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Comparative Study with Enoxacin and Netilmicin in a Pharmacodynamic Model To Determine Importance of Ratio of Antibiotic Peak Concentration to MIC for Bactericidal Activity and Emergence of Resistance

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An in vitro pharmacokinetic model was used to study the comparative antibacterial activities of multiple-dose regimens of enoxacin and netilmicin. Strains of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus* were exposed to changing drug concentrations, mimicking human two-compartment pharmacokinetics. Oral administration was simulated for the quinolone, and intravenous administration was simulated for the aminoglycoside. Similar ratios of peak concentration to MIC resulted in similar changes in bacterial concentrations over time with both compounds. Following the initial dose, a rapid bactericidal effect occurred, with a >99% reduction of the bacterial counts within 4 h at peak concentrations more than three times the MIC. However, bacterial regrowth occurred within 24 h unless the peak concentration/MIC ratio exceeded 8:1 ($P < 0.01$). For the regrowing bacteria, MICs were four- to eightfold higher, and little or no bactericidal effect occurred following the second and subsequent doses. These data demonstrate the equally potent bactericidal activity of orally administered enoxacin and intravenously administered netilmicin. Selection of resistant subpopulations was similar with each drug. The peak concentration/MIC ratio may be an important parameter in the clinical use of quinolone and aminoglycoside antibiotics.

Similar to other newly developed fluoroquinolones, enoxacin has a broad spectrum of activity, particularly against gram-negative bacterial pathogens, at concentrations which are achievable in humans following oral administration (17, 18). Clinical experience in the use of these compounds is still too limited to define their ultimate position relative to older antibiotics (11). In many situations these drugs may offer a potentially less toxic alternative to parenteral aminoglycoside therapy. This in vitro study compares the activity of a new fluoroquinolone and an aminoglycoside against major bacterial pathogens in a model which exposes bacteria to oscillating drug concentrations in a manner that mimics human pharmacokinetics.

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MATERIALS AND METHODS

Apparatus. An in vitro two-compartment model which allows exposure of bacteria to changing concentrations of antibiotics was used in this investigation. This pharmacokinetic model has been described previously in detail (5, 6). However, the simulation of oral drug administration required a modification of the original design. An absorption compartment into which the dose was given as a bolus injection to achieve first-order absorption kinetics in the central compartment was added (Fig. 1). Intravenous drug administrtion was simulated by omitting the absorption compartment and directly infusing the drug into the central compartment.

In brief, the model consisted of a sterile central compartment (central reservoir, tubing, and lumina of capillary bundles), representing the systemic circulation, and several peripheral compartments for bacteria, representing extravascular infection sites. Bacteria were cultured in artificial capillary units (Vitafiber; Amicon Corp., Lexington, Mass.) through which bundles of capillaries from the central compartment coursed. Selectively permeable capillary walls with a nominal retention of 10,000 daltons allowed for bidirectional diffusion of antibiotics and nutrients from the central compartment to the peripheral compartments but prevented the penetration of bacteria into the central compartment.

The system was filled with Mueller-Hinton broth supplemented with calcium (50 μg of Ca^{2+} per ml) and magnesium (25 μg of Mg^{2+} per ml). Peristaltic pumps circulated the medium at a rate of 3 ml/min from the central reservoir through the tubing and the capillary lumina and finally back into the central reservoir. Each bacterial chamber contained 10 ml of broth, bacteria, and antibiotics. These contents were circulated by peristaltic pumps to achieve homogeneous concentrations. The broth was continuously eliminated from the central compartment and replaced with fresh, antibiotic-free medium from a diluent reservoir to mimic human first-order elimination kinetics, with half-lives of 2.2 and 5 h for netilmicin and enoxacin, respectively.

Dosage regimens. Enoxacin (Warner-Lambert Co., Ann Arbor, Mich.) was administered to simulate the oral administration of 500-mg doses to an adult patient every 12 h with a peak concentration in serum of 3 mg/liter (q12h regimen). The same total daily dose also was administered as a single dose every 24 h (q24h regimen). The same total daily dose of netilmicin (Schering Corp., Bloomfield, N.J.) was adminis-

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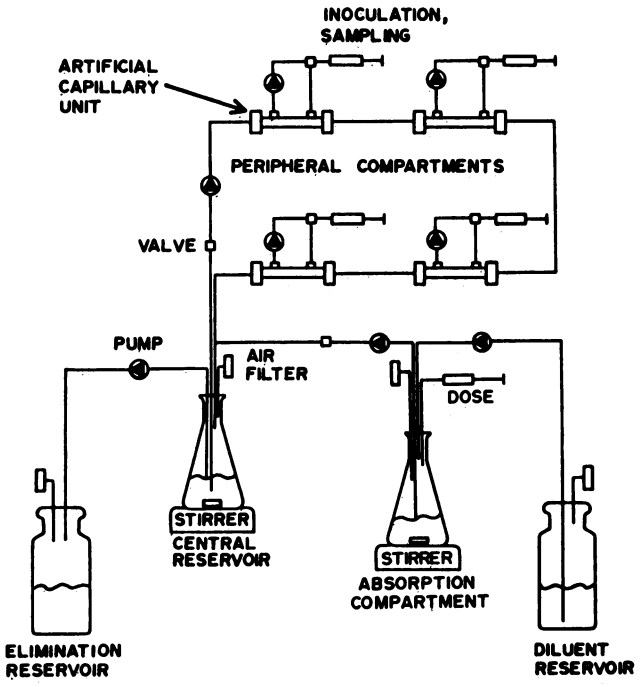

FIG. 1. Schematic drawing of the in vitro pharmacokinetic model. Bacterial cultures were placed into the peripheral culture chambers and exposed to changing drug concentrations according to two-compartment pharmacokinetics following first-order absorption. Drug was administered as described in Materials and Methods and distributed to the peripheral compartments. The drug-containing medium of the central and peripheral compartments was continuously eliminated and replaced with fresh, drug-free medium from the diluent reservoir.

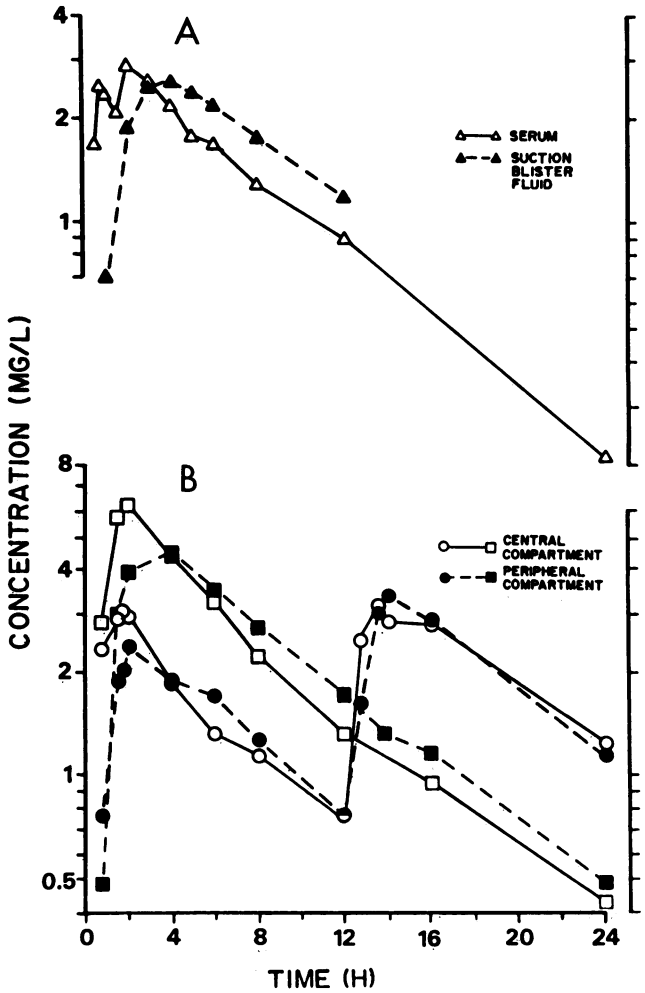

FIG. 2. Pharmacokinetics of enoxacin in the in vitro model versus human data. (A) Concentration-time curves in serum and blister fluid following oral administration of 600 mg of enoxacin to human volunteers (17). (B) Concentration-time curves in the central and peripheral compartments of the in vitro model during the q24h (squares) and q12h (circles) dose regimens.

tered as a continuous infusion over 24 h or as intermittent 1-h infusions every 8, 16, or 24 h. The pharmacokinetics of netilmicin in the model have been reported previously (6). In brief, the concentration-time curve in the peripheral compartments mimicked the kinetics of interstitial fluid as represented experimentally by the fluid in small suction blisters. All experiments with enoxacin and netilmicin were continued for a 28-h treatment period. Netilmicin was assayed by homogeneous enzyme-linked immunoassay (EMIT; Syva Corp., Palo Alto, Calif.), and enoxacin was assayed by high-performance liquid chromatography (14).

Bacteria. The growth chambers of the in vitro model were inoculated to obtain exponentially growing cultures of 10^6 CFU/ml in a 10-ml culture volume in 2 h at the beginning of treatment. Each of the following five organisms was tested in the pharmacokinetic model against enoxacin and netilmicin (the respective agar dilution MICs [10^4 CFU/10-μl spot] for enoxacin and netilmicin are given [in micrograms per milliliter] in parentheses): *Staphylococcus aureus* ATCC 29213 (1.5 and 0.25); *Klebsiella pneumoniae* ATCC 13883 (0.25 and 0.125); *Escherichia coli* ATCC 25922 (0.125 and 1.0); *Pseudomonas aeruginosa* ATCC 27853 (8 and 4); and a clinical strain of *P. aeruginosa*, A-10 (1 and 8). MICs and MBCs for the strains studied were also determined by a microdilution technique with inocula of 10^6CFU/ml and calcium (50 μg of Ca^{2+} per ml)- and magnesium (25 μg of Mg^{2+} per ml)-supplemented Mueller-Hinton broth (9). The MICs determined by agar dilution and microtiter dilution techniques either were the same or differed by one twofold dilution step. The MBCs were defined as concentrations resulting in >99.9% killing of inocula of 5×10^5 CFU/ml. The MBCs were either equal to the MICs or twofold higher than the MICs.

The emergence of resistance was monitored by the agar dilution technique. Postexposure samples were taken from the regrown cultures after 28 h of treatment. Pre- and postexposure samples were diluted appropriately on the basis of optical density, and 10-μl drops containing 10^4 CFU were placed side by side on antibiotic-containing agar plates.

Quantification of bacterial growth. A total of 12 to 14 samples of 0.3 ml each were drawn from the bacterial cultures before and during the 28-h exposure to the antibiotics. Bacteria were counted by making 10-fold dilutions of the samples with a sterile, chilled 0.9% NaCl solution and then plating 20 μl in triplicate on Mueller-Hinton agar. In addition, 100 μl of each sample with $<10^7$ CFU/ml was filtered through a 0.45-μm-pore-size filter (HA; 47-mm diameter; Millipore Corp., Bedford, Mass.) and cultured on agar. Plates and filters were read after sufficient incubation time to develop colonies at 37°C. For bacterial population analysis,

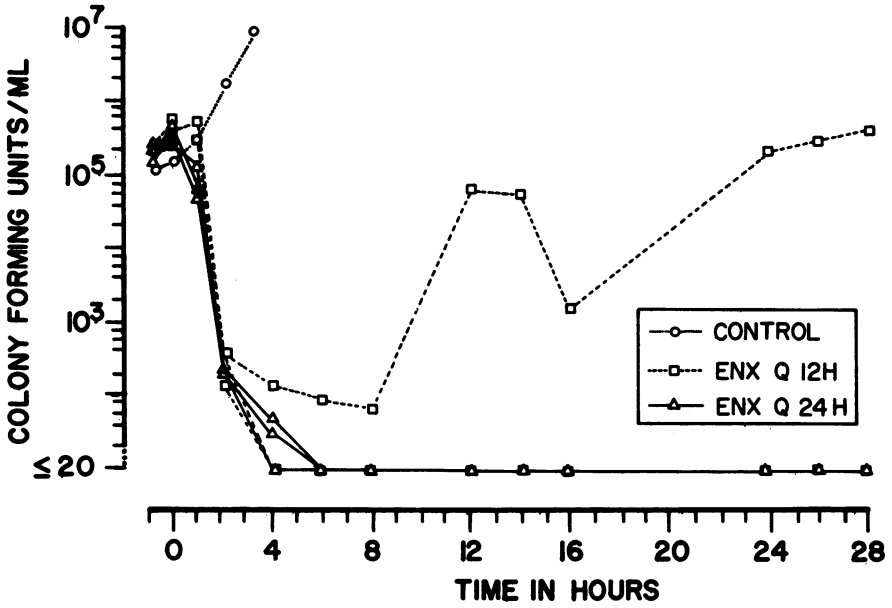

FIG. 3. Bactericidal activity of two enoxacin regimens against *K. pneumoniae* in the pharmacokinetic model. Cultures were eradicated in three of three experiments when the total daily dose was given as one dose q24h but in only two of three experiments when the same total daily dose was given as equal doses q12h.

CFU counts were determined simultaneously on drug-free Mueller-Hinton agar plates and on plates which contained various concentrations of the antibiotic studied.

RESULTS

The concentration-time curves of enoxacin in the central and peripheral compartments of the model mimicked closely the two-compartment pharmacokinetics observed in humans in serum and in blister fluid following oral administration. Figure 2 compares data determined in the in vitro model with data obtained by Wise et al. in serum and in the exudate of blisters which were induced by cantharis-impregnated plasters (17). Following the initial dose of enoxacin, the peak concentrations in the central compartment were 3 and 6 μg/ml for the q12h and the q24h regimens, respectively. During the q12h regimen, drug accumulation was observed as a result of residual concentrations still present at the end of the dosage interval. The peak concentrations in the peripheral compartments increased from 2.5 to 3.6 to 4.0 μg/ml for the q12h regimen following the first, second, and third doses, respectively. A similar close agreement of the kinetics in the model and the time-concentration curves in human serum and suction blister fluid has been reported with netilmicin (6).

Rapid and profound bacterial killing of susceptible strains occurred following the initial dose of enoxacin (Fig. 3 and 4). The initial bactericidal effect increased with increasing ratios of peak concentrations to MIC ($P < 0.01$ as determined by the Wilcoxon test; Table 1 and Fig. 5). All inocula were reduced during treatment with either drug by more than 99% within 4 h if peak concentrations exceeded the MIC by at least a factor of three. The bactericidal effect of the initial oral dose of enoxacin was equivalent to the bactericidal effect of the first intravenous dose of netilmicin at similar ratios of peak concentrations to MIC (Fig. 5 and 6).

The peak concentration/MIC ratios were also predictive of the final outcome of treatment with enoxacin and netilmicin. Regrowth occurred in all cultures during enoxacin treatment at ratios of less than eight, despite the administration of subsequent doses (Fig. 4 and 5). At higher ratios no regrowth was observed ($P < 0.01$ as determined by the chi-square test), except for one experiment with *K. pneumoniae*. This experiment was replicated three times (Fig. 3). Regrowth occurred only once, and the culture was sterilized with enoxacin in the other two replicate experiments during the q12h regimen. During these experiments the peak concentration following the first dose exceeded the MIC by a factor of 10. No regrowth was observed during the q24h regimen, which provided a ratio of 20. In this model an enoxacin peak concentration/MIC ratio of eight represents a threshold value, with regrowth very unlikely at ratios above this threshold and very likely at ratios below this threshold. Almost identical results were obtained with netilmicin (Fig. 5 and 6). Figure 6 shows the antibacterial activity of netilmicin against *E. coli* at two dose levels, providing peak concentration/MIC ratios of eight and one, respectively. With the higher-dose regimen, the cultures were ultimately sterilized, whereas regrowth occurred with the lower-dose regimen.

In contrast to the bactericidal effect of the initial dose, little or no killing was seen following the second and third doses against regrowing cultures during treatment with enoxacin (Fig. 3 and 4). Figure 3 demonstrates the decreasing effect of the first, second, and third doses of enoxacin against a regrowing culture of *K. pneumoniae*. At 4 h following the respective doses, the bacterial concentration changed by −3.4, −1.6, and +0.3 $\log_{10}$ CFU/ml. No bactericidal effect was obtained following the second enoxacin dose against *P. aeruginosa* (Fig. 4) and *S. aureus* (19), in contrast to a more than 100-fold reduction of the inoculum following the initial enoxacin dose. A similar reduction of the bactericidal effect from the first to the second and subsequent doses was also found with netilmicin (6) (Fig. 6).

Figure 4 shows a population analysis of the regrowing culture of *P. aeruginosa* during treatment with enoxacin.

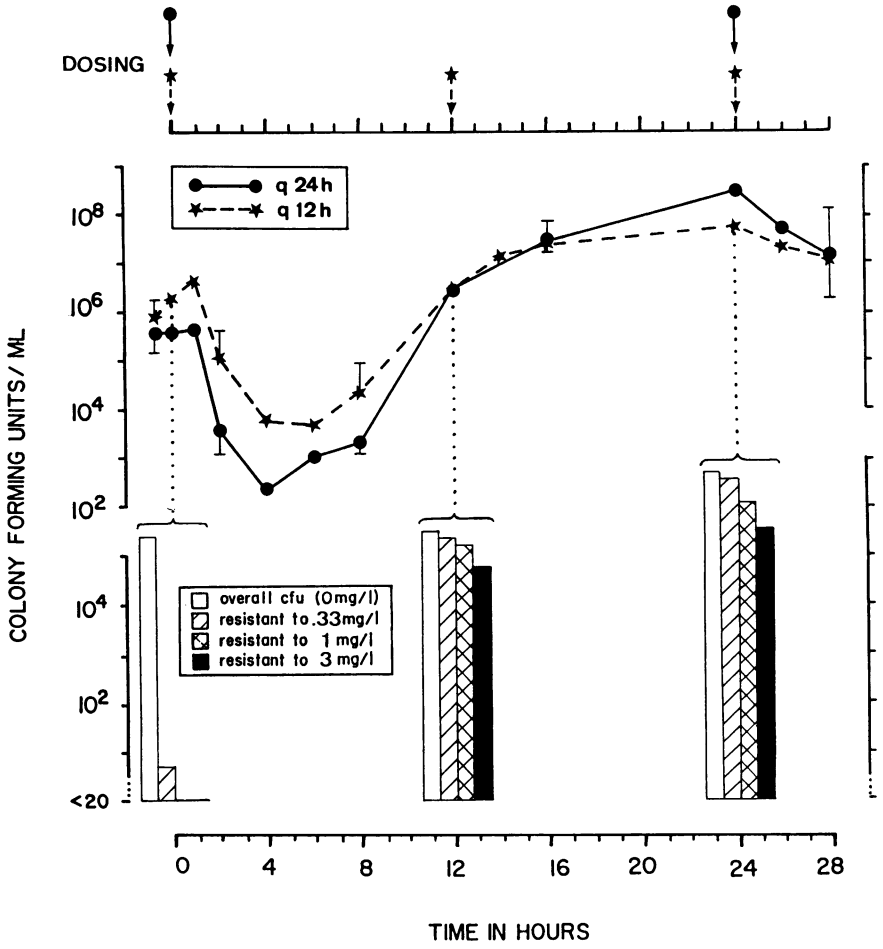

FIG. 4. Efficacy of enoxacin against *P. aeruginosa* A-10 (MIC, 1 μg/ml). Oral administration of enoxacin was simulated in the pharmacokinetic model. The same total daily dose was given q12h or q24h (top panel). The central panel shows the bacterial response to the two regimens. Geometric means and ranges of duplicate experiments are plotted. The population analyses shown in the bottom panel document the development of resistance within the first dosage interval.

This analysis documents the selection of resistant subpopulations within the first dosage interval and provides an explanation for the decreased effect of the second and third doses. Very similar results were obtained with enoxacin against *S. aureus* (19) and also with netilmicin (6). All regrowing cultures became resistant, as determined by an average increase of the MIC during enoxacin treatment by a factor of eight (range, 4 to 16) and during netilmicin treatment by a factor of four (range, 2 to 4).

DISCUSSION

The bacterial killing obtained during treatment of large inocula with enoxacin was strikingly similar to that obtained with netilmicin. A rapid and profound bactericidal effect occurred with both drugs following the first dose. The magnitude of the initial killing was similar with both drugs at comparable ratios of peak concentration to MIC. However, with both drugs, bacterial regrowth resulting from the selection of resistant subpopulations was prevented only at peak concentrations which exceeded the MIC by a factor of eight or more. Regrowth occurred despite the fact that the number of bacteria at the time of the second dose often was lower than that in the original inoculum at the time of the first dose (6) (Fig. 3 and 4). Also, following the second dose of enoxacin, the bacterial cultures were exposed to drug concentrations which were 44% higher than those present after the first dose, owing to drug accumulation in the peripheral compartment (Fig. 2). The reduced antibacterial effect of the second dose could not be explained by altered milieu conditions. Measurements of the pH and of the partial O_2 pressure after 8 h of treatment revealed unchanged conditions when compared with the beginning of the experiment. However, anaerobic conditions were present after 24 h in cultures of very high bacterial densities.

The reduced antibacterial effect of the second and subsequent doses coincided with the selection of resistant subpopulations. Resistance became apparent within a few hours during treatment with either drug of both gram-positive and gram-negative pathogens. In the pharmacokinetic model, bacterial regrowth during treatment with the aminoglycoside or the quinolone was prevented only when the bacteria studied were highly susceptible. However, selection of resistance occurred only infrequently during treatment with ceftazidime, azlocillin, or netilmicin in combination with one of these beta-lactams (4; J. Blaser, B. B. Stone, D. H. Gilbert, and S. H. Zinner, Proc. 14th Int. Congr. Chemother., p. 1547–1548, 1985).

Sanders et al. studied the frequency of selection of resistance to these drugs in vitro with an agar dilution technique

TABLE 1. Changes in bacterial concentrations between 0 and 4 h of treatment with various dose regimens of netilmicin and enoxacin

Organism	Change in bacterial concn between 0 and 4 h[a] with:			
	Netilmicin[b]		Enoxacin[c]	
	CI	q24h	q12h	q24h
P. aeruginosa ATCC 27853	+1.0	−3.9	+1.7	+0.9
P. aeruginosa A-10	+1.6	−3.1	−2.5	−3.1
K. pneumoniae ATCC 13883	<−4.0	<−4.0	−4.0	<−4.0
E. coli ATCC 25922	−2.2	<−4.0	<−4.0	<−4.0
S. aureus ATCC 29213	−3.2	<−4.0	−1.4	−2.7

[a] Given as change in $\log_{10}$ CFU per milliliter (geometric mean of duplicate experiments).

[b] Netilmicin was administered as a continuous infusion (CI) (steady-state concentration, 3.7 μg/ml) or as 1-h infusions q24h (peak concentration, 24 μg/ml).

[c] Enoxacin was administered q12h (peak concentration, 3 μg/ml) or q24h (peak concentration, 6 μg/ml).

and found mutational frequencies of 10^{-7} to 10^{-8} with quinolones and aminoglycosides and 8- to 16-fold increases in MICs (16). In the present study resistant bacteria were not found if the peak antibiotic concentration exceeded the MIC by a factor of eight. However, the selection of resistant subpopulations was very reproducible at peak concentration/MIC ratios of one to eight, as observed in 8 of 8 enoxacin experiments and in 10 of 11 netilmicin experiments (Fig. 5). These data suggest a mutational frequency of less than 10^{-7} after challenge with appropriate drug concentrations, since inocula of 10^6 CFU/ml in a volume of 10 ml were exposed in the model.

The bacterial response was analyzed as a function of the ratio of peak concentration to MIC (Fig. 5). Trough concentration/MIC ratios were less correlated with bacterial response than were peak concentration/MIC ratios (Fig. 4). The q24h regimen provided better killing of *P. aeruginosa* than did the q12h regimen, owing to the higher peak concentrations with the former regimen. The low trough concentrations with the q24h regimen were not critical to the appearance of bacterial regrowth, because regrowth had already begun 4 to 6 h after the initial high peak enoxacin concentration was reached. Furthermore, bacterial regrowth was not affected by the second dose in the q12h regimen.

The MICs used for the calculation of the peak concentration/MIC ratios were determined by standard methods with recommended bacterial inocula. Although relatively minor inoculum effects have been noticed during in vitro tests of aminoglycosides and quinolones, particularly when compared with data obtained with some beta-lactams, changes in the inoculum may affect the MIC and therefore also the ratio of peak concentration to MIC. For example, the median MIC of enoxacin and ciprofloxacin for *P. aeruginosa* was increased twofold by an increase in the inoculum from 10^5 to 10^7 CFU/ml (3). However, the peak concentration/MIC ratio was found to be a major determinant of the clinical response to aminoglycoside therapy in a recent clinical study (13). An elevated peak concentration/MIC ratio was strongly associated with a favorable clinical response. At maximum peak concentration/MIC ratios of 6:1 or less, the average response rate was less than 70%, as opposed to a clinical response rate of approximately 90% at ratios of 8:1 or higher.

The data presented in Fig. 5 are limited to one fluoroquinolone and one aminoglycoside in the pharmacokinetic model.

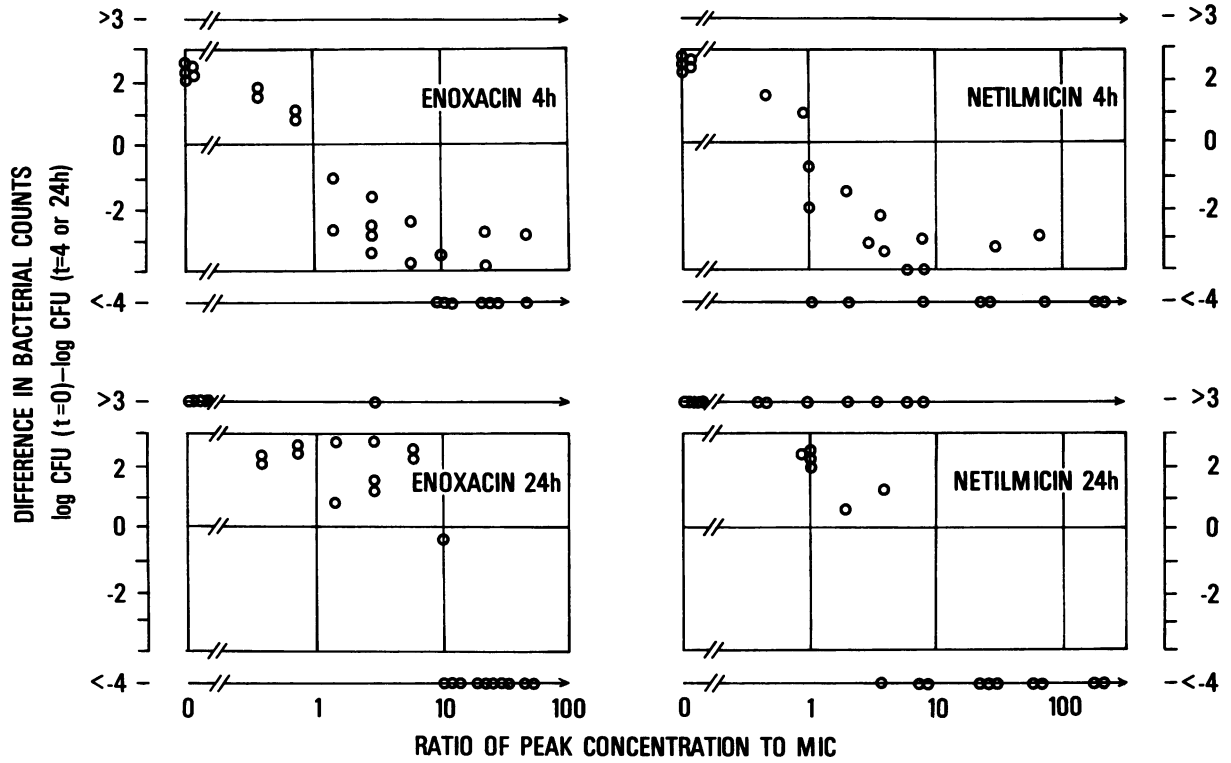

FIG. 5. Antibacterial effect of multiple-dose regimens of enoxacin and netilmicin against five organisms. Changes in bacterial numbers during treatment periods of 4 and 24 h are plotted against the ratios of peak concentration to MIC. t = time.

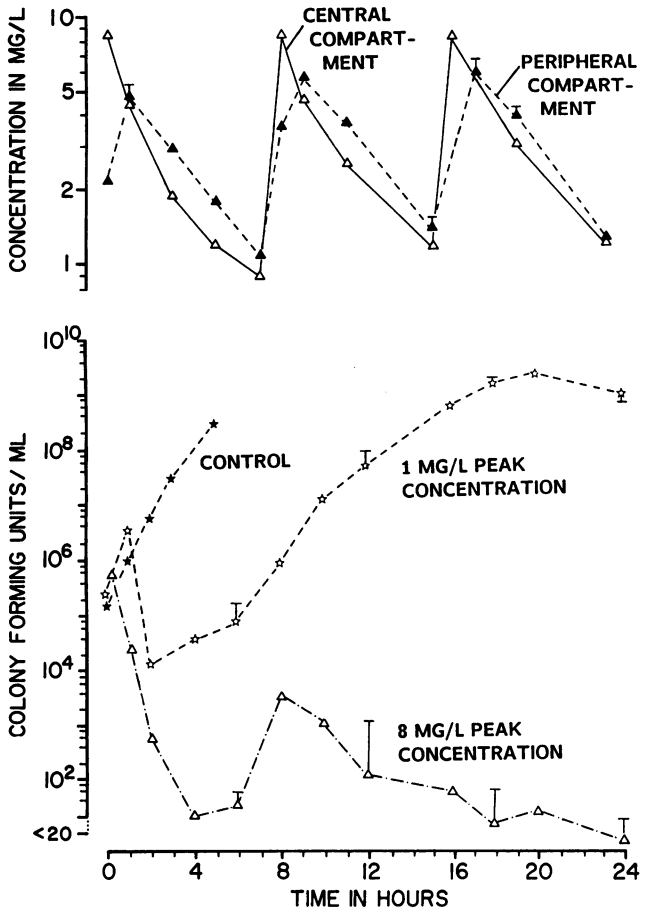

FIG. 6. Antibacterial effect of netilmicin against *E. coli*. Bacteria cultured in the peripheral chambers of an in vitro model were exposed to oscillating drug concentrations. Human pharmacokinetics were mimicked during the administration of 60-min infusions every 8 h at two dose levels. Netilmicin was given either at doses corresponding to peak levels in serum of 8 mg/liter (concentrations achieved in the pharmacokinetic model during this regimen are shown in the top panel) or at doses corresponding to one-eighth these levels. Also, control growth in the absence of any drug is shown. Geometric means and ranges of duplicate experiments are shown.

However, results obtained with this model during multiple-dose regimens with ciprofloxacin (M. N. Dudley, J. Blaser, D. Gilbert, and S. H. Zinner, Rev. Infect. Dis., in press) and amikacin (20) were similar with respect to the rapid initial killing and regrowth of resistant subpopulations at ratios of peak concentration to MIC of less than 8:1. Bauernfeind et al. found similar results in an in vitro study of bacterial killing and selection of resistant mutants by 4-quinolones during exposure to stepwise decreasing concentrations, mimicking serum kinetics (1).

The selection of resistant subpopulations during treatment with quinolones and aminoglycosides is not limited to in vitro experiments. Similar observations were reported in a leukopenic mouse model with breakthrough growth of resistant subpopulations of *P. aeruginosa* 6 h after the institution of aminoglycoside treatment (10). Resistance to amikacin emerged during treatment of experimental *P. aeruginosa* endocarditis in 79% of rabbits treated with amikacin (2). Olson et al. reported that the clinical use of aminoglycosides was associated with increasing aminoglycoside resistance in serial surveillance isolates and that mortality was higher in patients infected with increasingly resistant subpopulations (15). Follath et al. reported the emergence of nine bacterial strains with decreased susceptibility to ciprofloxacin in 7 of 30 patients during treatment with ciprofloxacin (8). Desplaces et al. reported the selection of resistant mutants during monotherapy with fluoroquinolones in 8 of 131 treated patients (7), and Wolff et al. observed a dramatic increase in the resistance of *P. aeruginosa* and *S. aureus* to pefloxacin in an intensive-care unit (M. Wolff, J. P. Pathe, B. Pangon, A. Bure, B. Regnier, and F. Vachon, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 654, 1985).

Despite reports of the emergence of resistant bacteria during antibiotic therapy, this phenomenon does not occur clinically as frequently as predicted in this study. This discrepancy may reflect in part the unstable resistance of the selected subpopulations. A partial loss of aminoglycoside resistance may occur in vivo within hours or days after the end of antibiotic treatment and after passage in antibiotic-free medium in vitro (2, 6, 10).

In the pharmacokinetic model successful treatment depends exclusively on the ability of the antibiotic to kill the pathogens, representing an even greater challenge for antibiotic efficacy than treatment in the presence of host defenses during clinical infections. Regrowth has been observed in a neutropenic animal model as breakthrough growth of resistant subpopulations during treatment of infected leukopenic mice, whereas normal mice recovered rapidly (10). Experiments in which leukocytes were added to the bacterial growth chambers of the pharmacokinetic model suggest suppression of bacterial regrowth by leukocytes (J. Blaser, D. Gilbert, and S. H. Zinner, Rev. Infect. Dis., in press). However, the leukocytes were active in vitro only for approximately 2 h. Some in vitro data suggest alterations in the susceptibility of bacteria to phagocytosis by human polymorphonuclear leukocytes during or after exposure to aminoglycoside or quinolone antibiotics (12). Leukocyte killing activity was enhanced against resistant bacterial subpopulations selected during aminoglycoside and quinolone exposure, as compared with untreated bacteria (F. Schlaeffer, J. Blaser, and S. H. Zinner, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 400, 1985).

This study demonstrates the equivalent bactericidal activities of simulated oral enoxacin administration and simulated intravenous netilmicin administration, as well as the similar selection of resistant subpopulations by each drug. The data generated in this model that simulates infection in a neutropenic site suggest that the ratio of peak concentration to MIC may be an important parameter in the clinical use of quinolone and aminoglycoside antibiotics. The data also emphasize the importance of host defense mechanisms during treatment of infections caused by bacteria of borderline antibiotic susceptibility.

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