] Ampicillin resistant

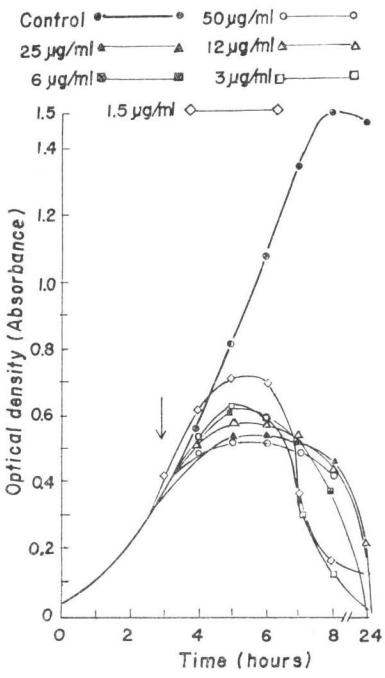
Table 3. MIC values of SK&F 80303 and controls against fastidious organisms.

Strain	No. of strains	Mean MIC's (μg/ml)		
		SK&F 80303	Penicillin G	Ampicillin
Neisseria gonorrhoeae[1]	15	≤0.15	0.75	ND
*Neisseria gonorrhoeae**	5	0.14	8.7	ND
Neisseria meningitidis[1]	15	0.10	0.15	ND
Haemophilus influenzae[1]	15	0.23	ND	0.4
*Haemophilus influenzae**	7	0.12	ND	12.0
Streptococcus pneumoniae[2]	15	0.36	0.06	ND
Streptococcus pyogenes[2]	15	0.40	0.025	ND

* β-Lactamase producing strains (Nitrocefin Color Test)[11]
[1] Fortified MUELLER-HINTON agar
[2] Supplemented TODD-HEWITT agar
ND: Not determined.

Fig. 2. Lytic action of SK&F 80303 on *Staphylococcus aureus* No. 674 (Tour-U strain)

Arrow shows time of drug addition to culture.



coccus strains is compared with those of penicillin G or ampicillin in Table 3. While the median MIC values for the strains of *S. aureus* that produce β-lactamase is only two-fold greater than for the benzylpenicillin-sensitive strains (Table 2), the differences between the strains of *E. coli* that produce β-lactamase and those that do not was greater (median MIC values of 1.6 versus 12.5 μg/ml). However, all of the MIC values were within achievable serum concentrations. The more fastidious *Neisseria, Haemophilus* and *Streptococcus* strains were very sensitive to SK&F 80303 with mean MIC values <0.5 μg/ml (Table 3). SK&F 80303 was found to be resistant to β-lactamase-producing strains of *N. gonorrhoeae* and *H. influenzae*.

Bacteriolytic Properties

SK&F 80303 was lytic to *S. aureus* (Fig. 2) and non-β-lactamase producing *P. mirabilis*, *E. coli* and *K. pneumoniae* strains at concentrations of 1.5~3.0 μg/ml. It was also lytic at concentrations of 12~50 μg/ml to *E. coli*, *P. mirabilis* and *K. pneumoniae* strains that produce low levels of β-lactamases. Partial or no lysis was observed with Gram-negative bacilli which produce high levels of β-lactamases. This observation is in agreement with the finding that SK&F 80303 displays inoculum-dependent MIC values against these Gram-negative bacilli.

Pharmacokinetics and Metabolism in Mice

The pharmacokinetic behavior of the three hydroxyiminocephalosporins in mice injected subcutaneously with 20 mg/kg of compound is indicated in Table 4. SK&F 79433 and SK&F 80000 produced serum levels similar to those of cefazolin whereas SK&F 80303 produced levels that were higher (approximately twice) and significantly longer in duration (half-life of 230 minutes). While SK&F 79433 and SK&F 80000 bind plasma proteins approximately 50%, SK&F 80303 was highly bound to mouse serum proteins (91%), a factor that may be responsible for its extremely long serum half-life.

All three cephalosporins showed lower urinary recovery than cefazolin. No biologically active metabolites were detected in the urine of mice dosed with SK&F 80000 and 80303. The urine from SK&F 79433 treated mice contained two bioactive components (bioautography), the parent compound and a bioactive metabolite.

Serum levels of SK&F 80303 were also determined in rabbit and squirrel monkey (Table 5). The peak serum levels in both species were higher than in the mouse, although the biological half-lives were somewhat shorter. The binding of SK&F 80303 to the serum proteins of these species was also high

Table 4. Antibiotic serum concentration in mice, half-lives and urinary recovery following subcutaneous injections of 7-(2-hydroxyiminophenylacetamido)cephalosporins at 20 mg/kg and their *in vitro* binding to serum proteins compared to those of cefazolin.

Compounds	Average serum concentration (μg/ml)				$T_{1/2}$ (min.)	Urinary recovery (%) at 4 hours	Serum protein binding (%)
	15 min.	30 min.	60 min.	120 min.			
SK&F 79433	51.5	31.6	10.0	1.0	18	19	53
SK&F 80000	52.7	40.8	15.5	2.0	23	29	55
SK&F 80303	102.2	99.0	82.6	77.5	230	13	91
Cefazolin	52.0	37.0	15.0	2.6	23	57	24

Table 5. Average serum levels (μg/ml) of SK&F 80303 and cefazolin (CEZ) in mice, rabbits, and squirrel monkeys; Half-lives and *in vitro* serum protein bindings.

Animal	Compound	Sampling-time (min) and serum levels (μg/ml)				$T_{1/2}$ (min.)	Serum protein binding (%)[c]
		15	30	60	120		
Mouse[a]	80303	102	99	83	78	230	91
	CEZ	52	37	15	3	23	24
Rabbit[b]	80303	177	184	137	115	135	97
	CEZ	78	63	32	7	30	69
Squirrel monkey[b]	80303	151	164	146	104	160	92
	CEZ	92	99	97	48	75	42

[a] 20 mg/kg SC; [b] 20 mg/kg IM; [c] Binding to human serum proteins is 96% for SK&F 80303 and 72% for cefazolin.

(rabbit, 97%; squirrel monkey, 92%). Again, the serum protein binding would seem to play a role in the serum level profiles in these species. Corresponding data for cefazolin are also presented.

Comparative *In Vivo* Studies

The experimental chemotherapeutic effectiveness against acute bacterial infections in mice of the three test compounds are compared with that of cefazolin in Table 6. In general, the ED_{50} values against the Gram-positive and Gram-negative bacteria used in the test were inferior to those of cefazolin in spite of relatively equivalent MIC values and favorable pharmacokinetic behavior.

Table 6. Subcutaneous antibacterial activity of three 7-(2-hydroxyiminophenylacetamido)cephalosporins and cefazolin in experimental acute bacterial infections of mice.

Test organism	ED_{50} (mg/kg)				Challenge LD_{50}
	SK&F 79433	SK&F 80000	SK&F 80303	Cefazolin	
Staphylococcus aureus #127[a]	1.8	—	—	1.0	26
	—	—	15.0	1.6	50
Escherichia coli #12140[b]	25.0	—	—	4.4	45
	—	18.0	—	6.3	100
	—	—	25.0	6.3	66
Klebsiella pneumoniae #4200	40.0	—	—	6.3	66
	—	12.5	—	4.4	428
	—	—	4.4	7.2	416

[a] Inducible β-lactamase producer
[b] Weak β-lactamase producer (Nitrocefin Color Test)[11]

Experimental Prophylactic Chemotherapy

The serum level profile in mice suggests that SK&F 80303 should be effective when used prophylactically. To test this, a single dose of SK&F 80303 or cefazolin was injected subcutaneously at the time intervals indicated in Table 7, before the mice were infected with either *S. aureus* 127 or *K. pneumoniae* 4200. The ED_{50} values against *S. aureus* were comparable for SK&F 80303 and cefazolin when the agents were injected one hour prior to infection. However, a significant difference in ED_{50} values was seen as the time interval between dosing and infection was increased. When injected 24 hours before infection, SK&F 80303 protected the mice, whereas cefazolin did not. Even more striking differences between SK&F 80303 and cefazolin were observed when the mice were infected with *K. pneumoniae*. These data indicate that the high and prolonged blood levels of SK&F 80303 are reflected in prolonged (prophylactic) antibacterial efficacy.

Table 7. Effect of a single dose in mice of SK&F 80303 or cefazolin injected subcutaneously prior to challenge.

Dosage time (hours)	ED_{50} (mg/kg)			
	Staphylococcus aureus #127*		*Klebsiella pneumoniae* #4200**	
	SK&F 80303	Cefazolin	SK&F 80303	Cefazolin
−1	4.5	3.5	5.5	25.0
−4	10.0	22.5	12.5	>25.0
−6	9.3	25.0	12.5	>25.0
−24	13.0	>25.0	ND	ND

* LD_{50}=5.6 and 45 for the 1~6 hours and 24 hours challenges, respectively.
** LD_{50}=150.
ND: Not done.

Stability of SK&F 80303 in Aqueous Solution

An aqueous solution of SK&F 80303 showed no loss of potency when held at 4°C for 6 months.

Conclusions

The capacity of the 3-sulfomethyltetrazolethiomethyl substituent to increase the peak and duration of serum levels attained by cephalosporins carrying this grouping is demonstrated by cefonicid (SK&F 75073)[3]. The unalkylated hydroxyimino grouping on SK&F 80303 (a grouping also contained in the nocardicin A structure)[4] appears to potentiate the effect of the sulfomethyltetrazole substituent, resulting in a cephalosporin analog that is highly bound to serum proteins, but which exhibits exceptionally high and prolonged serum levels in several animal species. This behavior, along with good *in vitro* activity against Gram-positive and many Gram-negative bacteria suggests the potential for continuous prophylactic applications, as for example, in rheumatic fever and bacterial endocarditis. Its use in this way would resemble that of suramin[7] another highly serum-bound drug which has been used in treatment and long-term prophylaxis against trypanosomiasis and onchocerciasis.

References

1) ACTOR, P.; J. V. URI, J. R. GUARINI, I. ZAJAC, L. PHILLIPS, C. S. SACHS, R. M. DEMARINIS, J. R. E. HOOVER & J. A. WEISBACH: A new parenteral cephalosporin. SK&F 59962; *in vitro* and *in vivo* antibacterial activity and serum levels in experimental animals. J. Antibiotics 28: 471~476, 1975
2) ACTOR, P.; J. V. URI, L. PHILLIPS, C. S. SACHS, J. R. GUARINI, I. ZAJAC, D. A. BERGES, G. L. DUNN, J. R. E. HOOVER & J. A. WEISBACH: Laboratory studies with cefatrizine (SK&F 60771), a new broad-spectrum orally-active cephalosporin. J. Antibiotics 28: 594~601, 1975
3) ACTOR, P.; J. V. URI, I. ZAJAC, J. R. GUARINI, L. PHILLIPS, D. H. PITKIN, D. A. BERGES, G. L. DUNN, J. R. E. HOOVER & J. A. WEISBACH: SK&F 75073, new parenteral broad-spectrum cephalosporin with high and prolonged serum levels. Antimicr. Agents & Chemoth. 13: 784~790, 1978
4) AOKI, H.; H. I. SAKAI, M. KOHSAKA, T. KONOMI, J. HOSODA, Y. KUBOCHI & E. IGUCHI: Nocardicin A, a new monocyclic β-lactam antibiotic. I. Discovery, isolation and characterization. J. Antibiotics 29: 492~500, 1976
5) BUCOURT, R.; R. HEYMES, A. LUTZ, L. PÉNASSE & J. PERRONNET: Cephalosporines a chains amino-2-thiazolyl-4 acetyles. Influence de la presence et de la configuration d'un groupe oxyimino sur l'activité antibacterienne. Tetrahedron 34: 2233~2243, 1978
6) HAMILTON-MILLER, J. M. T.; W. BRUMFITT & A. V. REYNOLDS: Cefotaxime (HR 756) a new cephalosporin with exceptional broad-spectrum activity *in vitro*. J. Antimicr. Chemoth. 4: 437~444, 1978
7) HAWKING, F.: Suramin: with special reference to onchocerciasis. *in* Advances in Pharmacology and Chemotherapy. Vol. 15, pp. 289~322, Academic Press, New York, San Francisco, London, 1978
8) HEYMES, R.; A. LUTZ & E. SCHRINNER: Experimental evaluation of HR 756, a new cephalosporin derivative: Pre-clinical study. Infection 5: 259~260, 1977
9) LITCHFIELD, J. T., Jr. & F. WILCOXON: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99~113, 1949
10) NUMATA, M.; I. MINAMIDA, S. TSUSHIMA, T. NISHIMURA, M. YAMAOKA & N. MATSUMOTO: Synthesis of a new cephalosporin with potent antibacterial activities. Chem. Pharm. Bull. 25: 3117~3119, 1977
11) O'CALLAGHAN, C. H.; A. MORRIS, S. KIRBY & A. H. SHINGLER: Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicr. Agents & Chemoth. 1: 283~288, 1972
12) O'CALLAGHAN, C. H.; R. B. SYKES, D. M. RYAN, R. D. FORD & P. W. MUGGLETON: Cefuroxime—a new cephalosporin antibiotic. J. Antibiotics 29: 29~37, 1976
13) O'CALLAGHAN, C. H. & G. I. GREGORY: Structure activity relationships amongst the 7-oximinocephalosporins. Abst. 79, 17th Intersci. Conf. Antimicr. Agents & Chemoth., New York, Oct. 12~14, 1977
14) OCHIAI, M.; O. AKI, A. MORIMOTO, T. OKADA & Y. MATSUSHITA: New cephalosporin derivatives with high antibacterial activities. Chem. Pharm. Bull. 25: 3115~3117, 1977
15) SCHOLTAN, W. & J. SCHMID: Die Bindung der Penicilline an die Eiweisskörper des Serums und des Gewebes. Arzneimit. Forsch. 12: 741~750, 1962
16) STEERS, E.; E. L. FOLTZ, B. S. GRAVES & J. RIDEN: An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. & Chemoth. 9: 307~311, 1959
17) URI, J. V.; P. ACTOR & J. A. WEISBACH: A rapid and simple method for detection of β-lactamase inhibitors. J. Antibiotics 31: 787~791, 1978
18) URI, J. V.; P. ACTOR, J. R. GUARINI, L. PHILLIPS, D. PITKIN, R. M. DEMARINIS & J. A. WEISBACH: Biological and chemotherapeutic studies on three semisynthetic cephamycins. J. Antibiotics 31: 82~91, 1978