ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1973, p. 40-48

Vol. 3, No. 1
Printed in U.S.A.

Microbiological Properties of a New Cephalosporin, BL-S 339: 7-(Phenylacetimidoylaminoacetamido)-3-(2-Methyl-1,3,4-Thiadiazol-5-Ylthiomethyl)Ceph-3-em-4-Carboxylic Acid

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Received for publication 28 September 1972

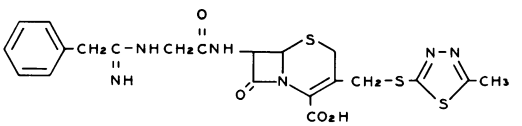
BL-S 339 is a new broad-spectrum, parenterally effective cephalosporin whose expression of antibacterial activity in vitro is markedly affected by the nature of the assay medium. When assayed in nutrient agar, BL-S 339 was more active than cephalothin against strains of *Diplococcus pneumoniae*, *Streptococcus pyogenes*, *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter*, and indole-positive *Proteus* sp. However, when assayed in Mueller-Hinton medium, its activity, especially against gram-negative bacteria, was reduced substantially, whereas the activity of cephalothin was virtually unaffected by the assay medium. The in vivo activity of BL-S 339 correlated well with its activity in nutrient agar; when administered subcutaneously to mice, it was therapeutically more efficacious than cephalothin in infections caused by both gram-positive and gram-negative bacteria. When BL-S 339 was administered intramuscularly to mice, the concentrations achieved in the blood were three times those achieved with cephalothin. BL-S 339 was bound to human serum proteins to the same extent as cephalothin. Recovery of BL-S 339 in the urine within the 24-hr period after intramuscular administration to rats was three times that of cephalothin.

BL-S 339 is a new broad-spectrum, parenterally effective cephalosporin (Fig. 1). It was selected from a general cephalosporin-screening program and represents one of the most active compounds of a series of 7-(substituted-imidoylaminoacetamido) derivatives synthesized by C. Holdrege and S. Baker of Bristol Laboratories. This report presents comparative data on the microbiological and pharmacological properties of BL-S 339 and cephalothin, including the effect of the assay medium on their activity against a variety of clinical isolates. Data on the parenteral absorption, urinary excretion, and therapeutic efficacy of these compounds in rodents are also presented.

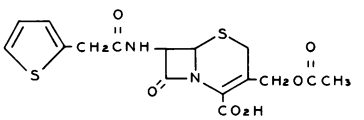
MATERIALS AND METHODS

Antibiotics. BL-S 339 was synthesized by S. R. Baker of the Bristol Laboratories Organic Chemistry Department. The crystalline form used in these studies was prepared by M. A. Kaplan of the Product Development Department and was more than 95% pure. Its solubility in water was limited to 5 mg/ml and in phosphate buffer (pH 7) to 10 mg/ml. When administered subcutaneously to mice in experimental infections, BL-S 339 was suspended in a polysorbate-carboxymethylcellulose formulation. Commercially available cephalothin (Keflin, Eli Lilly & Co.) was used as the control compound.

In vitro antibacterial activity. The susceptibility of a number of clinical strains of *Diplococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Proteus* sp., *Klebsiella pneumoniae*, *Enterobacter* sp., *Serratia marcescens*, *Salmonella* sp., and *Escherichia coli* to BL-S 339 and cephalothin was determined by a twofold agar dilution method. The antibiotics were incorporated into Mueller-Hinton medium (MHM, Difco) and into nutrient agar (NA) which was prepared by the addition of 1% Ionagar (Colab Laboratories, Inc.) to dehydrated nutrient broth (Difco). The agar surface was inoculated with 10^{-2} dilutions of overnight broth cultures (*D. pneumoniae* and *S. pyogenes* were used undiluted) by means of the inoculum-replicating apparatus de-



BL-S 339



CEPHALOTHIN

FIG. 1. *Structures of BL-S 339, 7-(phenylacetimidoylaminoacetamido)-3-(2-methyl-1,3,4-thiadiazol-5-ylthiomethyl)ceph-3-em-4-carboxylic acid, and cephalothin.*

scribed by Steers et al. (4). Susceptibility of *D. pneumoniae* and *S. pyogenes* to the antibiotics was determined on the above-described media supplemented with 4% defibrinated sheep blood. Minimal inhibitory concentrations (MIC) were determined after overnight incubation at 37 C.

The influence of inoculum size on the inhibitory activity of BL-S 339 and cephalothin was determined as above, except that the cell concentration of the inoculum was varied by using undiluted samples as well as 10^{-2} and 10^{-4} dilutions of overnight broth cultures of two strains of *S. aureus* and one each of *E. coli* and *K. pneumoniae*.

The bactericidal activity of BL-S 339 was compared with that of cephalothin as follows. Tubes of nutrient broth and Mueller-Hinton broth (Difco) containing various concentrations of the drugs were inoculated with a standard number of cells of each of the four strains listed above, and were then incubated over a period of 24 hr at 37 C. After incubation, 0.1 ml of appropriately diluted samples of broth containing the drug-exposed cells was plated on NA so that the number of viable cells present could be estimated. The minimal bactericidal concentration (MBC) was the lowest concentration of compound permitting the survival of <100 cells/ml.

Stability in solution. The stability of BL-S 339 and cephalothin in 0.005 M phosphate buffer, *p*H 7.4, was determined. Solutions containing 100 μg of compound/ml were incubated at 37 C for 23 hr. Samples were removed at various intervals, and their residual activity was measured by bioassay (agar diffusion-cylinder plate method with *Bacillus subtilis* ATCC 6633 as the assay organism). The half-life of the compound was determined graphically.

Serum binding. The extent to which BL-S 339 and cephalothin are bound to human serum proteins was determined by the agar diffusion method of Scholtan and Schmid (3). Solutions of the antibiotics were prepared over a range of concentrations in 0.001 M phosphate buffer (*p*H 7.4) and in 95% human serum. These solutions were then bioassayed with *B. subtilis* as the test organism. Any reduction in the expected potency of the drug in the "spiked" serum samples was considered to be attributable to protein binding.

Absorption in mice. Drug concentrations in the blood of male Swiss-Webster mice weighing about 20 g each were determined at 0.25, 0.5, 1, and 1.5 hr after intramuscular administration of the antibiotics at a dose of 10 mg/kg of body weight. The techniques used for dosing, bleeding, and assaying were essentially those described by Price et al. (2). Eight mice were dosed with each compound and bled via the orbital sinus. Blood samples were assayed with *B. subtilis* as the test organism.

Urinary excretion in rats. The percentage of the antibiotics recovered in the urine of rats was determined after intramuscular administration of a 10 mg/kg dose of each. Four fasted, individually housed, Sprague-Dawley rats were used for each compound. Urine specimens were collected over dry ice during intervals of 0 to 6 and 6 to 24 hr postadministration. These were assayed against a standard prepared by spiking normal urine with known quantities of drug. Urine samples were assayed by the agar diffusion-cylinder plate method with *B. subtilis* as the assay organism.

Acute toxicity in mice. The drug dosage producing 50% mortality (LD_{50}) in normal male Swiss-Webster mice was determined after subcutaneous administration of BL-S 339 and cephalothin. Survivors on the fourth day after administration of a single dose were noted, and the LD_{50} was calculated by the method of Litchfield and Wilcoxon (1).

Activity in experimental mouse infections. The dose of BL-S 339 and cephalothin protecting 50% of the mice from death (PD_{50}) was determined in animals infected with various bacteria. Male Swiss-Webster mice weighing about 20 g were infected intraperitoneally with sufficient bacterial cells to kill untreated mice within 72 hr. *D. pneumoniae*, *S. pyogenes*, and *K. pneumoniae* were suspended in brain heart infusion broth (Difco), and challenge doses of *E. coli*, *Proteus mirabilis*, *P. morganii*, *Enterobacter* sp., and *S. aureus* were in broth containing 4% mucin. The drugs were administered subcutaneously 1 and 3.5 hr postinfection. A log-probit plot of the percentage of survivors 72 hr after infection versus the dose (in milligrams per kilogram of body weight per treatment) was used to estimate the PD_{50}.

RESULTS AND DISCUSSION

In vitro studies. The susceptibility of a number of clinical isolates of various bacterial species to BL-S 339 and cephalothin on NA and MHM is illustrated in Fig. 2–7, in which the cumulative percentage of strains inhibited is plotted against the MIC of drug in micrograms per milliliter. The data show that the activity of both antibiotics was affected by the assay medium, but BL-S 339 was affected to a greater extent than cephalothin.

As can be seen from the data in Fig. 2, BL-S 339 was about twice as active as cephalothin against *D. pneumoniae* on both media. Against

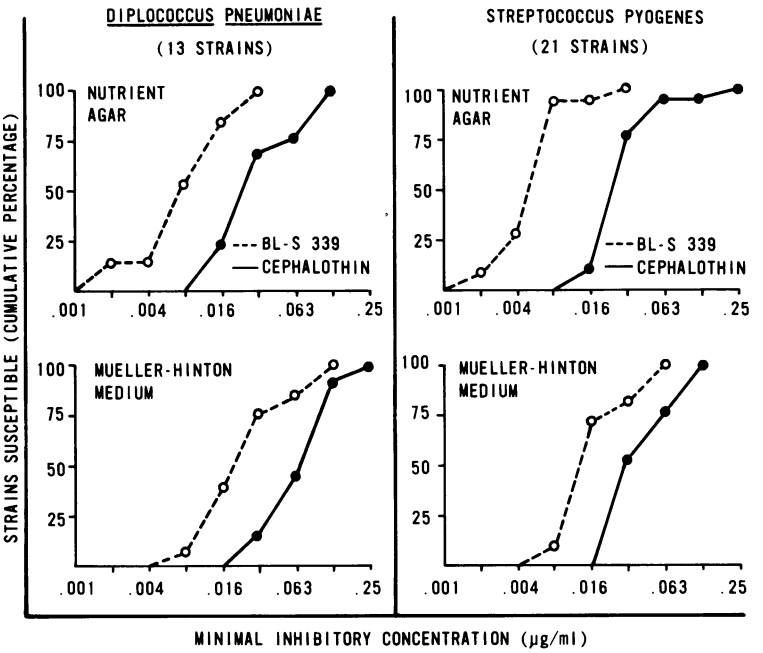


FIG. 2. *Effect of assay medium on the susceptibility of Diplococcus pneumoniae and Streptococcus pyogenes to BL-S 339 and cephalothin. Both media were supplemented with 4% sheep blood.*

S. pyogenes, BL-S 339 was four times as active as cephalothin on NA, but only twice as active on MHM. This change was primarily due to a reduction in the activity of BL-S 339, since cephalothin was almost equally active on both media.

In contrast to results obtained with the above organisms, *S. aureus*, as can be seen from the data in Fig. 3, was about twice as susceptible to cephalothin as to BL-S 339 on both media, regardless of the organism's penicillinase-producing ability. However, a much wider range of MIC values was obtained with penicillinase-producing strains than with nonproducers. In addition, the activity of the antibiotics was not entirely independent of the assay medium, as both compounds were somewhat less active on MHM than on NA.

As indicated by the data in Fig. 4, BL-S 339 was less active than cephalothin against *P. mirabilis* on both media: about 4-fold on NA and 32-fold on NHM. The higher degree of sensitivity of BL-S 339 to medium effects, relative to cephalothin, is strikingly illustrated by the MIC data obtained with indole-positive *Proteus* species. Here, BL-S 339 was markedly more effective than cephalothin on NA; for example, the percentage of strains susceptible to a 16 μg/ml concentration of drug was about 90 for BL-S 339 as compared to only 45 for cephalothin. However, when the compounds were assayed on MHM, the percentages of susceptible strains at the above concentration were 0 and 35, respectively, for BL-S 339 and cephalothin.

The response of *K. pneumoniae* and *Enterobacter* sp. to the two antibiotics when tested on NA and NHM is shown in Fig. 5. The activity of BL-S 339 against both organisms was substantially less on MHM than on NA, whereas that of cephalothin was virtually the same on both media. BL-S 339 was somewhat more effective than cephalothin against *K. pneumoniae* on NA, but the order of activity of the antibiotics was reversed on MHM. Against *Enterobacter* sp., BL-S 339 was about eight times as active as cephalothin on NA, but this superiority was virtually lost on MHM.

The susceptibility of *S. marcescens* and *Salmonella* sp. to the antibiotics in tests utilizing NA and MHM is shown in Fig. 6. *S. marcescens* was relatively insensitive to cephalothin on both media and to BL-S 339 on MHM. However, on NA, BL-S 339 inhibited about 50% of the strains at 16 μg/ml and over 90% at 63 μg/ml. Against *Salmonella* sp., BL-S 339 was slightly more active than cephalothin on NA, but significantly less effective than cephalothin on MHM. Once again, the influence of assay medium on MIC values was relatively small

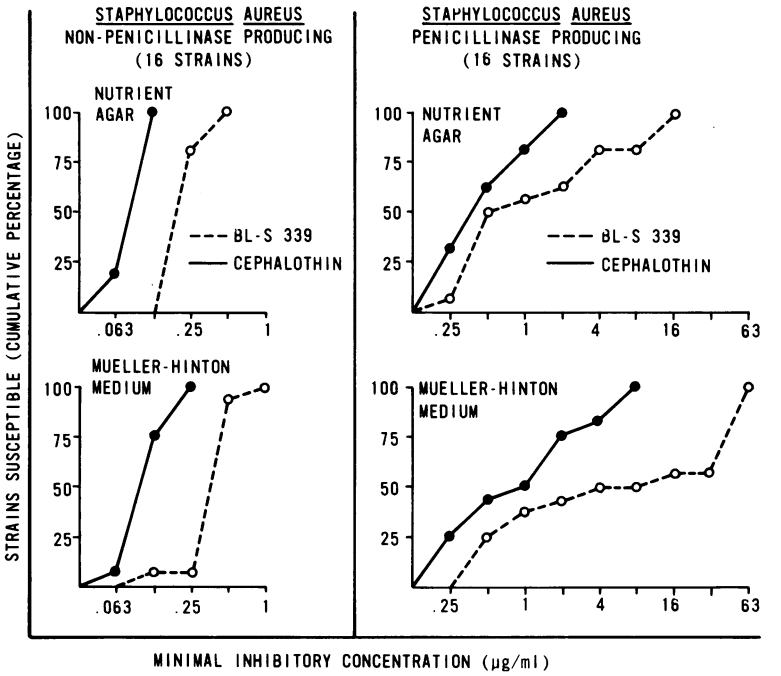


FIG. 3. *Effect of assay medium on the susceptibility of penicillinase-nonproducing and penicillinase-producing strains of Staphylococcus aureus to BL-S 339 and cephalothin.*

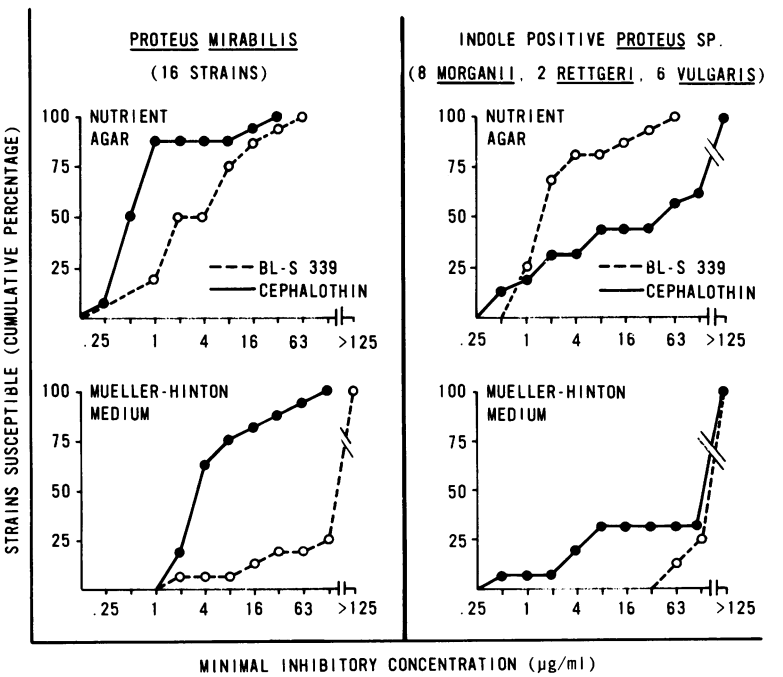


FIG. 4. *Effect of assay medium on the susceptibility of Proteus mirabilis and indole-positive Proteus sp. to BL-S 339 and cephalothin.*

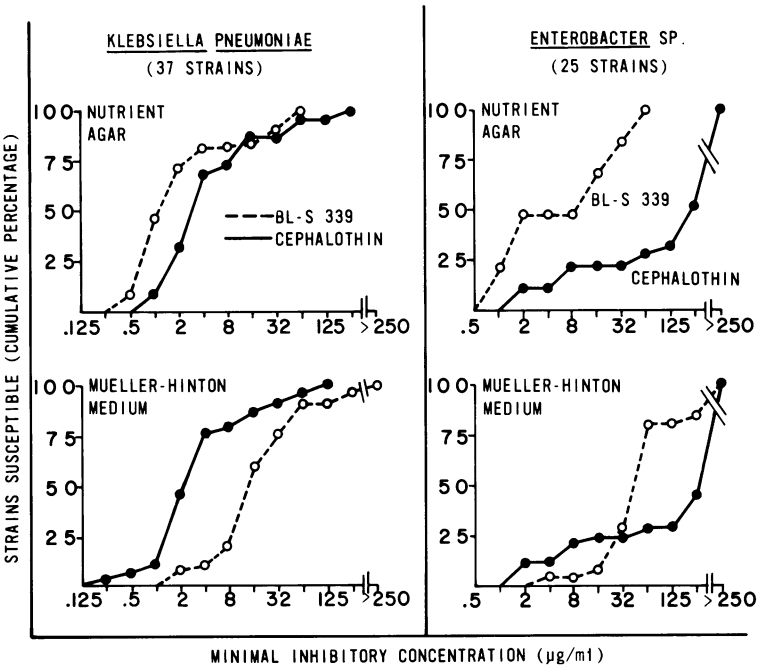


FIG. 5. *Effect of assay medium on the susceptibility of Klebsiella pneumoniae and Enterobacter sp. to BL-S 339 and cephalothin.*

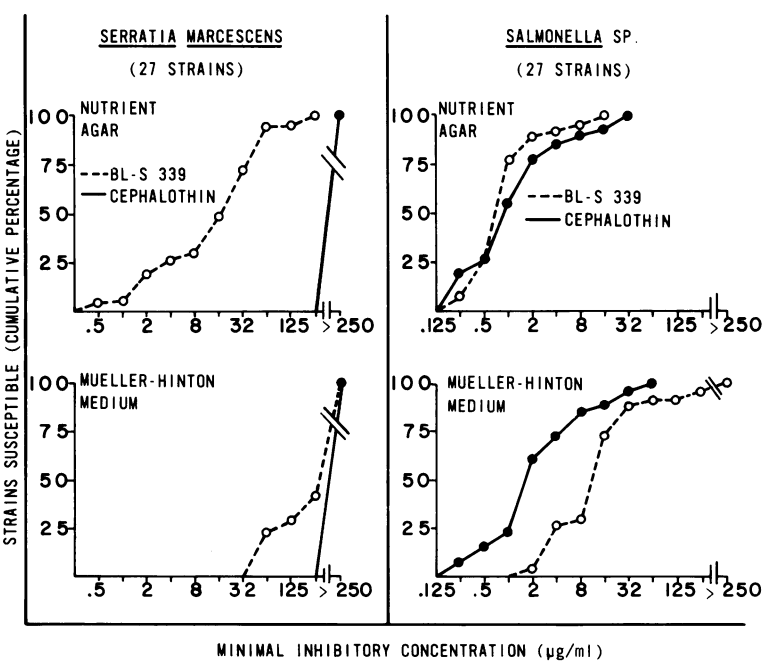


FIG. 6. *Effect of assay medium on the susceptibility of Serratia marcescens and Salmonella sp. to BL-S 339 and cephalothin.*

for cephalothin as compared to BL-S 339.

The comparative effect of medium on the susceptibility of *E. coli* to the two antibiotics is illustrated in Fig. 7. On NA, BL-S 339 was substantially more effective than cephalothin, whereas on MHM it was slightly less active than cephalothin. Again, this reversal in effectiveness was primarily due to a reduction in BL-S 339 activity as cephalothin was about equally active on both media.

Overall, when assayed on NA, BL-S 339 was more active than cephalothin against all organisms tested except *S. aureus* and *P. mirabilis*. However, the superiority in activity of BL-S 339 over that of cephalothin against *E. coli*, *K. pneumoniae*, *Salmonella* sp., *Enterobacter* sp., and indole-positive *Proteus* sp. observed on NA was lost on MHM.

The effect of inoculum size on the activity of BL-S 339 and cephalothin was determined on NA and MHM by use of 10^0, 10^{-2}, and 10^{-4} dilutions of 18-hr broth cultures of two strains of *S. aureus* and one each of *E. coli* and *K. pneumoniae*. The data in Table 1 show that changes in inoculum size had a similar effect on the MIC of each of the antibiotics on both media. A 10,000-fold increase in inoculum size (from 10^{-4} to 10^0 dilution) of two strains of *S. aureus*, one which produced penicillinase and one which did not, had virtually no effect on the inhibitory activity of BL-S 339 and cephalothin; increases in MIC values were no greater than twofold. However, the same increase in the size of the inoculum of *E. coli* and *K. pneumoniae* resulted in four- to eightfold increases in MIC values.

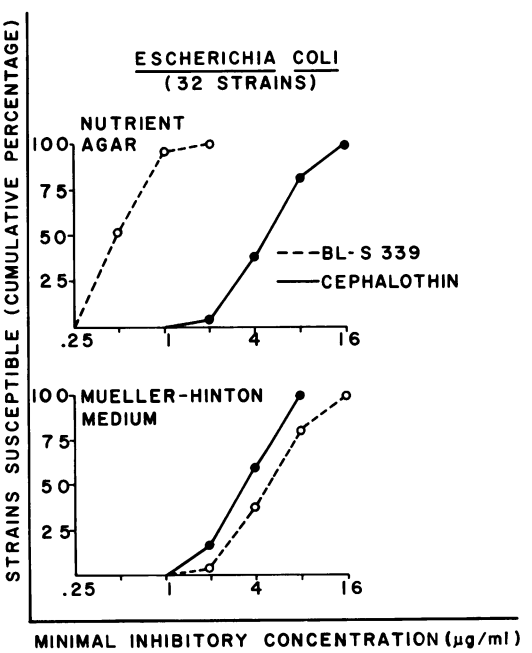


FIG. 7. *Effect of assay medium on the susceptibility of Escherichia coli to BL-S 339 and cephalothin.*

The relative bactericidal activity of BL-S 339 and cephalothin against the four strains listed above was determined in nutrient broth and Mueller-Hinton broth. The data in Table 2 show that the two antibiotics had comparable effectiveness against the two *S. aureus* strains in both media, the MBC being 2- to 4-fold higher than the MIC for the penicillinase-nonproducing strain and 8- to 16-fold higher for the penicillinase producer. Against *E. coli* and *K. pneumoniae*, however, the bactericidal activity of BL-S 339 differed markedly from that of cephalothin. The MBC of BL-S 339 was 8- to 63-fold higher than the MIC for the two strains, whereas the differences between the MBC and MIC values of cephalothin were negligible.

BL-S 339 was quite stable in buffer solution at *p*H 7.4 and 37 C. Its half-life was estimated to be about 11.5 hr, whereas that of cephalothin was >24 hr. Both antibiotics were bound to human serum proteins to about the same extent, 68 to 72%.

In vivo studies. The concentrations of BL-S 339 and cephalothin in the blood of mice were determined after intramuscular administration of a 10 mg/kg dose. The data in Fig. 8 show that BL-S 339 was well absorbed by this route, producing blood levels which were three times higher and more prolonged than those of cephalothin. The highest concentrations of both antibiotics were observed during the first of the four sampling periods (15 min postadministration). At 0.25, 0.5, 1, and 1.5 hr after administration, the concentrations of BL-S 339 in the blood were 16.6, 14.8, 8.7 and 5.0 μg/ml, respectively, whereas those of cephalothin were 5.6, 2.4, 1.6, and <0.7 μg/ml, at the same postadministration times. Data obtained in oral studies are not reported because both antibiotics were poorly absorbed when administered by this route.

The relative extent to which BL-S 339 and cephalothin are eliminated via the rat urinary tract after intramuscular administration of a 10 mg/kg dose is shown by means of a bar graph (Fig. 9). The data indicate that nearly all of the recoverable portion of both compounds was excreted during the first 6 hr after administration (57.3% of BL-S 339 and 18-1% of cephalothin). Only negligible amounts of each drug were recovered between 6 and 24 hr after administration. Recovery of BL-S 339 in the

TABLE 1. *Effect of inoculum size on the inhibitory activity of BL-S 339 and cephalothin determined on two assay media*

Test organism	Compound	Nutrient agar			Mueller-Hinton medium		
		10^{0a}	10^{-2}	10^{-4}	10^{0}	10^{-2}	10^{-4}
Staphylococcus aureus A9537 (penicillinase-nonproducer)	BL-S 339	0.5[b]	0.5	0.5	1	0.5	0.5
	Cephalothin	0.25	0.25	0.25	0.25	0.13	0.13
S. aureus A9606 (penicillinase producer)	BL-S 339	1	1	0.5	2	1	1
	Cephalothin	0.5	0.25	0.25	0.5	0.25	0.25
Escherichia coli A15119	BL-S 339	8	4	2	32	16	8
	Cephalothin	16	8	4	32	16	8
Klebsiella pneumoniae A15130	BL-S 339	32	4	4	32	8	4
	Cephalothin	32	8	4	32	4	4

[a] Dilution of culture. Undiluted broth culture contained 3.3×10^8 to 7.0×10^8 cells/ml.
[b] MIC, in micrograms per milliliter, determined by an agar dilution method.

TABLE 2. *Comparative bactericidal activity of BL-S 339 and cephalothin*

Compound	Assay medium	Test organism[a]							
		S. aureus A9537 (pen'ase −)		*S. aureus* A9606 (pen'ase +)		*E. coli* A15119		*K. pneumoniae* A15130	
		MIC[b]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC
BL-S 339	NB[c]	0.25	1	0.5	8	2	32	2	125
	MHB	0.25	0.5	1	8	16	125	8	>250
Cephalothin	NB	0.25	0.5	0.5	4	8	16	8	16
	MHB	0.13	0.5	0.25	2	16	16	4	8

[a] Initial cell counts ranged from 3.3×10^4 to 7.0×10^4 cells/ml. Pen'ase − and + indicate penicillinase-nonproducing and -producing strains, respectively.
[b] MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration), in micrograms per milliliter.
[c] NB = Nutrient broth; MHB = Mueller-Hinton broth.

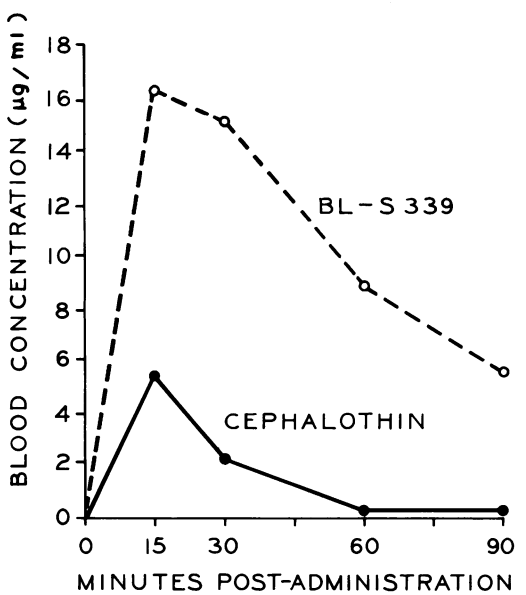


FIG. 8. *Average concentrations of BL-S 339 and cephalothin in the blood of mice after intramuscular administration of a 10 mg/kg dose.*

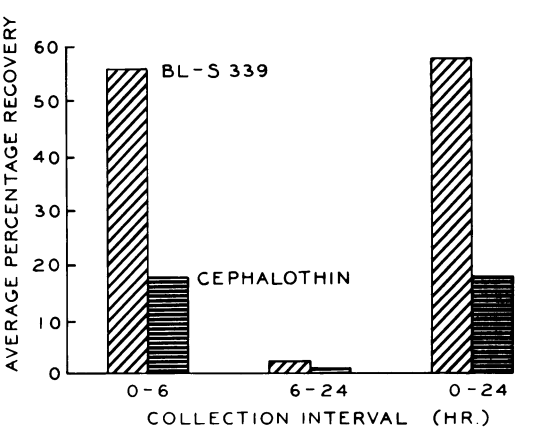


FIG. 9. *Percentage recovery of BL-S 339 and cephalothin from the urine of rats after intramuscular administration of a 10 mg/kg dose.*

urine within the 24-hr period after administration was three times that of cephalothin, or 59.3% of the administered dose versus 18.3% of cephalothin.

An indication of the relative acute toxicity

of BL-S 339 and cephalothin was obtained by administering each of the compounds subcutaneously to normal male mice weighing 18 g. The LD_{50} in mice by this route was in excess of 4,000 mg/kg for both drugs.

A comparative study was made of the therapeutic effectiveness of BL-S 339 and cephalothin in mice experimentally infected with a variety of pathogens. The results obtained with these drugs when administered subcutaneously in infections caused by gram-positive bacteria are shown in Table 3, and those obtained in infections caused by gram-negative organisms are presented in Table 4. Included in the tabulated data are the comparative MIC values of both drugs obtained on NA and MHM against these same organisms.

BL-S 339 was a more effective therapeutic agent than cephalothin in experimental infections caused by gram-positive organisms. Furthermore, the MIC values of BL-S 339 on NA predicted its degree of in vivo activity, relative to that of cephalothin, more accurately than did MIC values obtained on MHM. As can be seen in Table 3, BL-S 339 was about 40 times more active than cephalothin against infections caused by *D. pneumoniae* and *S. pyogenes*, whereas the antibiotics were about equally efficacious in infections caused by penicillinase-producing or nonproducing strains of *S. aureus*.

The data in Table 4 show that, in experimental infections of mice caused by gram-negative organisms, BL-S 339 was therapeutically more effective than cephalothin against the test strains of *K. pneumoniae* (2-fold), *E. coli* (≥20-fold), *P. morganii* (>4-fold), and *Enterobacter* sp. (>3-fold). The two antibiotics were equally active against *P. mirabilis*. Again, the antibacterial activity of BL-S 339 on NA reflected its degree of in vivo activity, relative to that of cephalothin, more realistically than

TABLE 3. *Comparative activity in vitro and in vivo of BL-S 339 and cephalothin against gram-positive bacteria*

Organism	MIC[a] (μg/ml)				PD_{50}[b] (mg/kg)	
	BL-S 339		Cephalothin			
	NA	MHM	NA	MHM	BL-S 339	Cephalothin
Diplococcus pneumoniae A9585	0.001	0.008	0.002	0.004	0.3	12
Streptococcus pyogenes A9604	0.002	0.063	0.016	0.032	0.25	9
Staphylococcus aureus A9537 (penicillinase-nonproducing)	0.25	0.5	0.13	0.13	0.1	0.1
S. aureus A9606 (penicillinase-producing)	0.25	1	0.25	0.25	7	9

[a] Minimal inhibitory concentrations on nutrient agar (NA) and Mueller-Hinton medium (MHM). Media seeded with *Diplococcus pneumoniae* and *Streptococcus pyogenes* were supplemented with 4% sheep blood.

[b] Dose required per treatment to protect 50% of infected mice from death. Mice were treated subcutaneously at 1 and 3.5 hr postchallenge.

TABLE 4. *Comparative activity in vitro and in vivo of BL-S 339 and cephalothin against gram-negative bacteria*

Organism	MIC[a] (μg/ml)				PD_{50}[b] (mg/kg)	
	BL-S 339		Cephalothin			
	NA	MHM	NA	MHM	BL-S 339	Cephalothin
Klebsiella pneumoniae A9977	0.5	16	2	2	136	280
Escherichia coli A15119	0.5	16	4	4	8	162
E. coli A15164	1	16	>250	>250	32	>800
Proteus mirabilis A9900	1	32	4	8	108	128
P. morganii A15153	2	>125	>125	>125	180	>800
Enterobacter sp. A20464	63	>250	>250	>250	240	>800

[a] Minimal inhibitory concentrations on nutrient agar (NA) and Mueller-Hinton medium (MHM).

[b] Dose required per treatment to protect 50% of infected mice from death. Mice were treated subcutaneously at 1 and 3.5 hr postchallenge.

did its activity on MHM.

In summary, BL-S 339, a 7-(substituted-imidoylaminoacetamido)cephalosporin C derivative, was found to be superior to cephalothin on the basis of its in vitro antibacterial activity on NA, its parenteral absorbability, and its therapeutic efficacy in experimental infections of mice.

ACKNOWLEDGMENTS

We thank M. C. Arnold, R. E. Buck, K. L. DenBleyker, T. Ingram, and G. E. Wright for capable technical assistance. We also extend our appreciation to Y. H. Tsai and D. R. Chisholm for conducting the in vivo studies and to E. Barenholtz for clerical assistance.

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