BICYCLOMYCIN, A NEW ANTIBIOTIC
III. *IN VITRO* AND *IN VIVO* ANTIMICROBIAL ACTIVITY

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Bicyclomycin is a new antibiotic active against Gram-negative bacteria such as *Escherichia coli*, *Klebsiella*, *Shigella*, *Salmonella*, *Citrobacter*, *Enterobacter cloacae*, and pathogenic group of *Neisseria*, but inactive against *Proteus*, *Pseudomonas aeruginosa*, and Gram-positive bacteria. This antibiotic shows no cross-resistance with any of the known antibacterial drugs such as streptomycin, kanamycin, chloramphenicol, tetracycline, ampicillin and nalidixic acid. Its activity is bactericidal, with little varieties of MIC values under various experimental conditions. Bicyclomycin is not degraded in culture broths of sensitive and resistant organisms or rat tissue homogenates, and the extent of binding with serum protein is very low. It was effective subcutaneously in curing *E. coli* experimental infections in mice with strains resistant to the usual antibiotics.

Bicyclomycin is a a new antibiotic developed in Fujisawa Research Laboratories, which is active against Gram-negative bacteria except *Proteus* and *Pseudomonas aeruginosa*. As shown in Fig. 1, bicyclomycin has a unique chemical structure, no chemical relation being noted to any groups of the known antibiotics.[1,2] It exhibits no cross-resistance to any of the currently available antibacterial drugs.

The present paper is mainly concerned with the *in vitro* and *in vivo* evaluation of this new antibiotic as compared with known antibacterial drugs.

Materials and Methods

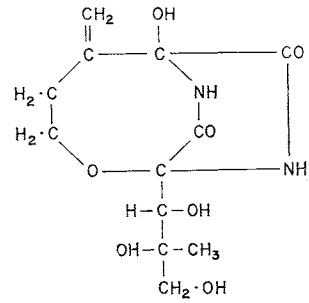
1. Antibacterial drugs tested

Bicyclomycin was prepared by Fujisawa Research Laboratories, and the following antibacterial drugs served as the controls: nalidixic acid (NA, Daiichi Pharmaceutical Co., Ltd.), chloramphenicol (CP, Fujisawa Pharmaceutical Co., Ltd.), tetracycline hydrochloride (TC, Takeda Chemical Industries, Ltd.), streptomycin sulfate (SM, Meiji Seika Co., Ltd.) and ampicillin (AB-PC, Beecham Research Laboratories).

2. Bacterial strains used

Standard strains stored in Osaka City Institute of Hygiene and Fujisawa Research Laboratories were used in this study. The clinical isolates were mainly supplied from Osaka Municipal Momoyama Hospital.

Fig. 1. Chemical structure of bicyclomycin



$C_{12}H_{18}N_2O_7$ M.W. 302.28

3. Method for *in vitro* sensitivity testing

The *in vitro* antimicrobial activity was tested by an agar dilution method. Two-fold serial dilutions of each test drug were prepared in melted heart infusion agar, made into agar plates, and were streaked with one loopful of an overnight culture of each test strain in Trypticase soy broth (BBL), and incubated at 37°C for 24 hours. Minimum inhibitory concentration (MIC) is expressed in terms of mcg per ml, at which the growth of test culture was completely suppressed.

4. Method for bioassay

Bicyclomycin is conveniently bioassayed in the usual manner as given below.

(1) Cylinder plate method:

Bacto-nutrient broth (Difco) 0.8 %, Powdered agar 0.8 %. Adjust the pH to 6.8.

One hundred ml of this medium was seeded with 0.2 ml of an overnight culture of *E. coli* ATCC-27166. Bicyclomycin was dissolved in 1 % phosphate buffer (pH 6.0), and bioassayed by the ordinary cylinder plate method. The lowest detectable concentration was 10 mcg/ml.

(2) Disk method:

Disks used were Toyo's filter paper intended for assaying antibiotics (8 mm in diameter). The lowest detectable concentration was 20 mcg/ml.

5. Bactericidal activity

Nutrient broth, containing bicyclomycin in various concentrations, was inoculated with *E. coli* ATCC-27166 (final cell count; 10^6/ml). The inoculated medium was incubated at 37°C, with shaking for the initial 6 hours and then with stationary culture for subsequent 18 hours, and sampled for estimation of viable cell counts at intervals.

6. Development of resistance *in vitro*

E.coli ATCC-27166 was serially subcultured 8 times in Trypticase soy broths containing increasing levels of bicyclomycin. The inoculation in each transfer was made after 24 hours incubation from a tube which contained the antibiotic in the highest concentration and showed visible growth.

7. Extent of binding to serum protein

One volume of 1,000 mcg/ml bicyclomycin solution was added to 9 volumes of fresh human serum or five different animal sera. Each sample was ultrafiltered through Visking tubing (8/32 in size) and followed by the assay of the antibiotic concentration in protein-free filtrates.

8. Stability in various aqueous phases

Bicyclomycin was studied for its stability in various aqueous phases, including buffer solutions of varying pH values, liquid media, human serum, and urine. Bicyclomycin was added to 0.1 M phosphate buffer (pH 6.0, 7.0 and 8.0) at a concentration of 1,000 mcg/ml, and allowed to stand for 1~5 days at varying temperatures ranging from about 5°C to 37°C. The residual antibiotic in the solution was assayed by the cylinder plate method, which was also applied to liquid media, serum and urine samples.

9. Stability in tissue homogenates

Male rats of SD-strain, weighing about 150 g, were used after 24 hours' fast. Twenty per cent tissue homogenates of liver, kidney, and small intestine were prepared with KREBS-RINGER phosphate (pH 7.2), and the stomach homogenate with saline (pH 2.0). One ml of 1,000 mcg/ml bicyclomycin solution was mixed with 1 ml each of these homogenates, and incubated at 37°C. Three hours afterwards, 2 ml of 95 % ethanol was added to terminate enzymatic reactions, and the supernatant fluid obtained by centrifugation was assayed by the disk method for the antibiotic content.

10. Protection against infection in mice

Male mice of ICR-strain, weighing 20~30 g and divided into groups of 10 animals each, were used. Strains of *E. coli* resistant to the control antibiotics were cultured overnight in BHI-broth at 37°C. A 0.5-ml aliquot of a 4- and 8-fold dilution was intraperitoneally

injected. One hour after the challenge, bicyclomycin dissolved in distilled water was given subcutaneously. These animals were observed for survival or death for 7 days, and the ED_{50} values were calculated by the probit method.

Results

1. Antimicrobial Spectrum

As shown in Tables 1 and 2, bicyclomycin was inactive against Gram-positive organisms but completely supressed the growth of standard strains of Gram-negative bacteria including *E. coli*, various species of *Salmonella*, *Shigella*, and *Klebsiella*, and *Neisseria gonorrhoeae*, mainly at concentrations of 12.5~25 mcg/ml. Although the *in vitro* antimicrobial activity of bicyclomycin against sensitive organisms was not stronger than that of AB-PC and SM, none of the strains tested was highly resistant to this new antibiotic. However, bicyclomycin was not active against *Proteus vulgaris* IAM-1025 and *Ps. aeruginosa* IAM-1095.

Table 1. Antibacterial spectrum of bicyclomycin (1) Gram-negative bacteria

Organism	MIC (mcg/ml)		
	Bicyclomycin	SM	AB-PC
E. coli NIHJ JC-2	25	6.25	12.5
Kl. pneumoniae NCTC-418	100	3.13	50
Sh. flexneri 1 a EW-8	25	3.13	3.13
〃 〃 1 b Showa 15	12.5	6.25	1.56
〃 〃 2 a EW-10	12.5	3.13	3.13
〃 〃 2 a Komagome BIII	12.5	3.13	1.56
〃 〃 3 a EW-14	12.5	3.13	3.13
〃 〃 4 a Saigon-Arai	12.5	0.78	1.56
〃 〃 5 Komagome A	25	3.13	3.13
Sh. sonnei I EW-33	50	6.25	3.13
〃 〃 Ohara	12.5	3.13	3.13
Salm. typhosa T-287	25	25	1.56
〃 〃 O-901	25	50	1.56
Salm. paratyphi A 1015	25	50	1.56
〃 〃 B 8006	25	100	6.25
Salm. typhimurium 1406	25	25	0.39
Salm. enteritidis 1891	12.5	3.13	0.39
Pr. vulgaris IAM-1025	>800	50	100
Ps. aeruginosa IAM-1095	>800	50	>100
N. gonorrhoeae Matuura*	25	1.56	< 0.05
N. meningitidis 68*	>800	6.25	< 0.05

Agar dilution method (Heart infusion agar, *GC-agar : 10^8/ml, 37℃, 20 hours)

Table 2. Antibacterial spectrum of bicyclomycin (2) Gram-positive bacteria

Organism	MIC (mcg/ml)		
	Bicyclomycin	SM	AB-PC
Staph. aureus 209-P JC-1	>800	6.25	0.1
〃 〃 Newman	>800	12.5	0.2
〃 〃 Terashima	>800	3.13	0.39
〃 〃 Smith	>800	6.25	0.1
Strept. hemolyticus S-23*	>800	25	0.05
〃 *faecalis* 6733*	>800	100	1.56
Dipl. pneumoniae I*	>800	12.5	0.1
〃 〃 II*	>800	12.5	0.1
〃 〃 III*	>800	25	0.05
B. subtilis ATCC-6633	>800	0.78	0.1
S. lutea PCI-1001	250	1.56	0.05
Coryn. diphtheriae PW 8	800	0.78	0.1
〃 〃 A-7	>800	3.13	0.2
〃 〃 AK O-222	>800	3.13	0.2
〃 〃 M 406 MGL	>800	1.56	0.39
〃 〃 AK O-167	>800	6.25	0.78
Mycob. tuberculosis 607	>800	0.1	>800

Agar dilution method (Heart infusion-agar : 10^8/ml, 37℃, 20 hours.)
* enriched with 10% rabbit blood

2. Sensitivity of Clinical Isolates

The antimicrobial activity of bicyclomycin against clinical isolates of some groups of enterobacteria was compared with that of NA, CP, SM, KM, TC, and AB-PC. The results are shown in Tables 3, 4 and 5. The MIC of bicyclomycin against 30 strains of *E. coli* was as follows: 25 mcg/ml in 19 strains (63.3%), 50 mcg/ml in 10 strains (33.3%), and 6.25 mcg/ml in 1 strain (3.3%). It is clear that no strain is so highly resistant to bicyclomycin as to require an MIC as high as 100 mcg/ml. The pattern of bicyclo-

Table 3. Distribution of sensitivity of clinical isolates

Organism	Antibiotic	MIC (mcg/ml)											
		>400	400	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39
*E. coli** 30 strains	Bicyclo.					10	19		1				
	NA							3	8	17	2		
	CP	3	7		4		5	4	6	1			
	SM	11	2	2	3			1	5	4	2		
	TC		8	6	1	1	1	10	3				
	KM	1					13	15	1				
	AB-PC	1						8	19	2			
*Klebsiella** 30 strains	Bicyclo.	6		1	10	12	1						
	NA				4	3	10	7	6				
	GP	9	4	2	1	2	3	5	3	1			
	SM	11	3	2					1	12		1	
	TC		10	8			2	9	1				
	KM							1	23	5	1		
	AB-PC	16	10	1	3								

Each figure indicates number of strains which showed the appropriate MIC (mcg/ml).
* Inoculum suspension : 10^8/ml.

Table 4. Distribution of sensitivity of clinical isolates

Organism	Antibiotic	MIC (mcg/ml)											
		>400	400	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39
*Shigella** 36 strains	Bicyclo.					1	23	12					
	NA			2				2	1	30	1		
	CP	20	8	3						1	3		1
	SM	30	1					1	4				
	TC		10	11		1	5	4		5			
	KM	6					2	14	14				
	AB-PC							2	19	13	2		
*Salmonella** 35 strains	Bicyclo.				9	9	17						
	NA				1		2	17	15				
	CP	1					3	9	22				
	SM	4		3	14	6	4	1	2		1		
	TC	4		1	2		2	14	12				
	KM						8	11	6	9	1		
	AB-PC	3			1			1	3	20	2	5	

Each figure indicates number of strains which showed the appropriate MIC (mcg/ml).
* Inoculum suspension : 10^8/ml.

mycin's activity resembled that of KM. Although bicyclomycin was less active than NA or AB-PC, it was proved to be active against organisms which were highly resistant to CP, SM, or TC.

The MIC of bicyclomycin for 30 strains of *Klebsiella* was 25~100 mcg/ml for 23 strains (76.6 %) and 200 mcg/ml or more for 7 strains (23.4 %). The activity of bicyclomycin against *Klebsiella* was lower than that of KM and NA, but was higher than that of AB-PC.

For 36 strains of *Shigella*, the MIC was 12.5~25 mcg/ml for 35 strains and 50 mcg/ml for 1 strain, and bicyclomycin was somewhat less active than NA or AB-PC. However, no strain was highly resistant to bicyclomycin, in spite of the high resistance to the control antibiotics.

Table 5. Distribution of sensitivity of clinical isolates

Organism	Antibiotic	MIC (mcg/ml)									
		>800	800	400	200	100	50	25	12.5	6.25	3.13
*Citrobacter** 22 strains	Bicyclo.				1	9	9	3			
	NA	7	3	1	4				4	3	
	CP	3	10	1	2	3	2	1			
	SM	6	5	5			5	1			
	TC			6	11	2			3		
	KM	9	1		1					10	1
	AB-PC	20	1	1							
*Enterobacter cloacae** 8 strains	Bicyclo.				1	2	4	1			
	NA						1	4	1	2	
	CP		3						5		
	SM	3								2	3
	TC			2	1				5		
	KM	3								3	2
	AB-PC	7	1								

Each figure indicates number of strains which showed the appropriate MIC (mcg/ml).
* Inoculum suspension : 10^8/ml.

For 35 strains of *Salmonella*, the MIC was 25 mcg/ml for 17 strains (48.6 %), 50 mcg/ml for 9 strains (25.7 %), and 100 mcg/ml for 9 strains (25.7 %).

For 22 strains of *Citrobacter*, the MIC was 25~100 mcg/ml for 21 strains (95.0 %) and 200 mcg/ml for 1 strain (5.0 %). The activity of bicyclomycin against *Citrobacter* was higher than that of SM, CP and AB-PC. In addition, there was no strain highly resistant to bicyclomycin, in spite of the high resistance to KM, TC and NA.

For 8 strains of *Enterobacter cloacae*, the MIC was 25 to 100 mcg/ml for 7 strains (87.5 %) and 200 mcg/ml for 1 strain (12.5 %). The activity of bicyclomycin against *Enterobacter cloacae* was lower than that of NA. However, no strain was highly resistant to bicyclomycin, in spite of the high resistance to control antibiotics.

3. Cross-resistance

The MIC of bicyclomycin was determined with strains highly-resistant to SM, CP, TC, KM, NA, or AB-PC. As shown in Table 6, bicyclomycin proved to be as active against these strains as sensitive strains, with MICs from 6.25 to 25 mcg/ml. At a concentration of 100 mcg/ml, bicyclomycin completely suppressed the growth of a strain of *E. coli* that had been artificially induced high resistance to NA (MIC: 800 mcg/ml).

Table 6. Antibacterial activity of bicyclomycin against antibiotic-resistant strains of *E. coli*

Resistant strain	MIC (mcg/ml)		
	Antibiotics tested		Bicyclomycin
SM-resistant *E. coli* 312*	SM	>100	25
CP " *E. coli* 367*	CP	>100	6.25
TC " *E. coli* 328*	TC	>100	25
KM " *E. coli* 323*	KM	>100	25
AB-PC " *E. coli* 323*	AB-PC	>100	25
NA " *E. coli* **	NA	>800	100
Bicyclomycin-resistant *E. coli***	SM	3.13	800
	CP	12.5	
	TC	6.25	
	KM	12.5	
	AB-PC	12.5	
	NA	12.5	

* Clinical isolates
** Artificially induced resistant strains

4. Factors Affecting the Antimicrobial Activity

Seven different media were tested to determine the MICs of bicyclomycin

Table 7. MIC values of bicyclomycin on several different agar media

	MIC (mcg/ml)						
	A*	B	C	D	E	F	G
E coli NIHJ JC-2	50	50	50	50	25	50	50
Salm. typhosa T-287	25	25	50	25	25	50	25
Sh. flexneri 2 a	12.5	12.5	12.5	12.5	6.25	12.5	12.5
Ps. aeruginosa IAM-1095	>800	>800	>800	>800	>800	>800	>800
Staph. aureus 209-P JC-1	>800	>800	>800	>800	>800	>800	>800

* Medium A : Nutrient agar, B : Heart infusion agar, C : Brain heart infusion agar, D : Trypticase soy agar, E : 0.5% peptone agar, F : Antibiotic medium No. 3, G : Modified MUELLER-HINTON agar.
Inoculum suspension : 10^8/ml

against 3 sensitive strains and 2 resistant strains. As shown in Table 7, the composition of the media led to little or no difference in the resultant MICs for *E. coli* NIHJ JC-2, *Salm. typhosa* T-287, and *Sh. flexneri* 2 a. Although not presented in Table 7, a similar trend was noted in 20 strains of clinically isolated strains of *E. coli*. The MICs for 2 resistant strains, *Ps. aeruginosa* IAM-1095 and *Staph. aureus* 209-P JC-1, were independent of the kind of media tested.

As shown in Table 8, the antimicrobial activity of bicyclomycin was unchanged by pH of the test medium in th range from 5 to 8, and inoculum size of 10^4~10^8. To examine the possible effect of addition of serum on the activity of bicyclomycin, MICs of this antibiotic for 5 different organisms were determined on heart infusion agar containing rabbit serum at 3 different concentrations (10, 25, and 50 %). As shown in Table 9, the antimicrobial activity was not altered even at the highest serum concentration. According to these experimental results, it is

Table 8. Effect of inoculum size and pH of test medium on the antibacterial activity of bicyclomycin in heart infusion agar

Organism	Inoculum size (cells/ml)	MIC (mcg/ml)			
		pH 5	pH 6	pH 7	pH 8
E. coli NIHJ JC-2	10^8	50	50	25	50
	10^6	25	25	25	25
	10^4	25	25	25	25
Salm. typhosa T-287	10^8	25	25	25	25
	10^6	25	25	25	25
	10^4	25	25	25	25
Sh. flexneri 2 a	10^8	12.5	12.5	12.5	12.5
	10^6	12.5	12.5	12.5	12.5
	10^4	12.5	12.5	12.5	12.5
Ps. aeruginosa IAM-1095	10^8	>800	>800	>800	>800
	10^6	>800	>800	>800	>800
	10^4	>800	>800	>800	>800
Staph. aureus 209-P JC-1	10^8	>800	>800	>800	>800
	10^6	>800	>800	>800	>800
	10^4	>800	>800	>800	>800

Table 9. Effect of addition of rabbit serum to the test medium on the antibacterial activity of bicyclomycin in heart infusion agar

Organism	Inoculum size (cells/ml)	Serum concentration and MIC (mcg/ml)			
		0 %	10 %	25 %	50 %
E. coli NIHJ JC-2	10^8	50	50	25	50
	10^6	25	25	25	25
Salm. typhosa T-287	10^8	25	25	25	25
	10^6	25	25	25	25
Sh. flexneri 2 a	10^8	12.5	12.5	12.5	12.5
	10^6	12.5	12.5	12.5	12.5
Ps. aeruginosa IAM-1095	10^8	>800	>800	>800	>800
	10^6	>800	>800	>800	>800
Staph. aureus 209-P JC-1	10^8	>800	>800	>800	>800
	10^6	>800	>800	>800	>800

Fig. 2. Bactericidal activity of bicyclomycin against *E. coli* ATCC-27166
MIC : 12.5 mcg/ml. Nutrient broth (Difco).
0~6 hr. Shaking. 6~24 hr. Stationary.

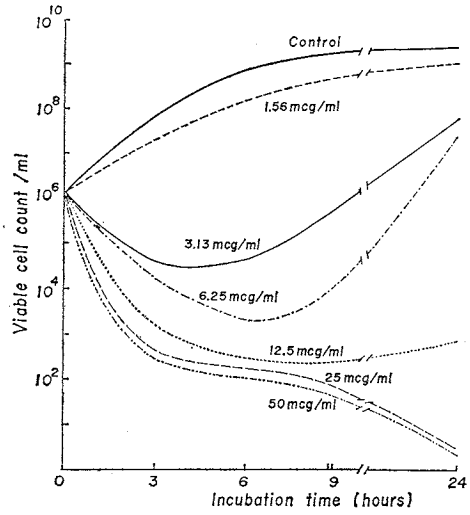
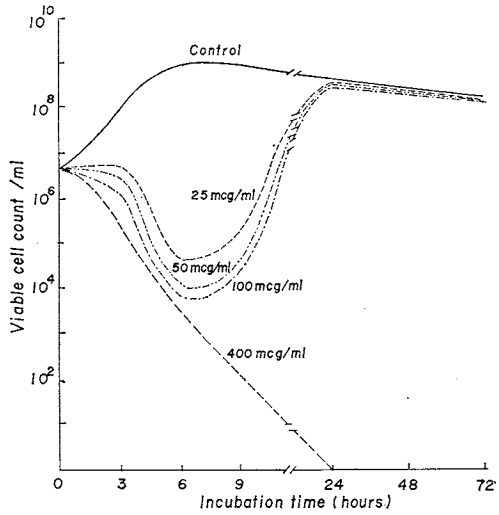


Fig. 3. Bactericidal activity of bicyclomycin against *E. coli* NIHJ JC-2
MIC : 50 mcg/ml. Nutrient broth (Difco).
0~6 hr. Shaking. 6~72 hr. Stationary



clear that the *in vitro* activity of bicyclomycin is not affected by various experimental conditions.

5. Bactericidal Activity

Bicyclomycin was tested for its bactericidal activity by the test procedures described above. The results are shown in Fig. 2 for *E. coli* ATCC-27166.

The lowest concentration of bicyclomycin, *i.e.* 1/8 MIC, or 1.56 mcg/ml, caused little suppression of the growth of the test organism. Concentrations of 1/4 and 1/2 MIC caused transient suppression lasting only 3~6 hours. At MIC level and above, bicyclomycin was bactericidal. A similar procedure was applied to *E. coli* NIHJ JC-2 (Fig. 3), the MIC for which was known to be 50 mcg/ml. After 24-hour incubation, bicyclomycin no longer suppressed the growth even at a concentration as high as 2 MIC, and gave viable cell counts nearly equal to those of the control. Thus, two test strains showed quite different responses.

6. Occurrence of Native Highly Bicyclomycin-Resistant Mutants

As described above, the MIC of bicyclomycin for *E. coli* NIHJ JC-2 is 25~50 mcg/ml. Bicyclomycin at these concentrations completely inhibited formation of visible colonies or turbidity of this organism on agar plates, in nutrient broth, and in heart infusion broth. However, as was described in the preceding section, the shaking cultures of this organism in nutrient broth, containing bicyclomycin at the MIC and 2 MIC, resulted in definite growth after 6-hour incubation, and proliferation comparable to that in the control media after 24~72 hours. In order to clarify the reason of this phenomena, the following experiments were conducted.

(1) Population analysis

A 0.1-ml aliquot of an overnight culture of *E. coli* NIHJ JC-2 in Trypticase soy broth (10^9 cells/ml) was spread on nutrient agar and desoxycholate agar each containing

Table 10. Population analysis of bicyclomycin-sensitivity of *E. coli* NIHJ JC-2

Bicyclomycin (mcg/ml)	Desoxycholate agar		Nutrient agar	
	24 hr.	48 hr.	24 hr.	48 hr.
800	0	0	0	0
400	1	2	0	2
200	3	3	2	2
100	4	4	2	3
50	7	46	18	N.D.
25	∞	∞	∞	∞

N.D.: not done. Inoculum suspension : 10^8/plate

Table 11. Distribution of cells resistant to bicyclomycin in clinical isolates of *E. coli*

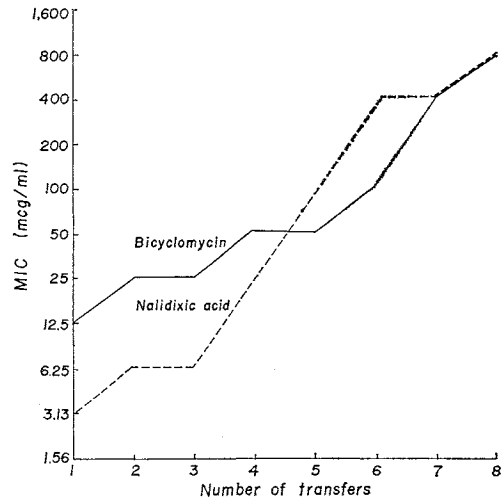
Strain No.	MIC (mcg/ml)	Concentration of bicyclomycin (mcg/ml)		
		100	200	400
13	25	0	0	0
15	25	0	0	0
22	25	1	0	0
23	25	1	0	0
24	25	1	2	0
26	25	0	0	0
27	25	3	1	0
35	25	0	0	0
36	50	1	0	0

Each figure indicates number of colonies grown on the agar medium including various concentrations of bicyclomycin.
Medium : Nutrient agar. Incubaction : 48 hours.

Table 12. Virulence of bicyclomycin-resistant sub-strains from *E. coli* NIHJ JC-2 in mice (ICR-mouse, ♂, 26~30 g, I.P.)

Strain	LD_{50} (I.P.)
E. coli NIHJ JC-2	3×10^7 cells/mouse
〃 200-No. 1	5×10^7 〃
〃 200-No. 3	6×10^7 〃
〃 400-No. 4	3×10^8 〃
〃 400-No. 5	2×10^8 〃

Fig. 4. Rate of development of resistance to bicyclomycin and nalidixic acid
E. coli ATCC-27166



graded concentrations of bicyclomycin, and incubated at 37°C for 24 and 48 hours to count the viable cell units.

The results are shown in Table 10. Although the MIC of bicyclomycin for *E. coli* NIHJ JC-2 has been found to be 50 mcg/ml, some of the 10^8 cells survived at a concentration higher than the MIC of this antibiotic, and formed colonies: 1 or 2 units at 400 mcg/ml, 2 or 3 units at 200 mcg/ml, and 2 to 4 units at 100 mcg/ml. At a concentration of 50 mcg/ml, the numbers of viable cell units after incubation were 7 (24 hours) and 46 (48 hours) on desoxycholate agar, and 18 (24 hours) on nutrient agar. These results indicate that a few mutant cells highly resistant to bicyclomycin occur in every 10^8 cells of this strain. Under such conditions where bicyclomycin is added to a medium to make a concentration of 2 MIC, selected resistant cells are likely to proliferate after prolonged incubation. The same procedure was applied to 9 clinical isolates of *E. coli*. The results are shown in Table 11. When 10^7 or 10^8 cells were inoculated, no clone highly resistant to bicyclomycin was found in any medium containing 400 or 800 mcg/ml of this antibiotic. However, 2 strains, *i.e.* Nos. 24 and 27, formed 2 and 1 colonies, respectively on media containing 200 mcg/ml of bicyclomycin.

(2) Virulence of the resistant cells

Colonies of the resistant strains, derived from *E. coli* NIHJ JC-2, were cultured

in nutrient broth. The virulence of these resistant strains was studied in ICR mice weighing 26~30 g in comparison to the original strain. Bacterial suspensions were injected intraperitoneally into these animals to determine LD_{50} values. No marked change occurred in the virulence of the culture (Table 12).

7. Development of Resistance *in vitro*

Fig. 4 shows the *in vitro* development of resistance of *E. coli* ATCC-27166 to bicyclomycin and NA. The MIC of bicyclomycin increased slowly from 12.5 to 800 mcg/ml, *i.e.*, 64-fold of the parent strain after 8 serial transfers. The rate of resistance development was similar to that of NA.

8. Effect of Binding of Bicyclomycin to Serum Protein

Binding of bicyclomycin with serum protein of man, calves, horses, dogs, rabbits and rats was studied by the centrifugal ultra-filtration method.

As shown in Table 13, the rate of binding to serum protein was generally weak, as evidenced by figures of 41.7 % for man and 16.4 % for horses, in contrast to those of NA (97 % for man and 67 % for horses). The low extent of protein binding for bicyclomycin agrees with the determination of MIC value in the presence of serum.

9. Microbial Degradation of Bicyclomycin

The ability of organisms to degrade bicyclomycin was tested with sensitive strains, including *E. coli* (2 strains) and *Kl. pneumoniae* (1 strain), and with resistant strains, including 1 strain each of *Staph. aureus* FDA 209-P, *Kl. pneumoniae*, and 2 strains of *Ps. aeruginosa*. The results are shown in Table 14. Under conditions specified above, no test organism highly degraded bicyclomycin. Highly resistant and sensitive strains did not differ in their ability to inactivate degradation ranging from 0 to 21 %.

Furthermore, bicyclomycin was not degraded by the resistant sub-strains of *E. coli* NIHJ JC-2. This finding indicates that the resistance of these strains to bicyclomycin is not due to the bacterial degradation of the antibiotic (Table 15).

10. Stability of Bicyclomycin in Tissue Homogenates

One ml of 1,000 mcg/ml bicyclomycin solution was mixed with 1 ml each of 20 % homogenates of liver, kidney, small intestine, and stomach of rats, and allowed to stand at 37°C. As shown in Table 16, no degradation occurred during incubation.

Table 13. Extent of binding of bicyclomycin to serum protein of different animals

Animal	% Bound	
	Bicyclomycin	Nalidixic acid
Human	41.7	97.0
Calf	21.6	88.8
Horse	16.4	66.9
Dog	31.6	67.5
Rabbit	31.7	89.6
Rat	34.8	86.3

One volume of 1,000 mcg/ml solution of bicyclomycin or nalidixic acid was added to 9 volumes of serum, incubated at 37°C for 1 hour, and ultra-filtered.

Table 14. Stability of bicyclomycin in broth cultures

Organism	MIC : mcg/ml	% Degradation
Staph. aureus 209-P JC-1	>1,000	21
E. coli NIHJ JC-2	25	0
〃 324	25	4
Kl. pneumoniae 420	800	19
〃 415	50	19
Ps. aeruginosa IAM-1095	> 800	3
〃 723	> 800	3
Control : Trypticase soy broth		0
〃 : Phosphate buffer		2

One ml of bicyclomycin solution (200 mcg/ml) was added to 9 ml of broth culture, and incubated at 30°C for 3 hours. The potency was assayed by the cup method with *E. coli* ATCC-27166.

Table 15. Degradation of bicyclomycin by resistant sub-strains from *E. coli* NIHJ JC-2

Strain	MIC : mcg/ml	Reaction system	% Degradation
E. coli NIHJ JC-2 native resistant sub-strain No. 1	800	Broth culture	0
		Sonicated culture	0
E. coli NIHJ JC-2 native resistant sub-strain No. 2	800	Broth culture	0
		Sonicated culture	0

One ml of bicyclomycin solution (200 mcg/ml) was added to 9 ml of broth culture or sonicated culture, and incubated at 30℃ for 3 hours. The potency was assayed by the disc method with *E. coli* ATCC-27166.

Table 16. Stability of bicyclomycin in rat tissue homogenates

Homogenate	% Degradation	
	30 min.	90 min.
Liver	0	0
Kidney	0	0
Stomach	0	0
Small intestine	0	0

One ml of bicyclomycin solution (100 mcg/ml) was added to 9 ml of 20% tissue homogenate, and incubated at 37℃. The potency was assayed by the disc method with *E. coli* ATCC 27166.

This suggests that bicyclomycin has satisfactory stability to tissue enzymes.

11. Stability in Liquid Phases

(1) 0.1 M Phosphate buffer (pH 6, 7, and 8)

Fig. 5 shows the residual activities of bicyclomycin in 0.1 M phosphate buffer after 5 days at 3 different temperatures. Bicyclomycin was more stable in acidic solution than in alkaline solution, and gradually degraded as the temperature increased. When kept at 5°C, bicyclomycin was stable for at least 5 days at pH 6, and for 24

Fig. 5. Stability of bicyclomycin in 0.1 M phosphate buffer (100 mcg/ml)

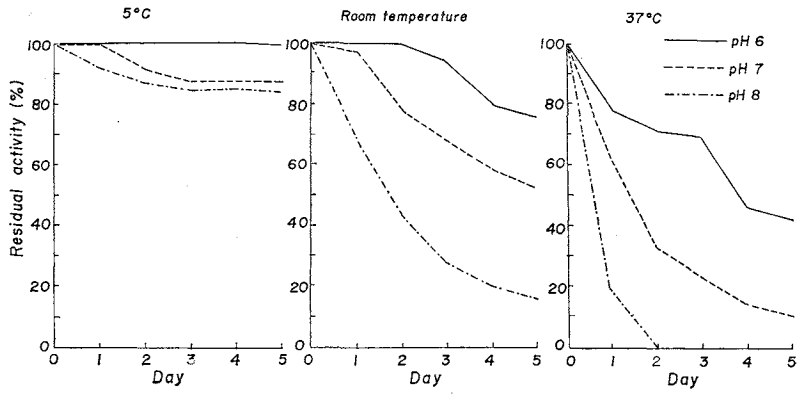
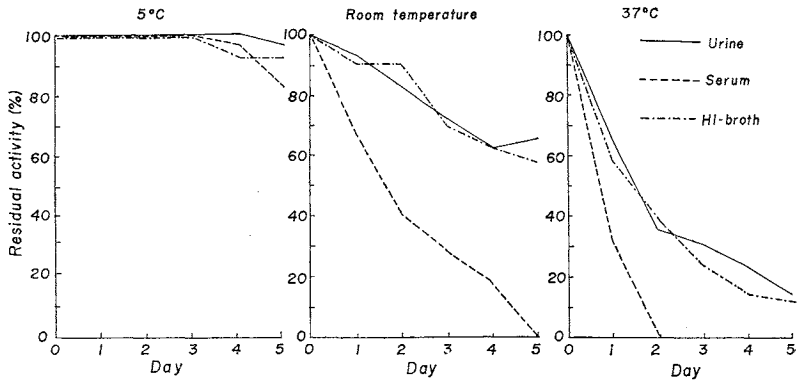


Fig. 6. Stability of bicyclomycin in heart infusion broth, human serum and urine (100 mcg/ml)



hours at pH 7. At pH 8, about 10 % of bicyclomycin was inactivated after 24 hours' storage.

(2) Heart infusion broth, human serum and urine

The results are shown in Fig. 6. When incubated at 37°C for 24 hours, about 40 % of bicyclomycin in heart infusion broth or urine and 70 % in serum was inactivated. At low temperatures, bicyclomycin was relatively stable: when kept at 5°C, it undergoes no degradation for 4 days in serum, 5 days in urine and 3 days in heart infusion broth.

12. Protective Effect against Experimental Infections in Mice with *E. coli*

Mice were challenged intraperitoneally with clinical isolates of *E. coli* that were resistant to any of the control antibiotics. The *in vivo* activity of bicyclomycin against these infections was expressed in terms of the ED_{50} values. Results are shown in Table 17.

Table 17. Protective effect of bicyclomycin against experimental infection of *E. coli* in mice

*E. coli**	Antibiotic**	MIC (mcg/ml)	ED_{50} (mg/mouse)
336	Bicyclomycin	25	0.95
	Ampicillin	3.13	0.55
312	Bicyclomycin	25	3.05
	Ampicillin	6.25	0.63
	Chloramphenicol	>100	> 28
324	Bicyclomycin	25	0.69
	Ampicillin	6.25	0.63
	Tetracycline	>100	>28
323	Bicyclomycin	25	4.05
	Ampicillin	>100	>28
	Kanamycin	>100	>28
320	Bicyclomycin	25	0.87
	Streptomycin	>100	17.09
335	Bicyclomycin	50	0.88
	Nalidixic acid	12.5	0.54

* Ten mice (ICR strain 27~30 g) of each group were challenged intraperitoneally with 0.5 ml of overnight culture.
** The antibtotics were administered subcutaneously once 1 hour after the challenge.

Although the *in vitro* activity of bicyclomycin was 1/4 to 1/8 that of AB-PC for 3 strains of *E. coli* (Nos. 312, 324, and 336) served as the challenging organisms, the ED_{50} values of this antibiotic for infections with the strains 336 and 324 fell within the range between 0.69 and 0.95 mg/mouse, and indicated satisfactory therapeutic efficacy almost comparable to that of AB-PC. In the case of No. 312 strain, however, the difference in the ED_{50} value paralleled the difference in MIC. Also, the therapeutic activity of bicyclomycin for infections with several strains of *E. coli* which are resistant to the control antibiotics was compared with that of the control antibiotics. In this experiment, bicyclomycin was more effective than the control antibiotics, as shown by the following figures. The ED_{50} of bicyclomycin for infection with CP-resistant *E. coli* 312 was as low as 3.05 (1.47~7.66) mg/mouse. The ED_{50} of CP for this infection exceeded 28 mg/mouse. Unlike AB-PC or KM, which gave ED_{50} values above 28 mg/mouse for infection with *E. coli* 323, bicyclomycin was effective at 4.05 (0.94~17.5) mg/mouse. Bicyclomycin also proved to be effective for infections with SM-resistant *E. coli* 320 and TC-resistant *E. coli* 324. In an infection with the NA-sensitive strain 335 (MIC : NA 12.5 mcg/ml), NA and bicyclomycin were almost equally effective, as is evidenced by similar ED_{50} values : 0.88 mg/mouse for bicyclomycin and 0.54 (0.26~1.12) mg/mouse for NA.

Discussion

The present study on bicyclomycin clarified the characteristic antimicrobial activity of this antibiotic. This antibiotic proved to be specifically active against Gram-negative organisms, including species of *E. coli*, *Klebsiella*, *Salmonella*, *Shigella*, *Neisseria*, *Citrobacter* and *Enterobacter cloacae*, but not active against species of *Proteus* and *Pseudomonas aeruginosa*. The MICs of bicyclomycin for various sensitive strains of *E. coli* are focused on a narrow range between 25 and 50 mcg/ml. This profile resembles that of KM in spite of the wide structural difference between the antibiotics. Although the mechanism of action of this antibiotic is not known, low toxicity in acute and subacute toxicological experiments with rats and dogs indicates that it affects specifically bacterial cell functions.

The fact that bicyclomycin-resistant cultures failed to degrade this substance, indicate that in these cultures it cannot penetrate the cell membrane. Unlike SM or rifampicin[3], bicyclomycin, did not exhibit a one-step development of resistance, but the occurrence of naturally resistant cells was occasionally observed. The occurrence of such resistant cells has also been reported in the case of rifampicin.[4,5]

One of the advantages of bicyclomycin over usual antibiotics is the absence of *in vitro* and *in vivo* cross-resistance between this antibiotic and other commercially available antibiotics for Gram-negative organisms.[6] Furthermore, bicyclomycin displayed effectiveness nearly equivalent to that of AB-PC for the treatment of experimental infections with *E. coli* Nos. 336 and 324, although its *in vitro* activity was apparently inferior to that of AB-PC. This must be explained by absorption and excretion, stability in the living system, or incorporation into the infected lesion.

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