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Research Paper

Use of *in Vitro* Critical Inhibitory Concentration, a Novel Approach to Predict *in Vivo* Synergistic Bactericidal Effect of Combined Amikacin and Piperacillin Against *Pseudomonas aeruginosa* in a Systemic Rat Infection Model

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Purpose. This study was undertaken to explore the use of *in vitro* critical inhibitory concentration (CIC) as a surrogate marker relating the pharmacokinetic (PK) parameters to *in vivo* bactericidal synergistic effect [pharmacodynamic (PD)] of amikacin + piperacillin combination against *Pseudomonas aeruginosa* in a systemic rat infection model.
Methods. The *in vitro* antibacterial activities of amikacin and piperacillin, alone and in combinations at various ratios of the concentrations, were tested against a standard [5×10^5 colony-forming units (CFU)/ml] and a large (1.5×10^8 CFU/ml) inoculum of *P. aeruginosa* ATCC 9027 using a modified survival-time method. The CIC of each individual antibiotic for the different combinations was determined using a cup-plate method. *In vivo* studies were performed on Sprague-Dawley rats using a systemic model of infection with *P. aeruginosa* ATCC 9027. PK profiles and *in vivo* killing effects of the combination at different dosing ratios were studied.
Results. An inoculum effect was observed with the antibiotics studied. Synergy was seen against both the inocula at the following concentration ratios: 70% C_{ami} + 30% C_{pip} and 75% C_{ami} + 25% C_{pip}, where C_{ami} and C_{pip} are the concentrations of amikacin and piperacillin to produce a 1000-fold decrease in bacterial population over 5 h, respectively. The CIC values determined corroborated with the order of *in vitro* bacterial killing observed for the antibiotic combinations. The dosing ratio of 12.6 mg/kg amikacin + 36 mg/kg piperacillin (a 70:30 ratio of the individual doses) exhibited the greatest killing *in vivo* when compared to the other ratios. The PK–PD relationships were described by simple, linear regression equations using the area under the *in vivo* killing curve as a PD marker and the $AUCIC_{ami}/CIC_{ami} + AUCIC_{pip}/CIC_{pip}$, $AUC_{ami}/CIC_{ami} + AUC_{pip}/CIC_{pip}$, $C_{max,ami}/CIC_{ami} + C_{max,pip}/CIC_{pip}$, and $AUCIC_{ami}/MIC_{ami} + AUCIC_{pip}/MIC_{pip}$ as PK markers for the amikacin + piperacillin combination.
Conclusion. The combination of amikacin and piperacillin exhibited synergistic killing effect on *P. aeruginosa* that could be modeled using CIC as a surrogate marker relating the PK parameters to *in vivo* bactericidal effect.

KEY WORDS: amikacin-piperacillin combination; concentration ratio; *in vitro* critical inhibitory concentration; *in vivo* bactericidal synergism; pharmacokinetic-pharmacodynamic relationship; *Pseudomonas aeruginosa* infection.

INTRODUCTION

Combinations of antimicrobial drugs have been commonly used in medical practice for specific reasons: to expand the bacterial coverage over a single agent, to prevent the emergence of resistant organisms, to decrease toxicity by allowing lower doses of both agents, to treat polymicrobial infections, and/or for synergy (1,2). Synergy is one of the most common of these reasons, especially in serious infections.

The rational use of antimicrobial drugs as well as the design of effective dosage regimens is facilitated by the appreciation of the relationship between the administered dose of a drug, the resulting drug concentrations in body fluids accessible for measurements, and the intensity of the antimicrobial effects caused by these concentrations. Combined pharmacokinetic (PK) and pharmacodynamic (PD) models that describe the relationship between plasma and/or tissue drug concentrations and an antimicrobial effect are usually expressed as a biomarker or a surrogate end point. The relationship between dose, plasma concentrations, and antimicrobial effects is frequently complex, and hence, a

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quantitative relationship of pharmacodynamics to dose and/or pharmacokinetics is usually of interest. The ability to predict the *in vivo* efficacies of antibiotics through microbiological and PK data is one of the main objectives in antimicrobial chemotherapy (3–5).

Several surrogate relationships have been specified for various classes of antibiotics to obtain a specific and precisely defined correlation between the antimicrobial pharmacokinetics and the PD interaction between the antimicrobial agent and its bacterial target. These PK–PD surrogates relate various PK parameters to a measure of the PD interaction [e.g., minimum inhibitory concentration (MIC)]. One of such surrogate relationships examines the peak plasma concentration (C_{max}) of the antimicrobial agent relative to the MIC value (i.e., the C_{max}/MIC ratio) (4,6). This relationship has been used for aminoglycosides and fluoroquinolones (7). Another surrogate marker is the area under the inhibitory plasma concentration–time curve (AUIC), which may be defined as the area under the curve (AUC) for time points with concentrations above the MIC (4,8,9).

It has been accepted that for β-lactam antibiotics, the time that the levels in plasma exceed the MIC is the most significant parameter determining their *in vivo* efficacies. This is one of the most extensively used relationships, and there are many studies describing its use (3,4). The use of time above the MIC is, however, not without its flaws. It is well known that the MICs of β-lactam antibiotics for some organisms may vary with the method used to determine the results (10,11). One of the factors that may influence the MICs is the inoculum effect, where there is an increase in the MIC when the inoculum size is increased. MIC is also dependent on the concentration of β-lactam antibiotics when more resistant organisms are used. Moreover, there is an accepted optimum time during which the concentration has to be greater than the MIC. Hence, the time above the MIC is not a very reliable estimate in establishing a correlation between *in vitro* and *in vivo* efficacies.

All the above-mentioned surrogate relationships to establish a correlation between the PK and PD effects of antimicrobial agents are valid only when a single antimicrobial agent is used. There have not been any reports of a combined PK–PD model for use when two or more antimicrobial agents are used to combat an infection. This might be because when a combination of antibiotics is used, one of the considerations is to reduce the dose of the individual antibiotics to avoid toxicity. This reduced dosing leads to decreased blood levels of the antibiotics. This, in turn, leads to decreased C_{max} and AUC values. Therefore, the use of C_{max}/MIC ratio, AUIC, and time above MIC yields different values according to the dose used. Moreover, combination dosing often involves the use of an aminoglycoside and a β-lactam antibiotic, and the surrogate markers are different for the two different classes of antibiotics (3–5).

Pseudomonas aeruginosa is a common pathogen that is implicated in nosocomial infections in the hospitals and health-care centers in Singapore (12,13). *P. aeruginosa* has also been reported to be the second most common bacterium, after *Escherichia coli*, to be implicated in gram-negative infections worldwide (12,13). On the other hand, the emergence of resistance has been reported when antipseudomonal antibiotics is used alone against this organism (12,14). For these reasons, *P. aeruginosa* has been an important consideration in the development of effective combination therapy to produce rapid enhancement of bactericidal activity and to help prevent or delay the emergence of resistance.

Combinational antimicrobial therapy, involving an aminoglycoside and a β-lactam antibiotic, has often been used in the treatment of infections caused by gram-negative pathogens (15,16). To test for combination therapy, amikacin (an aminoglycoside) and piperacillin (a β-lactam antibiotic) that are commonly used against *P. aeruginosa* were chosen in this study. There have been varied reports on the killing effects of this combination. Whereas this combination has been shown to have *in vitro* synergy against some strains of bacteria, they have also shown additivity, indifference, or even antagonism against other strains of the same bacteria (17). These studies have also shown bacterial killing to be dependent on the concentration of the antibiotics used in the combination. In the present study, it was proposed to study combinations of antibiotics for their killing effects against bacteria by varying not only the concentrations of the individual antibiotics but also by varying the ratios in which the two antibiotics were used in a combination. This is to determine if bacterial killing and synergy are also dependent on the ratio of the antibiotics used in the combination.

The inoculum effect on MIC has been recognized as having therapeutic implications for β-lactams in treating infections caused by both gram-positive and gram-negative bacteria (18–20). But not much is known about the effect of the inoculum size on *in vitro* synergy. In this study, the effects of the inoculum size on the pattern of killing and the *in vitro* synergy of the antibiotic combinations mentioned earlier were also investigated. The *in vitro* critical inhibitory concentration (CIC) of each individual antibiotic for the different concentration ratios of the combination was determined.

In vivo studies on the antibiotic combination were carried out in a rat systemic model of infection to ascertain if the synergy that was seen *in vitro* was also exhibited *in vivo*. The area under the *in vivo* killing curve (AUKC) for the various combinations was chosen as the PD parameter. The use of *in vitro* CIC as a PK–PD surrogate was explored to relate PK parameters to *in vivo* bactericidal synergistic effect in this study.

MATERIALS AND METHODS

Chemicals and Bacterial Strains

Amikacin sulfate was obtained from Sigma (St. Louis, MO, USA). Piperacillin sodium was a gift from Wyeth Laboratories (Hampshire, England). *P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739, and *Bacillus subtilis* ATCC 6633 were obtained from American Type Culture Collection (Rockville, MD, USA). Nutrient agar no. 2 was obtained from Oxoid Ltd. (Hampshire, England). Penicillinase type I and cellulose phosphate were purchased from Sigma.

In Vitro Studies

Broth Dilution Technique

P. aeruginosa was inoculated onto the surface of nutrient agar no. 2 from a recently acquired stock culture and

incubated at 37°C for 3 days. Bacterial cells were harvested by using sterile water to wash the surface growth into a suitable receptacle. The stock solution was standardized by viable count method using McFarland standards and stored in the refrigerator.

Susceptibility testing of each antibiotic was performed in duplicate by a broth dilution technique using two bacterial inocula of 5×10^5 colony-forming units (CFU)/ml (standard inoculum) and 1.5×10^8 CFU/ml (large inoculum). The method essentially consisted of inoculating graded concentrations of the antibiotics with the test organism and incubating for 24 h to determine the minimum concentration preventing detectable growth (MIC).

Survival-Time Method

Bactericidal activity was evaluated by survival-time method. In this method, each sample concentration of the antibiotic was inoculated under aseptic conditions with sufficient quantity of *P. aeruginosa* suspension to achieve a desired level of antimicrobial count (standard or large). The inoculated samples were mixed thoroughly and maintained at ambient temperature of 25°C. Aliquot quantities of the inoculated samples were withdrawn aseptically at time intervals of 0, 1, 2, 3, 4, and 5 h for viable count determinations. Each withdrawn sample was diluted serially with sterile water to produce a count of about 100 cells/ml. One-milliliter aliquot of each diluted sample was used to prepare triplicate petri plates using nutrient agar no. 2 using a pour-plate technique, and these plates were incubated at 37°C for 36 h. Average CFU on each plate was determined, and from these values, the total number of CFUs per milliliter in the original sample was calculated. Survival-time studies were performed for both the antibiotics by varying the concentration until the lowest concentration required to produce a 1000-fold decrease in bacterial population over the 5-h period was determined. These concentrations are represented as C_{ami} and C_{pip} for amikacin and piperacillin, respectively. Similar survival-time studies were then performed for the antibiotic combination (amikacin + piperacillin).

Critical Inhibitory Concentrations

For the antibiotic combinations that showed synergy, the CIC of each individual antibiotic for the different concentration ratios of the combination was determined using a cup-plate method. In this method, molten agar medium inoculated with the test organism was allowed to solidify in petri dishes, and holes of 10 mm in diameter were cut into the medium with a sterile Oxford cylinder. For a given ratio of the antibiotic combination, equal volumes of graded concentrations at 20, 40, 60, 80, and 100% of the antibiotic solutions were placed directly into the holes, and the plates were incubated at 37°C for 20 h. The zone of inhibition developed for each concentration was measured, and the value of d, the distance from the edge of the hole to the edge of the zone of inhibition, was calculated. On plotting d^2 against $\log_e m_0$ (the concentration of the antibiotic solution at time zero), a straight line intercepting the $\log_e m_0$ axis at $\log_e m_c$ was obtained. The CIC value was obtained by taking the antilog of the $\log_e m_c$ value from the graph. Figure 1 shows a typical plot of d^2 against $\log_e m_0$ of piperacillin and amikacin for a given ratio of the antibiotic combination.

In Vivo Studies

Rat Preparation and Infection

Adult, male Sprague–Dawley rats, weighing 230–270 g, obtained from the Animal Holding Unit, NUS, were used as experimental animals. The animals were housed in the laboratory in which the experiments were performed and were acclimatized for a day before the experiment. The animals were fasted overnight before the experiment, but water was supplied *ad libitum*. A systemic infection model was used to estimate the *in vivo* killing effects of the individual antibiotics and the combinations against *P. aeruginosa* ATCC 9027 (21). Briefly, the rats were made neutropenic (with white blood cell count less than 2000/mm^3) to eliminate natural host defenses by injecting intraperitoneally with a single dose of 300 mg/kg of cyclophosphamide 3 days before *P. aeruginosa* ATCC 9027 was inoculated. The rats were then injected intraperitoneally with 0.25 ml per 200 g of body weight of a suspension containing 1.5×10^7 CFU/ml of *P. aeruginosa* ATCC 9027 in saline. Antibiotic solution for injection (0.2 ml per 200 g of body weight) was administered intraperitoneally, 4–5 h (preincubation time) after injection of the microorganisms. This preincubation time of 4–5 h was to allow microorganisms to produce and reach an exponential *in vivo* culture of 5×10^5 CFU/ml (21). The research adhered to the principles of laboratory animal care (NIH publication #85-23, revised 1985).

Antibiotics Pharmacokinetics

Single-dose PK studies of the antibiotics were performed in systemic-infected rats. A dose of 18 mg/kg amikacin and 120 mg/kg piperacillin, given alone, were used to obtain the PK parameters. PK studies for combinations of antibiotics were performed at ratios of the doses of the antibiotics that showed *in vitro* synergy. These ratios were in the same proportion for which *in vitro* synergy was observed. For each single or combined dosing group of three rats, a serial of blood samples was taken at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 h after intraperitoneal administration of the

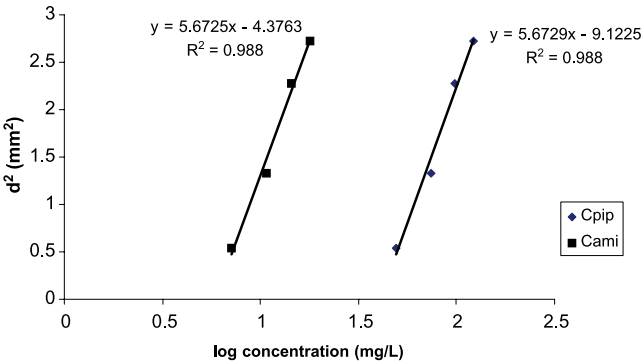

Fig. 1. Representative plot of d^2 (square of distance from the edge of hole to the edge of zone of inhibition) against $\log_e m_0$ (concentration at time zero) of amikacin (■) and piperacillin (♦) for a 70:30 ratio of the antibiotic combination.

antibiotics from each animal. Blood samples (about 400 μl) were obtained, by retro-orbital puncture, using heparinized capillary tubes, and drug concentrations were determined by agar-well microbiological assays. PK analyses were performed using the WinNonlin iterative curve-fitting program (Standard edition, 1.1, Scientific Consulting Inc., Lexington, KY, USA) based on nonlinear regression analysis. The plasma concentration–time profile of each antibiotic was analyzed using model-independent noncompartmental analysis. PK parameters [peak plasma concentration (C_{max}), peak time (t_{max}), total area under the plasma concentration time curve (AUC), and elimination half-life ($t_{1/2}$)] were estimated.

Microbiological Assays

Microbiological assays were performed to determine the concentrations of the antibiotics in plasma obtained by centrifuging the rat blood samples for 10 min at 3000 × *g*.

In plasma samples containing a mixture of amikacin and piperacillin, the β-lactam antibiotic was destroyed by penicillinase (300 U/ml of plasma), and plasma levels of amikacin were then determined using an agar-well microbiological assay with *B. subtilis* ATCC 6633 as the test organism (21,22).

Likewise, amikacin was inactivated by cellulose phosphate (10 mg/ml of plasma), and levels of piperacillin in plasma samples were determined using an agar-well microbiological assay with *E. coli* ATCC 8739 as the test organism (22).

A calibration curve for amikacin was obtained as follows. Molten agar medium inoculated with 1.5×10^8 CFU/ml *B. subtilis* ATCC 6633 was allowed to solidify in petri dishes, and wells 10 mm in diameter were cut into the medium with a sterile cork borer. Aliquots of 100 μl of 0.1, 1.0, 5.0, 15.0, and 25.0 mg/l of amikacin solutions in the blank rat plasma were placed in the wells. The solutions were allowed to diffuse into the agar medium for about 2 h at room temperature, and the plates were then incubated at 37°C for 20 h. The zone of inhibition developed for each antibiotic concentration was measured, and the value of x, the diameter of the zone of inhibition minus the diameter of the hole, was calculated. The calibration curve was obtained by plotting the log concentration against x.

Similar calibration curves were obtained for piperacillin at the following antibiotic concentrations: 0.1, 1.0, 2.0, 16.0, 64.0, and 100.0 mg/l using *E. coli* as the test organism.

Accuracy and precision of the assays were determined for calibration standards as a measure of the percent bias and the intra- and interassay coefficients of variation (% CV), respectively. Assay validations were performed for amikacin at 0.1, 5.0, and 25.0 mg/l in plates inoculated with *B. subtilis* ATCC 6633. These validations were performed in triplicate on 3 days and also on the days of the *in vivo* experiments. The lower limit of quantification (LLOQ) of this assay was 0.1 μg/ml. The % bias of the calculated values was lower than the acceptable range of 15%. Acceptable intra- and interassay precision were obtained with CV values of 5.14–7.78% at low, medium, and high concentration levels of amikacin.

Similar assay validations were performed for piperacillin at 0.1, 16.0, and 64.0 mg/l in plates inoculated with *E. coli* ATCC 8739. The LLOQ of this assay was 0.1 μg/ml. The % bias of the calculated values was well within the acceptable range of 15%. Acceptable intra- and interassay precision were obtained with CV values of 5.78–9.32% at low, medium, and high concentration levels of piperacillin.

Bacterial Quantification in Experimental Animals

Aliquots (20 μl) of the whole blood drawn at the sampling time points mentioned above were immediately mixed with 380 μl of a saline solution containing 300 U/ml of penicillinase and 10 mg/ml of cellulose phosphate to destroy any residual penicillin and amikacin that might prolong bacterial killing after the sample was drawn. After 10-fold dilution in saline, 10 μl of all dilutions was placed in duplicate plates of a nutrient agar no. 2 plate for CFU counts. After incubation at 37°C for 30 h, the colonies were counted on each plate, and the numbers of CFUs per milliliter of the samples were determined.

Statistical Analyses

Statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). For *in vitro* survival-time methods, comparisons of means of the differences of the reduction factors (ratio of the initial bacterial population to the population at 5 h) from the baseline value when exposed to a single antibiotic, for each concentration ratio, against the standard or the large inocula when exposed to the antibiotic combinations, were performed using one-way analysis of variance with the *post hoc* multiple comparisons made using Tukey's honestly significant difference test. At each concentration ratio, the reduction factors between the standard and the large inocula were compared using the two independent samples t test. The relationship between PK and PD parameters was examined using linear regression analysis. A value of $p < 0.05$ was taken to indicate statistical significance.

RESULTS AND DISCUSSION

In Vitro Studies

The *in vitro* killing effects of amikacin and piperacillin, alone and in combinations, were analyzed over a range of concentration ratios. Because the density of inoculum in an antimicrobial susceptibility assay is critical for the generation of reliable and reproducible susceptibility test results (18,19), two bacterial inocula, the standard inoculum (5×10^5 CFU/ml) established by the National Committee for Clinical Laboratory Standards (NCCLS) (23) and a large inoculum (1.5×10^8 CFU/ml), were used in this study.

The MIC ranges of the antibacterial agents against *P. aeruginosa* ATCC9027 are given in Table I. As the inoculum size was increased from standard to large, there was a 64-fold increase in the MIC values of piperacillin as well as a 32-fold increase in the MIC of amikacin, based on either the minimum or maximum values observed, indicating the existence of an inoculum effect for the antibiotics studied. The inoculum effect that was observed with the β-lactam piperacillin is quite expected, as the β-lactam antibiotics have

Table I. *In Vitro* Susceptibility of *P. aeruginosa* ATCC 9027 Strain to the Antibiotics Studied

	Standard inoculum[a]		Large inoculum[a]	
Antibiotic	MIC (mg/L)	C_{1000}[b] (mg/L)	MIC (mg/L)	C_{1000}[b] (mg/L)
Piperacillin	0.4–0.8	1.60	25.6–51.2	409.60
Amikacin	0.1–0.2	0.80	3.2–6.4	25.60

MIC = minimum inhibitory concentration.
[a] The standard inoculum is 5×10^5 CFU/ml; the large inoculum is 1.5×10^8 CFU/ml.
[b] C_{1000} represents the concentration required to produce a 1000-fold decrease in bacterial concentration after 5 h.

been known to show this effect extensively (24). An inoculum effect is not normally associated with aminoglycosides, but the present study showed an inoculum effect with amikacin.

A possible explanation for the observed inoculum effect is that an organism may become less susceptible when it is present in large numbers because of the combined production of β-lactamases or, alternatively, preferential affinity for some penicillin-binding proteins (PBPs) (1). This effect may also be because of a normal distribution of the MICs of individual bacterial cells instead of a homogenous progeny. Thus, with larger inocula, there is a greater probability that there will be some cells or variants from the more resistant end of the distribution curve. The cells from this extreme of the normal distribution are more likely to survive and grow (1).

The inoculum effect has been found in several bacterial species and is particularly widespread among the β-lactam antimicrobial agents when their activity is directed against β-lactamase-producing bacteria. Although the inoculum effect has been most widely studied in staphylococci, it has been shown to be associated with a variety of bacterial species and almost every class of antimicrobial agent, especially the β-lactam antimicrobial agents. The phenomenon of the effect is largely attributed to the inactivation of the antimicrobial agents by β-lactamase (1). Other possible explanations for the inoculum effect can be ascribed to the selection of resistant mutants or to drug breakdown by other drug-targeted inactivating enzymes; combined production of β-lactamases when an organism is present in large numbers making it less susceptible or a preferential affinity for some PBPs may be involved (25).

The clinical implication of the inoculum effect is uncertain. The inoculum standard established by NCCLS, final concentration of 5×10^5 CFU/ml for broth dilution, is not applicable to all clinical situations (6). At best, it represents a compromise between various clinical infections and the procedural manipulation for eliminating the potential for trailing end points.

The concentrations of the antibiotics, required to produce a 1000-fold decrease in bacterial population after 5 h, were 2.0 × MIC and 8.0 × MIC for piperacillin against the standard and large inocula, respectively, as well as 4.0 × MIC for amikacin against both the standard and large inocula (Table I). The increase in this concentration over the MIC for piperacillin for the large inoculum might once again be attributed to the inoculum effect. However, this effect was not seen with amikacin, which might be because of the fact that amikacin is an aminoglycoside and does not possess the β-lactam ring. As mentioned earlier, the inoculum effect is largely attributed to the inactivation of the antimicrobial agents by β-lactamase enzymes.

Ratios of the concentrations required to produce a rapid rate of killing (1000-fold decrease over 5 h) were used in this study, as the rate of killing may be important in clinical situations (17). These ratios were chosen to determine if the killing effects and synergy are dependent not only on the concentrations of the antibiotics but also on the ratio in which these antibiotics are used and to observe the pattern of killing over a range of these ratios. Because a universally accepted laboratory definition of *in vitro* antibiotic synergy and antagonism is not available (17), synergy for this study was defined as a 10-fold increase in bacterial killing at 5 h with an antibiotic combination as compared to the most active antibiotic alone, and antagonism was defined as a 10-fold decrease in bacterial killing at 5 h with an antibiotic combination as compared to the least active antibiotic alone with other interaction being indifference.

The different concentration ratios used for the amikacin + piperacillin combination against the standard inoculum and the large inoculum are given in Table II. Figure 2(A) shows the plot of the log of the reduction factors against the concentration ratios used in the amikacin + piperacillin combination against the standard inoculum. From this figure, it is evident that the order of killing for the combination is 25% C_{ami} + 75% C_{pip} < 10% C_{ami} + 90% C_{pip} < 90% C_{ami} + 10% C_{pip} < 100% C_{ami} < 80% C_{ami} + 20% C_{pip} < 100% C_{pip} < 50% C_{ami} + 50% C_{pip} < 75% C_{ami} + 25% C_{pip} < 70% C_{ami} + 30% C_{pip}. According to the definition of synergy provided, the 75% C_{ami} + 25% C_{pip} and the 70% C_{ami} + 30% C_{pip} concentrations exhibited synergy. All the other concentration

Table II. The Concentration Ratios of Amikacin + Piperacillin Combination used Against *P. aeruginosa* ATCC 9027 for Survival-Time Studies

	Standard inoculum		Large inoculum	
Combination	Amikacin (mg/l)	Piperacillin (mg/l)	Amikacin (mg/l)	Piperacillin (mg/l)
100% C_{ami}	0.80	–	25.60	–
90% C_{ami} + 10% C_{pip}	0.72	0.16	23.04	40.96
80% C_{ami} + 20% C_{pip}	0.64	0.32	20.48	81.90
75% C_{ami} + 25% C_{pip}	0.60	0.40	19.20	102.40
70% C_{ami} + 30% C_{pip}	0.56	0.48	17.92	122.88
50% C_{ami} + 50% C_{pip}	0.40	0.80	12.8	204.80
25% C_{ami} + 75% C_{pip}	0.20	1.20	6.40	307.20
10% C_{ami} + 90% C_{pip}	0.08	1.44	2.56	368.64
100% C_{pip}	–	1.60	–	409.60

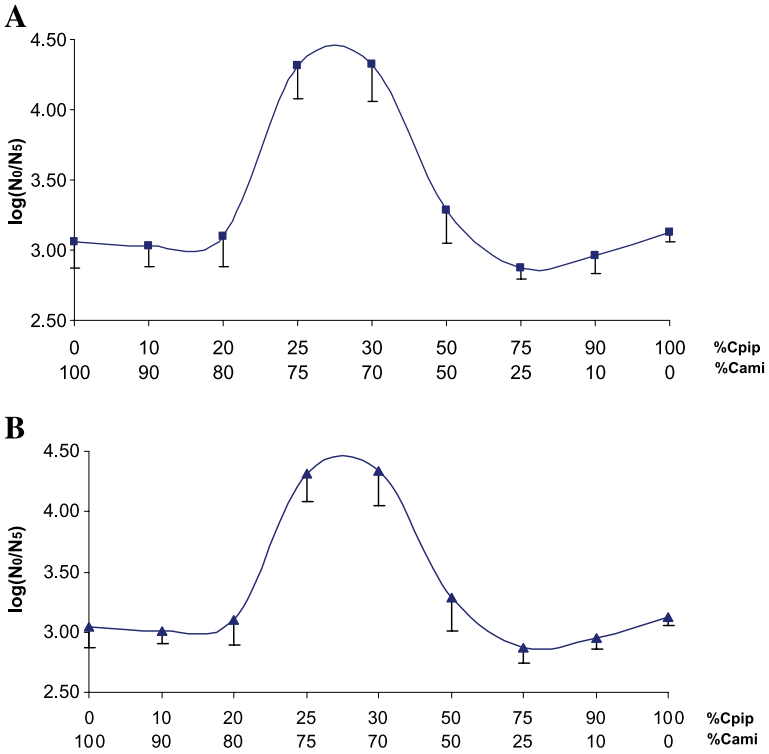

Fig. 2. Plot of the logarithm of reduction factors *vs*. the concentration ratios used in the amikacin + piperacillin combination against (A) a standard inoculum (■) and (B) a large inoculum (▲) of *Pseudomonas aeruginosa* ATCC 9027.

ratios exhibited indifference, whereas no concentration ratio showed antagonism.

Figure 2(B) shows the plot of the log of the reduction factors against the concentration ratios used in the amikacin + piperacillin combination against the large inoculum. The order of killing for the combination was similar to that seen against the standard inoculum with the 75% C_{ami} + 25% C_{pip} and the 70% C_{ami} + 30% C_{pip} concentrations showing synergy. Moreover, the means of the differences of reduction factors of 70:30 and 75:25 concentration ratios from the baseline value (which was arbitrarily set at 3) were significantly different from those of other concentration ratios of the amikacin + piperacillin combination ($p = 0.001$ or 0.002) for both the standard and the large inocula.

The CICs of amikacin and piperacillin for each concentration ratio of the amikacin + piperacillin combination against the standard and the large inocula are shown in Fig. 3(A) and (B), respectively. In both cases, the critical concentrations of each antibiotic decreased exactly in the same order as the reduction factors increased with the 70% C_{ami} + 30% C_{pip} concentration ratio, having the highest reduction factor, showing the least critical concentration. There is no critical concentration for amikacin at 100% C_{pip} and no critical concentration for piperacillin at 100% C_{ami}.

Results of the *in vitro* killing of the various antibiotic combinations against *P. aeruginosa* ATCC 9027 suggest that a definitive killing pattern is present over the range of concentration ratios, and this pattern is similar for both the standard and the large inocula. Synergy was seen only for the amikacin + piperacillin combination at the 70% C_{ami} + 30% C_{pip} and 75% C_{ami} + 25% C_{pip} concentration ratios (Fig. 2). Synergy has been reported for the amikacin + piperacillin combination against *P. aeruginosa* in the literature (26,27). This is the first study in which combination ratios of antibiotic combinations have been used to study the synergistic effect of the antibiotic combination.

Combinations of a cell-wall-active antimicrobial (e.g., penicillin) with an antimicrobial that acts intracellularly (aminoglycoside) are frequently synergistic. This mechanism of synergy is believed to result from the cellular uptake of the aminoglycoside by disruption of cell wall by the other antimicrobial. β-Lactam antibiotics have been shown to exert an effect on the permeability of the outer membrane of gram-negative bacteria (17,28). This increases the number of β-lactam molecules penetrating the periplasmic space, allowing greater saturation of their preferred PBPs and synergy. This mechanism of synergism probably describes the synergism seen between amikacin and piperacillin in this study.

For all the combinations tested in this study, at each concentration ratio, the reduction factors were not statistically significant ($p > 0.05$) between the standard and the large inocula. These findings suggest that while performing *in vitro* survival-time studies, either of the two inocula could be used. However, a better correlation has been reported between *in vivo* efficacy and *in vitro* activities determined with a large inoculum than a standard inoculum (6). In many human and animal infections, the number of organisms isolated from the infected tissue or organ exceeds the inoculum normally used in most *in vitro* studies. It is possible that higher doses of antibiotics may be necessary to cure an infection in

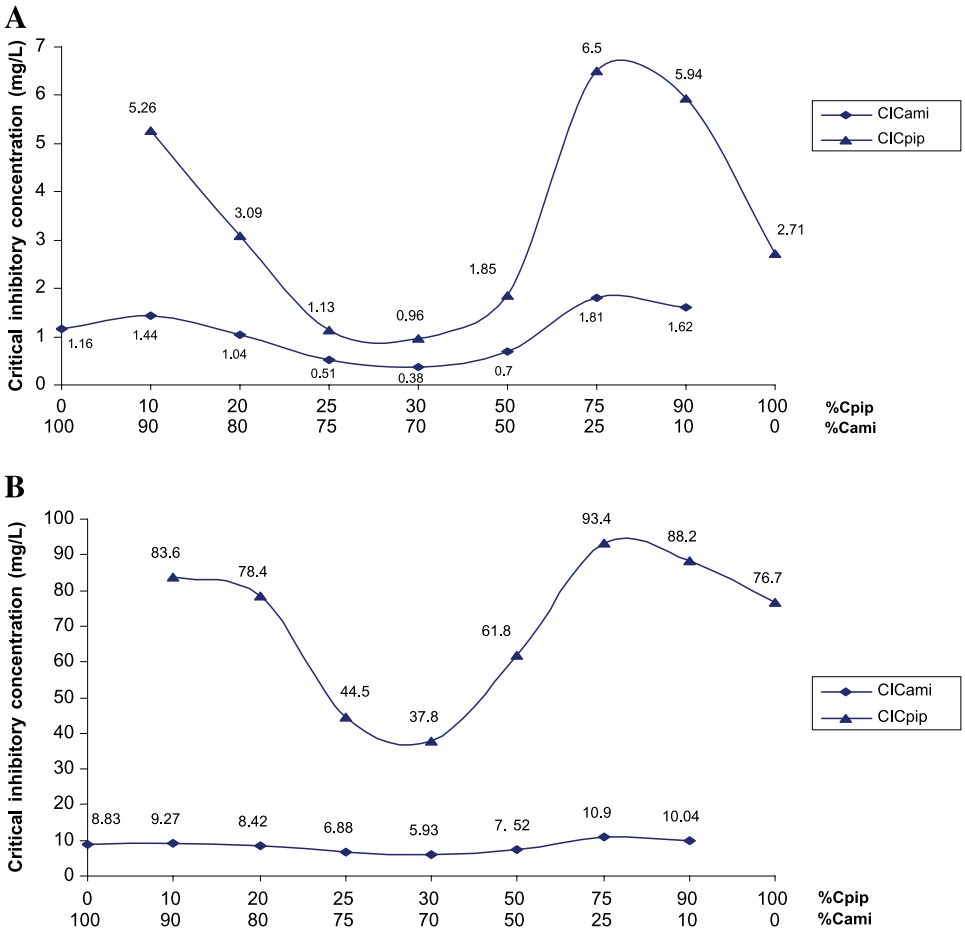

Fig. 3. Critical inhibition concentrations (mg/l) of amikacin (♦) and piperacillin (▲) of the various concentration ratios of the amikacin + piperacillin combination against (A) a standard inoculum and (B) a large inoculum of *P. aeruginosa* ATCC 9027.

which dense bacterial populations are involved, especially with those antibiotics that show a great inoculum effect (6).

The CICs of amikacin and piperacillin for each concentration ratio in the amikacin + piperacillin combination were determined to see if their pattern agreed with that of the killing rates. The CIC is not identical to the MIC, when antibiotics are used in a combination. Each concentration ratio in the combination has a unique critical concentration for a particular antibiotic. Therefore, a higher killing effect implies a lower CIC. This phenomenon was observed for the amikacin + piperacillin combination against both inocula, with the 70% C_{ami} + 30% C_{pip} concentration having the least critical concentrations (Fig. 3). This is consistent with the observed pattern of killing for amikacin + piperacillin combination.

The overall *in vitro* results suggest that the killing effect is not only concentration-dependent but is also dependent on the ratio of the antibiotics used in the combination. Because the ratio at which synergy is exhibited is independent of the inoculum size, this ratio might be unique for a particular combination of antibiotics against a strain of the bacteria. This ratio is also different between different species of bacteria, as synergy was also seen for the amikacin + piperacillin combination against *E. coli* ATCC 8739 at concentration ratios different from those against *P. aeruginosa* ATCC 9027 (data not shown). So far, there have been no reports in the literature of this phenomenon. So while performing *in vitro* survival-time studies to assess the activity of a combination of antimicrobials, it may be worthwhile to test a range of *in vivo*

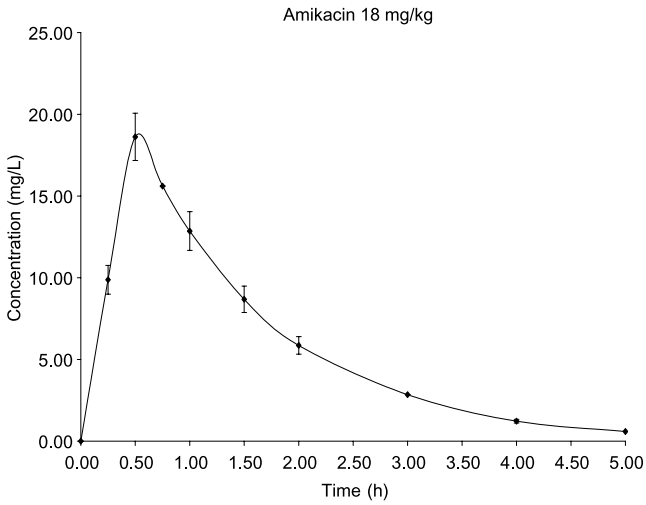

Fig. 4. Concentration–time profile of amikacin in rat plasma after intraperitoneal administration of 18 mg/kg amikacin.

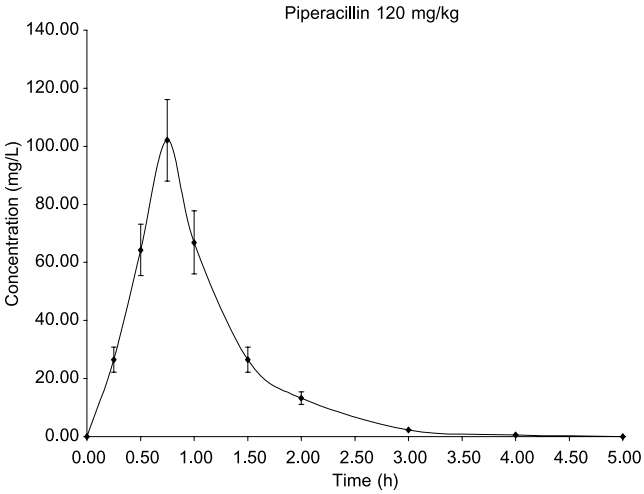

Fig. 5. Concentration–time profile of piperacillin in rat plasma after intraperitoneal administration of 120 mg/kg piperacillin.

plasma-attainable concentration ratios of the two antibiotics rather than to just test for the *in vitro* concentrations used to simulate those achieved *in vivo*, as antimicrobial synergism obtained *in vitro* may not be seen *in vivo* (29).

In Vivo Studies

In vivo studies were performed in rats to estimate the *in vivo* PK and PD parameters for the antibiotic combinations that showed *in vitro* synergy against *P. aeruginosa* ATCC 9027. Plasma concentrations of amikacin and piperacillin were determined with the validated agar-well microbiological assays using *B. subtilis* ATCC 6633 and *E. coli* ATCC 8739 as the test organism, respectively.

Microbiological assays have been used in this study for determining the plasma concentrations of amikacin and piperacillin in this assay because of the relative ease with which they can be performed. The microbiological assays in this study are specific, sensitive, accurate, and precise and therefore show good reproducibility. High-performance liquid chromatography (HPLC) assays have been reported for analyzing amikacin and piperacillin, but these involve complex derivatization steps, which could lead to loss of sensitivity (30,31). There have been other reports that use microbiological assays for analyzing amikacin and piperacillin using *B. subtilis* and *E. coli* as test organisms, respectively. These microbiological assays have been reported to compare well with HPLC techniques with respect to the validation parameters (22). Microbiological assays conducted after inactivating the interfering antibiotic have also been reported to have good agreement with values obtained after HPLC analysis (22). Taking all these factors into consideration, microbiological assays were used in this study.

Pharmacokinetic Parameters

In the present study, the PK parameters of amikacin and piperacillin were determined in adult male Sprague-Dawley rats after a single intraperitoneal dose of 18 and 120 mg/kg, respectively. The use of these doses has been mentioned in other studies (32–35). The plasma concentration–time profiles of amikacin and piperacillin at the doses mentioned above are shown in Figs. 4 and 5, respectively. Amikacin showed peak concentrations between 15 and 45 min after administration, whereas piperacillin was absorbed more slowly and showed peak concentrations between 30 min and 1 h. The PK parameters were also determined for the ratios of the doses of the two antibiotics that were used in this study. These ratios, along with the peak plasma concentrations, elimination half-lives, and AUCs for the two antibiotics, are shown in Table III. The values of the PK parameters obtained were in good agreement with other studies (32,34,35).

In Vivo Pharmacodynamics

The bacterial killing curves for the individual antibiotics as well as the combinations are shown in Fig. 6. This figure shows that, after 5 h, the amikacin at a dose of 18 mg/kg produces a log reduction factor of 1.20, whereas piperacillin at a dose of 120 mg/kg produces a log reduction of 1.08. Figure 6 also shows that the dosing combination of 12.6 mg/kg amikacin and 36 mg/kg piperacillin shows the greatest killing producing a log reduction factor of 2.10. This is nearly eight times greater than the killing seen with amikacin alone and more than ten times greater than the killing with piperacillin alone after 5 h. This dosing combination corresponds to a 70:30 ratio of the individual doses (18 mg/kg amikacin and 120 mg/kg of piperacillin).

Similarly, the 50:50 ratio of the individual doses, 9 mg/kg amikacin and 60 mg/kg piperacillin, showed a slightly greater

Table III. Dose, Dosing Ratio, AUKC, C_{max}, AUC, and $t_{1/2}$ for Amikacin and Piperacillin Combinations used in *in Vivo* Studies

Antibiotics	Dose (mg/kg)	Dosing ratio (amikacin/piperacillin)	AUKC (h)	C_{max} (mg/l)	AUC (mg h/l)	$t_{1/2}$ (h)
Amikacin	18	100:0	2.5852 ± 0.2608	18.62 ± 1.44	29.00 ± 4.06	2.07 ± 0.22
Piperacillin	–			–	–	–
Amikacin	12.6	70:30	4.2375 ± 0.3048	14.33 ± 1.10	22.45 ± 3.34	2.19 ± 0.25
Piperacillin	36			29.58 ± 0.14	29.26 ± 5.67	1.67 ± 0.17
Amikacin	9	50:50	2.9217 ± 0.2422	10.12 ± 0.75	15.98 ± 3.28	2.23 ± 0.31
Piperacillin	60			46.96 ± 0.25	50.40 ± 8.43	1.50 ± 0.13
Amikacin	–	0:100	2.2360 ± 0.2855	–	–	–
Piperacillin	120			102.05 ± 14.10	99.35 ± 10.39	1.33 ± 0.16

AUKC = area under the *in vivo* killing curve; AUC = area under the curve.

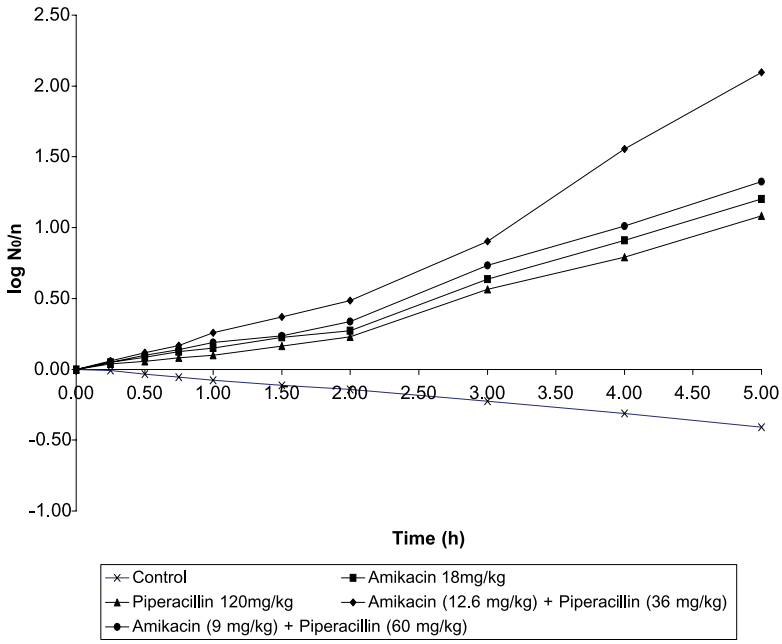

Fig. 6. *In vivo* killing curves for the various dosing combinations against *P. aeruginosa* ATCC 9027.

killing effect than the individual antibiotics alone. This dosing combination produced a log reduction factor of 1.32. The doses that were used are summarized in Table III.

Correlation of PK Parameters to *in Vivo* Efficacy

Because killing curves were used in this study, it is rational to use a killing parameter as a PD marker. Therefore, the AUKC for the various combinations was chosen as the PD marker (Table III). The choice of a proper PK–PD relationship is difficult in the case of this study, as ratios of combination of antibiotics that showed *in vitro* synergy were used. Combination of the 70% C_{ami} + 30% C_{pip} showed synergy and exhibited the greatest killing when compared to the individual antibiotics. When this ratio, i.e., 70:30, was used as the dosing ratio of the antibiotics to determine the *in vivo* bacterial killing vis-a-vis the bacterial killing when the same ratio was used *in vitro*, similar results were obtained. The dosing ratio of 70:30 (corresponding to 12.6 mg/kg amikacin and 36 mg/kg of piperacillin) showed almost ten times greater killing than when either 18 mg/kg amikacin or 120 mg/kg piperacillin was given alone. However, the plasma concentrations of amikacin and piperacillin when administered as this combination are obviously lesser than plasma concentrations when these antibiotics are administered alone at the normal dose. This results in lower C_{max} and AUC values for the antibiotics in a combination (Table III). Moreover, the use of antibiotic combinations also reduces the AUIC and AUCIC (area under CICs, defined as the area under the curve for time points where concentrations are above the CICs) for the antibiotics in the combination. The AUIC and AUCIC for the different dosing ratios of the amikacin + piperacillin combination are shown in Tables IV and V, respectively. The AUIC and AUCIC values for piperacillin in the case of the large inoculum were either 0 or close to 0 for a 70:30 dosing combination. Yet, this dosing ratio exhibited the highest killing. This could be because the concentration of bacteria that was targeted to be obtained *in vivo* represents a standard inoculum, and therefore, the AUIC and AUCIC values for the standard inoculum may be the true representatives. However, this situation does reflect on the ambiguity of using AUIC or AUCIC alone as PK markers. As expected, poor relationships were seen between AUKC and AUC (i.e., AUC_{ami} + AUC_{pip}; r^2 = 0.130), and AUKC and C_{max} (i.e., $C_{max,ami}$ + $C_{max,pip}$; r^2 = 0.144), as well as between AUKC and AUIC (i.e., $AUIC_{ami}$ +

Table IV. AUIC Values for Amikacin and Piperacillin for Different Dosing Ratios of the Amikacin + Piperacillin Combination

	AUIC when MIC values for standard inocula are used		AUIC when MIC values for large inocula are used	
%Ami/%Pip	Amikacin (mg h/L)	Piperacillin (mg h/L)	Amikacin (mg h/L)	Piperacillin (mg h/L)
100:0	28.52 ± 4.06	–	17.17 ± 3.66	–
70:30	21.97 ± 3.34	27.56 ± 5.67	11.38 ± 3.02	0.995 ± 0.341
50:50	15.49 ± 3.28	48.72 ± 8.43	5.935 ± 1.61	10.80 ± 5.69
0:100	–	96.13 ± 10.39	–	44.35 ± 8.63

AUIC = area under the inhibitory plasma concentration–time curve.

Table V. AUCIC Values for Amikacin and Piperacillin for Different Dosing Ratios of the Amikacin + Piperacillin Combination

	AUCIC when critical inhibitory concentration values for standard inocula are used		AUCIC when critical inhibitory concentration values for large inocula are used	
%Ami/%Pip	Amikacin (mg h/l)	Piperacillin (mg h/l)	Amikacin (mg h/l)	Piperacillin (mg h/l)
100:0	23.70 ± 3.48	–	6.234 ± 1.76	–
70:30	20.60 ± 3.34	25.89 ± 5.67	5.713 ± 1.68	0
50:50	12.71 ± 3.15	44.02 ± 7.81	0.9237 ± 0.2676	0
0:100	–	89.98 ± 10.31	–	64.37 ± 8.51

AUCIC = area under critical inhibitory concentration.

$AUIC_{pip}$; r^2 = 0.140 and 0.479) and between AUKC and AUCIC (i.e., $AUCIC_{ami}$ + $AUCIC_{pip}$; r^2 = 0.114 and 0.316) for either the standard or the large inocula. Therefore, AUIC, AUCIC, C_{max}, and AUC values were normalized with respect to either the MICs or the CICs: AUIC/MIC, AUCIC/MIC, AUC/MIC, C_{max}/MIC, AUIC/CIC, AUCIC/CIC, AUC/CIC, and C_{max}/CIC, and these were used as PK markers. The CICs represent the inhibitory concentrations of the individual antibiotics when used in a combination, whereas the MICs represent the inhibitory concentrations of the antibiotics when used alone.

The relationship between AUKC and AUIC/MIC (i.e., $AUIC_{ami}/MIC_{ami}$ + $AUIC_{pip}/MIC_{pip}$) was found to be poor (r^2 = 0.448 and 0.0342) for either the standard or the large inocula. Poor relationships were also seen between AUKC and AUC/MIC (i.e., AUC_{ami}/MIC_{ami} + AUC_{pip}/MIC_{pip}; r^2 = 0.518 and 0.250), and AUKC and C_{max}/MIC (i.e., $C_{max,ami}/MIC_{ami}$ + $C_{max,pip}/MIC_{pip}$; r^2 = 0.047 and 0.317), for either the standard or the large inocula, as well as between AUKC and AUCIC/MIC (i.e., $AUCIC_{ami}/MIC_{ami}$ + $AUCIC_{pip}/MIC_{pip}$) for the large inoculum (r^2 = 0.042). A strong relationship (r^2 = 0.976) between AUKC and AUCIC/MIC, however, was seen for the standard inoculum (Fig. 7). It is clear that there is a poor correlation between the PD marker, AUKC, and the PK parameters (AUC, C_{max}, and AUIC) normalized with respect to the MIC. The reason for these poor relationships might be because there is a small decrease or little change in the sum of C_{max}/MIC, AUC/MIC, and AUIC/MIC values of individual antibiotics, which are proportionately decreased with reduced dosing, and yet there is a synergistic increase in bactericidal effect for a 70:30 dosing combination. In contrast, the corresponding sum (270.75 h) of AUCIC/MIC values of individual antibiotics is the highest compared to that of other dosing combination (237.05 h when amikacin given alone or 225.0 h when piperacillin given alone) for the standard inoculum that could account for a synergistic increase in bactericidal effect observed (Fig. 7). Such a good relationship was not seen for the large inoculum, perhaps because of no contribution of piperacillin to the overall sum of AUCIC/MIC for the dosing combinations (AUCIC = 0, Table V). For Fig. 7, the linear regression equation obtained is as follows:

$$AUKC = 0.0438\left(AUCIC_{ami}/MIC_{ami} + AUCIC_{pip}/MIC_{pip}\right) - 7.637, \quad (1)$$

r^2 = 0.976 (Fig. 7) when CIC and MIC for the standard inoculum are used.

The relationships between AUKC and AUIC/CIC (i.e., $AUIC_{ami}/CIC_{ami}$ + $AUIC_{pip}/CIC_{pip}$; r^2 = 0.370), and AUKC and AUCIC/CIC (i.e., $AUCIC_{ami}/CIC_{ami}$ + $AUCIC_{pip}/CIC_{pip}$), were found to be poor (r^2 = 0.370 and 0.072, respectively) for the large inoculum only, but were good (r^2 =0.661 for the former and, in particular, 0.872 for the latter; Fig. 8) for the standard inoculum. In addition, when AUC/CIC and C_{max}/CIC were chosen as the PK parameters, plots of the AUKC *vs.* AUC_{ami}/CIC_{ami} + AUC_{pip}/CIC_{pip} and AUKC *vs.* $C_{max,ami}/CIC_{ami}$ + $C_{max,pip}/CIC_{pip}$ show good correlation between the parameters even when CICs of both

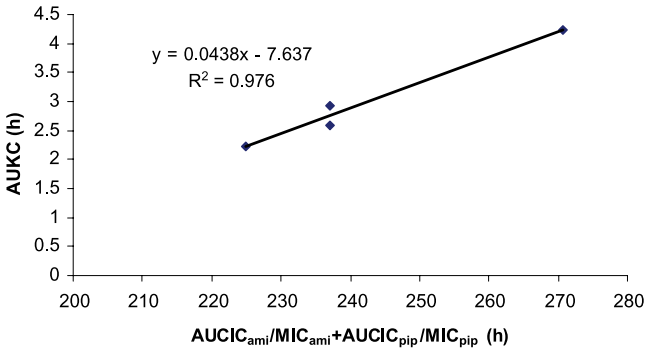

Fig. 7. Plot of AUKC *vs.* $AUCIC_{ami}/MIC_{ami}$ + $AUCIC_{pip}/MIC_{pip}$, when critical inhibitory concentrations (CICs) and minimum inhibitory concentrations (MICs) for a standard inoculum are used. AUKC = area under the *in vivo* killing curve; AUCIC = area under critical inhibitory concentration.

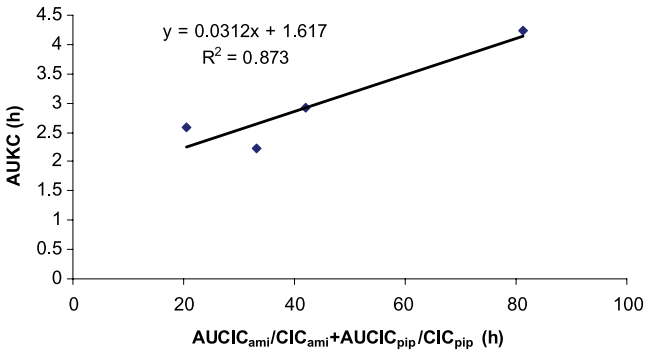

Fig. 8. Plot of AUKC *vs.* $AUCIC_{ami}/CIC_{ami}$ + $AUCIC_{pip}/CIC_{pip}$, when CICs for a standard inoculum are used.

the standard and the large inocula are used. The linear regression equations are as follows:

$$\mathrm{AUKC} = 0.0312\left(\mathrm{AUCIC_{ami}}/\mathrm{CIC_{pip}} + \mathrm{AUCIC_{pip}}/\mathrm{CIC_{pip}}\right) + 1.617, \quad (2)$$

$r^2 = 0.872$ (Fig. 8) when CIC for the standard inoculum is used.

$$\mathrm{AUKC} = 0.0294\left(\mathrm{AUC_{ami}}/\mathrm{CIC_{ami}} + \mathrm{AUC_{pip}}/\mathrm{CIC_{pip}}\right) + 1.518, \quad (3)$$

$r^2 = 0.891$ (Fig. 9) when CIC for the standard inoculum is used.

$$\mathrm{AUKC} = 0.577\left(\mathrm{AUC_{ami}}/\mathrm{CIC_{ami}} + \mathrm{AUC_{pip}}/\mathrm{CIC_{pip}}\right) + 1.253, \quad (4)$$

$r^2 = 0.785$ (Fig. 9) when CIC for the large inoculum is used.

$$\mathrm{AUKC} = 0.0338\left(C_{\mathrm{max,ami}}/\mathrm{CIC_{ami}} + C_{\mathrm{max,pip}}/\mathrm{CIC_{pip}}\right) + 1.625, \quad (5)$$

$r^2 = 0.694$ (Fig. 10) when CIC for the standard inoculum is used.

$$\mathrm{AUKC} = 1.098\left(C_{\mathrm{max,ami}}/\mathrm{CIC_{ami}} + C_{\mathrm{max,pip}}/\mathrm{CIC_{pip}}\right) + 0.597, \quad (6)$$

$r^2 = 0.932$ (Fig. 10) when CIC for the large inoculum is used.

The PK–PD relationships described by Eqs. (1)–(6) are simple linear regressions, and it may be possible to predict the killing effects (AUKC) of the amikacin + piperacillin combination *in vivo* using AUCIC/MIC, AUCIC/CIC, AUC/CIC, or C_{max}/CIC as the PK parameter. The relationships have better correlations as compared to the other PK–PD relationships dealt with earlier. By estimating the CFU per milliliter of bacteria obtained from the infected site and determining whether it represents a standard or a large inoculum, it is possible to use the corresponding CIC value for the PK–PD relationships. In this study, the *in vivo* concentration of bacteria obtained from blood represented the standard inoculum. Thus, Eqs. (1)–(3) and (5) in which the critical concentrations for a standard inoculum are used may be the most appropriate in the case of this study.

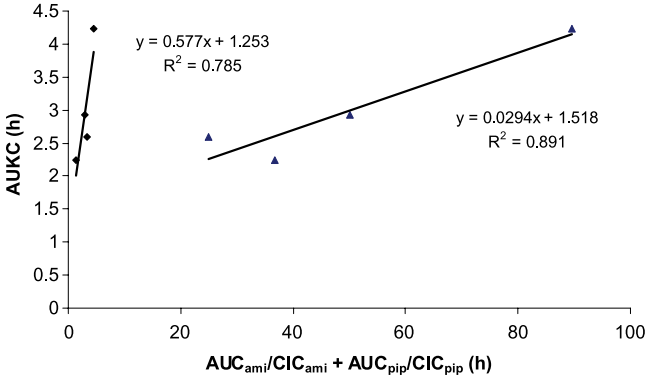

Fig. 9. Plot of AUKC *vs.* $\mathrm{AUC_{ami}}/\mathrm{CIC_{ami}}$ + $\mathrm{AUC_{pip}}/\mathrm{CIC_{pip}}$, when CICs for a standard inoculum (▲) and a large inoculum (♦) are used.

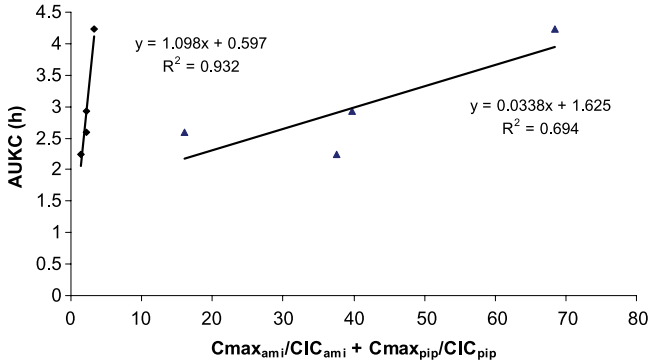

Fig. 10. Plot of AUKC *vs.* $C_{\mathrm{max,ami}}/\mathrm{CIC_{ami}}$ + $C_{\mathrm{max,pip}}/\mathrm{CIC_{pip}}$, when CICs for a standard inoculum (▲) and a large inoculum (♦) are used.

However, it needs to be mentioned that the linear equations describing the PK–PD relationships may be valid only for the dosing combinations, route of administration, model of infection, CICs, and the strain of *P. aeruginosa* used in this study. But it is clear that when an antibiotic combination is used in concentration ratios, as shown in this study, it is possible to obtain a good PK–PD model using AUKC as the PD parameter and either AUCIC/MIC, AUCIC/CIC, AUC/CIC, or C_{max}/CIC as the PK parameter. This model seems to be the first of its kind for antibiotic combinations.

CONCLUSION

The antibacterial activities of amikacin and piperacillin, alone and in combinations, were tested against a standard (5×10^5 CFU/ml) and a large (1.5×10^8 CFU/ml) inocula of *P. aeruginosa* ATCC 9027 using a modified survival-time method. An inoculum effect was observed with the antibiotics studied. Synergy was seen against both the inocula with the amikacin + piperacillin combinations at the following concentration ratios: 70% C_{ami} + 30% C_{pip} and 75% C_{ami} + 25% C_{pip}, where C_{ami} and C_{pip} are the concentrations of amikacin and piperacillin to produce a 1000-fold decrease in bacterial population over 5 h, respectively. CICs determined for these combinations corroborated with the pattern of killing observed.

In vivo studies were performed on Sprague–Dawley rats using a systemic model of infection with *P. aeruginosa* ATCC 9027. PK parameters and *in vivo* killing effects of the amikacin + piperacillin combination at different dosing ratios were studied. The dosing ratio of 12.6 mg/kg amikacin + 36 mg/kg piperacillin corresponding to a 70:30 ratio of the individual doses exhibited the greatest killing *in vivo* when compared to the other ratios.

The bacterial killing *in vivo* observed was consistent with that *in vitro* as the 70:30 dosing (or concentration) ratio of the amikacin + piperacillin combination showed the

greatest killing in both tests. The common PK–PD relationships that are used for antibiotics involve the MIC as a PK–PD surrogate marker, e.g., time above MIC, AUC/MIC ratio, and C_{max}/MIC ratio. These relationships are valid only when a single antibiotic is used and where there is no inoculum effect. Inoculum effect invalidates the use of the MIC as a surrogate marker. The AUKC was used as the PD marker in this study. Either C_{max}, AUC, and AUCIC values normalized with respect to the CICs or AUCIC values normalized with respect to the MICs, $C_{max,ami}/CIC_{ami}$ + $C_{max,pip}/CIC_{pip}$, AUC_{ami}/CIC_{ami} + AUC_{pip}/CIC_{pip}, $AUCIC_{ami}/CIC_{ami}$ + $AUCIC_{pip}/CIC_{pip}$, and $AUCIC_{ami}/MIC_{ami}$ + $AUCIC_{pip}/MIC_{pip}$ were chosen as the PK parameters. There seems to be definitive linear relationships between AUKC and the PK parameters, and these relationships can be used to predict the killing effects of the antibiotic combination if the doses of the antibiotics are known. These relationships seem to be the first PK–PD models reported for an antibiotic combination.

This study shows that the amikacin + piperacillin combination used against *P. aeruginosa* ATCC 9027 achieves two objectives of an ideal antibiotic combination: synergy and reduced dosing when the combination is used.

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