# THE POSTANTIBIOTIC EFFECT OF THE CARBAPENEM ANTIBIOTICS ON GRAM-NEGATIVE BACTERIA

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Doctor of Philosophy

ASTON UNIVERSITY

September 1998

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## SUMMARY

Postantibiotic effect (PAE) describes the suppression of microbial growth occurring after a short exposure to an antimicrobial agent. PAE appears to be a property of the majority of antimicrobial agents and is demonstrated by a wide variety of microorganisms. At present, carbapenems and penems are the only members of the ß-lactam group of antimicrobial agents that exhibit a significant PAE on Gram-negative bacilli.

A standardised method was developed to evaluate the *in vitro* PAE of three carbapenems; imipenem, meropenem and biapenem on Gram-negative bacteria under reproducible laboratory conditions that partially mimicked those occurring *in vivo*. The effects on carbapenem PAE of the method of antimicrobial removal, concentration, exposure duration, inoculum size, inoculum growth phase, multiple exposures and pooled human serum were determined. Additionally, the reproducibility, susceptibility prior to and after PAE determination and inter-strain variation of carbapenem PAE were evaluated. The method developed determined PAE by utilising viable counts and demonstrated carbapenem PAE to be reproducible, constant over successive exposures, dependent on genera, concentration, duration of exposure, inoculum size and growth phase. In addition, carbapenem PAE was not significantly affected either by agitation, the antimicrobial removal method or the viable count diluent.

At present, the mechanism underlying PAE is undetermined. It is thought to be due to either the prolonged persistence of the antimicrobial at the cellular site of action or the true recovery period from non-lethal damage. Increasing the L-lysine concentration and salinity at recovery decreased and increased the carbapenem and imipenem PAE of *Pseudomonas aeruginosa*, respectively. In addition, no apparent change was observed in the production of virulence factors by *P. aeruginosa* in PAE phase. However, alterations in cell morphology were observed throughout PAE phase and the reappearance of normal cell morphology corresponded to the duration of PAE determined by viable count. Thus, the recovery of the penicillin binding protein target enzymes appears to be the mechanism behind carbapenem PAE in *P. aeruginosa*.

Keywords : Imipenem, Meropenem, Biapenem,
*Pseudomonas aeruginosa*, *Escherichia coli*.

## ACKNOWLEDGEMENTS

I am very grateful to Mrs Jenny Andrews for her invaluable support and all the other staff of the Antimicrobial Chemotherapy Research Department at The City Hospital for their help and encouragement. I would also like to thank Professor R. Wise for his constant supervision and funding.

I would especially like to thank Dr P. Lambert of Aston University for his constant encouragement and helpful advice.

I am also grateful to Mr P. Stanley of Birmingham University for the preparation of samples for examination by scanning electron microscopy.

The constant support during the writing of this thesis of James Mundye is also gratefully acknowledged.

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## ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| AUC | Area under concentration curve |
| CERT | Control related effective regrowth time |
| CFU | Colony forming units |
| $C_{max}$ | Peak serum concentration |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| CV | Coefficient of variance |
| °C | Degrees centigrade |
| DHP I | Dehydropeptidase I |
| EOS | Effect of serum |
| ERT | Effective regrowth time |
| Etest | Epsilometer test |
| $E_{50}$ | AUC at 50% maximal PAE |
| g | gram (s) |
| *g* | acceleration due to gravity (9.81 $ms^{-1}$) |
| h | hour (s) |
| $IC_{50}$ | the amount of unlabelled antibiotic which reduces radiolabelled benzyl penicillin binding to a PBP by 50% |
| IV | Intravenous |
| IM | Intramuscular |
| kDa | kiloDalton |
| L | litre (s) |
| LPS | lipopolysaccharide |
| M | mole (s) per litre |
| MBC | minimum bactericidal concentration |
| mg | milligram (s) |
| MHB | Mueller-Hinton broth |
| MIC | minimum inhibitory concentration |
| $MIC_{50}$ | minimum inhibitory concentration of 50% of isolates tested |
| $MIC_{90}$ | minimum inhibitory concentration of 90% of isolates tested |

| | |
|---|---|
| mL | millilitre (s) |
| mM | millimole (s) per litre |
| mm | millimetre (s) |
| µm | micrometre (s) |
| MOPS | 3-(*N*-Morpholino) propane-sulphonic acid |
| mOsm/kg | milliOsmoles/kilogram |
| µg | microgram (s) |
| µL | microlitre (s) |
| PAE | Postantibiotic effect |
| $PAE_{MAX}$ | Maximal PAE |
| PALE | Postantibiotic leucocyte enhancement |
| PA SME | Postantibiotic sub-MIC effect |
| PBP | Penicillin binding protein |
| PBS | Phosphate buffered saline |
| PLIE | Post ß-lactamase inhibitor effect |
| RAC | Residual antimicrobial control |
| SD | Standard deviation |
| SOS-response | Response due to induction of damage-inducible genes |
| $T_{1/2}$ | Serum half-life |

# 1. INTRODUCTION

## 1.1 Postantibiotic effect.

The term postantibiotic effect (PAE) is used to describe the phenomenon of the persisting suppression of microbial growth that occurs after a short exposure to an antimicrobial agent (McDonald *et al.*, 1977). It is due to a prior antimicrobial exposure rather than any persisting subinhibitory concentration of antimicrobial and is attributed to either a lag period of growth, an increase in microorganism generation time or a combination of both (MacKenzie and Gould, 1993). PAE was first observed in 1944 by Bigger who demonstrated that staphylococci exposed to penicillin for a short period of time did not resume normal growth for several hours. Parker and Luse in 1948, and Eagle and Musselman in 1949 also demonstrated the PAE of penicillin on staphylococci. Since the 1970s interest in PAE has increased, Rolinson in 1973 and McDonald and coworkers in 1977 studied other antimicrobial agents and Gram-negative organisms. PAE has since been observed with a wide variety of microorganisms and appears to be a property of the majority of antimicrobial agents in clinical use.

PAE has been determined using various *in vitro* and *in vivo* methods, the majority of which involve the determination of microbial growth kinetics. At present, there is neither an accepted standardised method for PAE determination nor defined criteria for its reproducibility.

The microorganisms studied, their growth phase, the concentration and duration of antimicrobial exposure, the method of antimicrobial removal and the quantification of PAE are all highly variable, therefore, data comparison between investigators is difficult. In addition, the observed variations in PAE duration have been partially attributed to the use of different growth kinetic monitoring methods (MacKenzie and Gould, 1993).

### 1.1.1 Rapid methods of antimicrobial agent removal.

For the *in vitro* determination of PAE it is essential to rapidly transfer the exposed and possibly damaged but surviving microorganisms (after the required duration of exposure) to antimicrobial-free media. Several methods of rapid antimicrobial removal have been used, these include: repeated washing; dilution; antimicrobial inactivation and filtration. With all methods of antimicrobial removal a non-exposed control culture must be included and treated in the same manner as the exposed test culture.

By the definition of PAE, the antimicrobial should be entirely eliminated after completion of the selected removal procedure, although usually a small subinhibitory amount persists. In addition, an antimicrobial that may be bound to the microorganism will not be removed by either dilution, washing or filtration methods and therefore, it is not always possible to distinguish a true PAE from a subinhibitory effect (Daikos, 1991). However, to ascertain that the PAE observed is not due to the extracellular persisting subinhibitory concentration of antimicrobial, a residual antimicrobial control (RAC) should be used.

Repeated washing has been used by many investigators, all of whom use at least two washes to ensure considerable antimicrobial removal (Craig and Gudmundsson, 1996). Washing is usually performed by centrifuging broth cultures at 1200 *g* or greater for 5 to 10 minutes, the supernatant is then removed and the pellet resuspended in fresh antimicrobial-free media. With removal of 90% of the supernatant, washing twice will reduce the antimicrobial concentration one hundred-fold (Craig and Gudmundsson, 1996). With complete removal of the supernatant, washing twice will reduce the antimicrobial concentration by ten thousand-fold (Craig and Gudmundsson, 1996). The washing procedure itself can have an effect on microbial growth due to a decrease in temperature, mechanical forces such as hydrostatic pressures and shearing stresses, decompression and resuspension which grossly distort surface characteristics (Gilbert *et al.*, 1991), osmotic effects and depletion of nutrients. Washing is a universal method that can be used for all classes of antimicrobial agent, but it is not particularly rapid as the entire procedure can take at least 30 minutes. A RAC for this method of removal has been described, whereby 0.1 mL of supernatant following the third centrifugation was added to a non-exposed culture, which then grew at same rate as the growth controls (Karlowsky *et al.*, 1993a).

Dilution is another universal removal method which is more rapid and more widely used than the washing method (Craig and Gudmundsson, 1996). This method involves the addition of a small volume of the antimicrobial exposed culture to a large volume of antimicrobial-free media. An adequate dilution must be performed to ensure that the antimicrobial concentration in the diluted culture fails to affect the growth of the microorganisms.

At exposure concentrations near the minimum inhibitory concentration (MIC), one hundred-fold dilution is sufficient, although at higher concentrations one thousand- or ten thousand-fold dilution is required (Craig and Gudmundsson, 1996). When using this method an initial microorganism inoculum of at least $10^5$ or $10^6$ colony forming units (CFU)/mL is necessary as the microorganisms are diluted to the same extent as the antimicrobial. The major disadvantage of the dilution method becomes apparent when the antimicrobial being investigated is rapidly bactericidal, because the number of microorganisms will fall below the lower limit of detection. A RAC can be easily achieved with this method by constantly exposing the culture to a concentration equivalent to the exposure concentration diluted by the dilution factor used to remove the antimicrobial. For example, if an exposure concentration of 2 mg/L is investigated followed by a thousand-fold dilution to remove the antimicrobial, the RAC contains culture constantly exposed to a concentration of 0.002 mg/L.

Another rapid method of antimicrobial removal is inactivation, this is not a universal method and has been confined largely to the ß-lactams due to simplicity although, it has occasionally been used with aminoglycosides (Gerber *et al.*, 1982; den Hollander *et al.*, 1996). Inactivation of the ß-lactams is usually performed by incubation with commercially available broad spectrum ß-lactamases. Microbiological plate assays have been performed to determine the antimicrobial concentration after inactivation and hence verify that inactivation was effective (Odenholt *et al.*, 1989a). Aminoglycosides, however, are inactivated by the addition of cellulose phosphate powder which then requires centrifugation to remove the powder-antimicrobial complex (Gerber *et al.*, 1982).

Recently an enzymatic method of aminoglycoside removal has been described using an aminoglycoside-acetylating enzyme extracted from a clinical isolate (den Hollander *et al.*, 1996).

Filtration using membrane filters as a method of antimicrobial removal has been utilised by Baquero and coworkers (1986). This method removes virtually all antimicrobial agent with minimum mechanical disturbance, although the removal of microorganisms from the membrane filter for enumeration is more time consuming than sampling from liquid media. Another application for membrane filters is to facilitate the exposure of microorganisms to antimicrobial by diffusion from the surface of an agar plate containing an antimicrobial agent, removal is then by repeated transfer of the filters to antimicrobial-free agar plates (Lorian *et al.*, 1989). Using this method the microorganisms are less affected by the mechanical effects associated with liquid media, although antimicrobial removal is awkward and difficult to control compared to the previously described methods.

Antimicrobial removal by ion-exchange resin could be used for many antimicrobials however, it has only been used for the cephalosporin, cefotetan (Ravizzola *et al.*, 1983).

### 1.1.2 *In vitro* methods of quantification of PAE.

Methods used for determining PAE differ in the techniques used for quantifying both the number of viable microorganisms and the PAE itself.

The majority of investigators have used viable counts of CFU and this is also the comparator method normally used for novel growth kinetic measurement methods (MacKenzie and Gould, 1993). However, viable counting methods are laborious, tedious and time consuming, so investigators usually only perform one or two experiments for each antimicrobial-microorganism combination and this does not enable the determination of reproducibility or the investigation of strain variation. In addition, any viable counting method is fundamentally flawed due to the assumption that there is a one to one relationship between a single bacterial genome and a single CFU (Lorian *et al.*, 1989).

Viable counts can also be inaccurate when aberrant cell forms are present. For example, problems occur with viable counts of Gram-negative bacteria because they undergo filamentation when exposed to ß-lactams that act on penicillin binding protein (PBP) 3. Rod shaped filaments of >10 µm in length result from bacilli growing and multiplying but not dividing into new individual bacterial cells (Lorian and Gemmel, 1991). However, after antimicrobial removal each filament can divide into many microorganisms, as one filament can contain more than 20 microorganisms (Hanberger *et al.*, 1990). This gives the impression that there is a more rapid increase in CFUs in the antimicrobial exposed cultures compared to the non-exposed cultures and therefore a negative PAE is observed. Conversely, if division does not occur, 20 microorganisms will be counted as 1 CFU.

Consequently, it has been recommended that for the calculation of PAE of ß-lactams and Gram-negative bacteria, after CFU data is plotted, the regrowth curve of the exposed culture should be extrapolated from the portion of curve demonstrating conventional regrowth kinetics to a new intercept at time zero (Kroeker *et al.*, 1995).

Microorganisms are also affected by the transfer from broth to solid media as this represents a major change in both the physical and chemical environment. Some defects that may not be lethal in broth might prevent growth on solid media (Yourassowsky *et al.*, 1981). These sub-lethally damaged bacteria are an artefact of *in vitro* testing and can give a false impression of low microbial numbers and therefore, an underestimation of PAE. This is observed with ß-lactams that act on PBP 2 of Gram-negative bacilli causing the formation of spherical cells which then lyse on solid media (Hanberger *et al.*, 1990). Also, microorganisms exposed to antimicrobials adhere to each other more readily than non-exposed microorganisms, therefore, viable counts appear artificially low and PAE is underestimated (MacKenzie and Gould, 1993).

Initially, PAE was defined as the period of stationary growth observed after antimicrobial removal (Eagle and Musselman, 1949), although a gradual increase or decrease in viable count can be observed before normal growth rates are achieved.

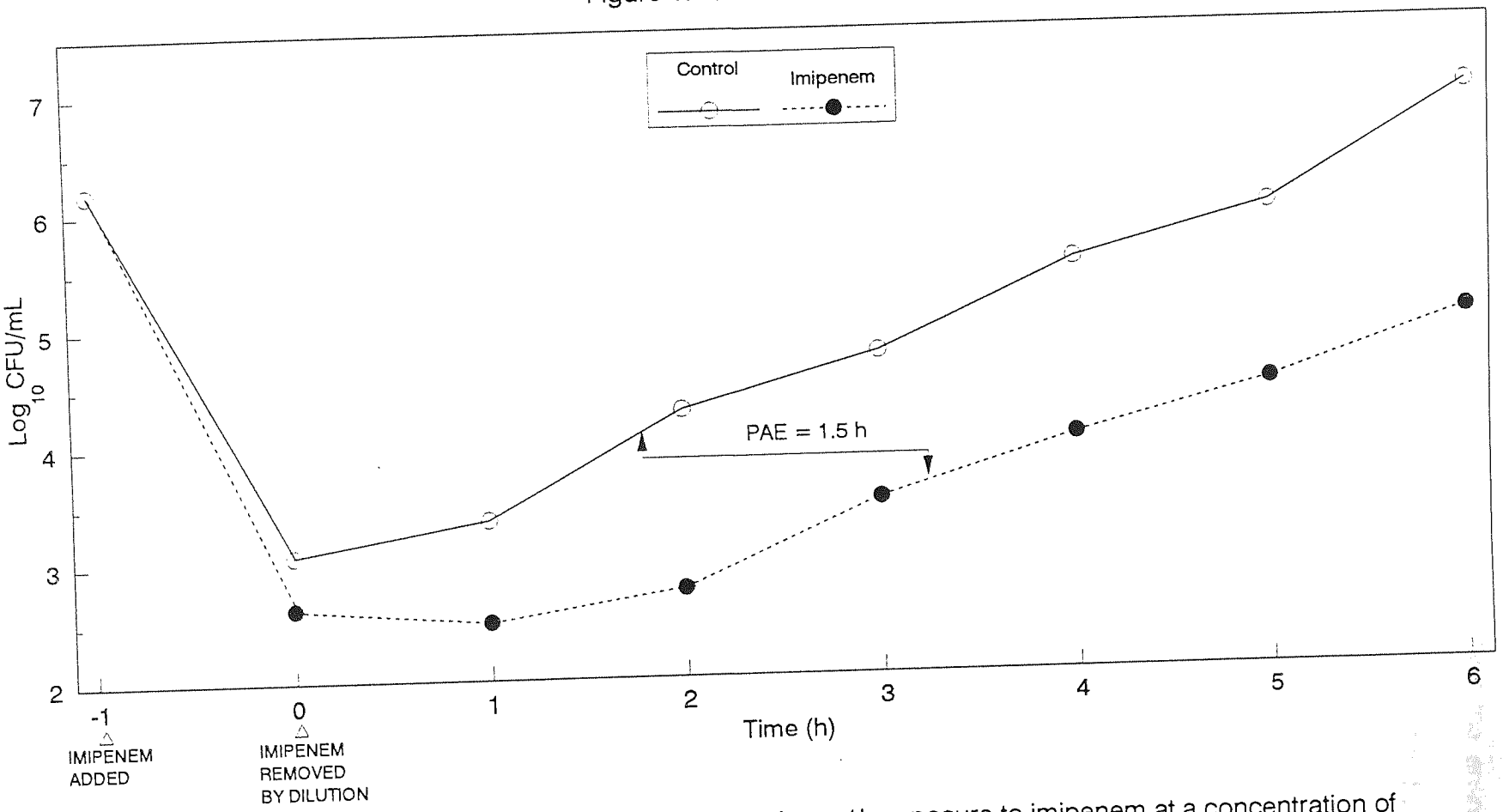
To allow definition and enable the comparison between different experiments an equation was developed :

$$PAE = T - C$$

where $T$ is the time (h) required for the count of CFU in the exposed test culture to increase by 1 $\log_{10}$ above the count observed immediately after antimicrobial removal and $C$ is the time (h) required for the count of CFU in the non-exposed control culture to increase by 1 $\log_{10}$ above the count observed immediately after completion of the same procedure used on the test culture for antimicrobial removal (Bundtzen *et al.*, 1981). The 1 $\log_{10}$ increase in CFU was chosen because beyond this the growth rate of the previously exposed culture was identical to that of the non-exposed control culture (Bundtzen *et al.*, 1981). With this method of quantification, antimicrobial removal method comparison studies gave PAE values that differed by 30% or less (Bundtzen *et al.*, 1981). Figure 1 demonstrates the quantification of imipenem PAE on *Pseudomonas aeruginosa* strain G404 using the above equation.

There are criticisms of the PAE = $T$ - $C$ equation, mainly because it emphasises an arbitrarily defined point with no consideration for the manner in which the microbial population reaches the defined point. Another fault is that the initial inocula of the test and control cultures could be different and therefore their growth rates may also be different. This difference can be overcome by altering the control inoculum to be the same as that of the test (Odenholt-Tornqvist, 1993). Despite these flaws it is still the most commonly used method of quantification of PAE.

Figure 1. Quantification of PAE.




Growth curves of *P. aeruginosa* strain G404 following a 1h exposure to imipenem at a concentration of four times the MIC. Imipenem was removed by a 1000-fold dilution into fresh broth. Arrows (▲) denote the time for the number of bacteria to increase by 1 $\log_{10}$ above the number present immediately after dilution, the difference in time between the arrows represents the duration of PAE.

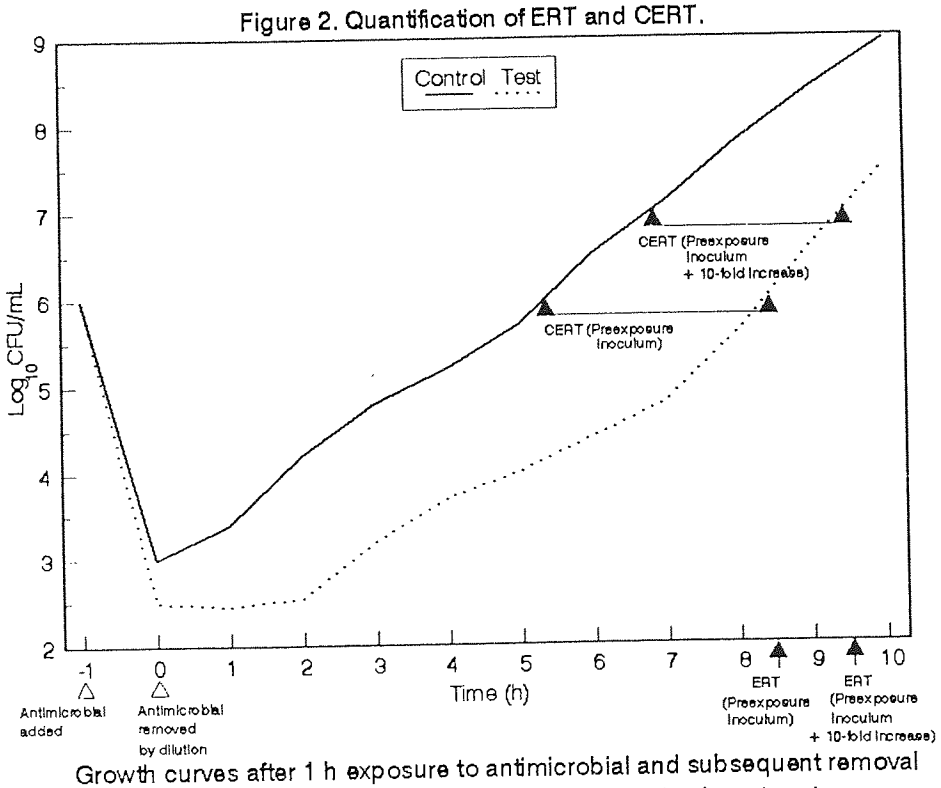
Other methods have been used to quantify PAE, Meng and coworkers (1991, 1991a, 1994) used the mean values determined over the entire experimental period. This allowed the mean recovery-time to be determined by measuring the area above the growth curve of a microbial population following antimicrobial exposure. Therefore, this method is less likely to be influenced by the initial portion of the growth curve and is less dependent on the lower limit of detection and other influences such as morphology. The quantification of PAE by the mean recovery-time gives comparable PAE results with spectrophotometrically and viable count measured growth curves (Meng, 1991a). However, this method is currently not widely used due to the need for stationary phase test and control cultures and identical test and control post exposure counts, the latter being almost impossible in practice (Jason *et al.*, 1994). In addition, the method of determining the mean PAE is tedious and regrowth needs to be followed for longer than usual (Jason *et al.*, 1994).

In addition, Gould (1997) has derived PAEs by measuring the time after antibiotic removal for the culture to reach peak growth rate and by measuring the prolongation of lag phase. Jason and coworkers (1994) using impedence observed that the most precise method of measuring PAE was based on the time for the culture to reach peak growth rate.

A two-phase mathematical regression model for PAE estimation has also been designed which allows a more consistent estimate of PAE and its standard error (Brunden *et al.*, 1991). However, this method of determination requires considerably more cultures than the conventional method.

Effective regrowth time (ERT) has also been used to quantify PAE. It has been inconsistently defined as either the time required for the microbial population in the test culture to return to the preexposure inoculum (Totsuka and Shimizu, 1993), or the preexposure inoculum plus a 10-fold increase (MacKenzie *et al.*, 1995), or an inoculum of $10^7$ CFU/mL (Baquero *et al.*, 1986)(Figure 2). Control-related effective regrowth time (CERT) has also been described, CERT = $T$ - $C$, where $T$ is the time required for the microbial population in the test culture to return to preexposure inoculum (Nilsson *et al.*, 1995)(Figure 2) or the pre-exposure inoculum plus a 10-fold increase (Hanberger *et al.*, 1995a) after elimination of the antimicrobial and $C$ is the corresponding time for the non-exposed control culture (Figure 2). CERT allows the antimicrobial activity to be assessed independently of method and temporary differences in growth phase and accounts for microbial killing as well as PAE (Hanberger *et al.*, 1995).

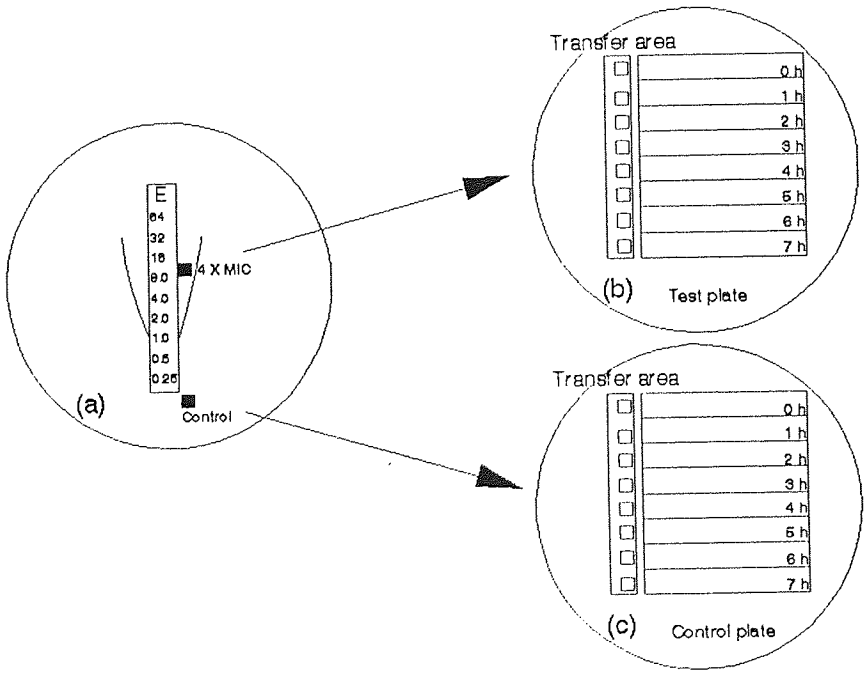
**Figure 2. Quantification of ERT and CERT.**




Growth curves after 1 h exposure to antimicrobial and subsequent removal by dilution. Arrows ( ↑ ) denote the time for exposed culture to return to preexposed inoculum and preexposure inoculum plus a 10 fold increase. Arrows ( ▲ ) denote the difference between the time required for exposed culture to return to preexposure inoculum (or preexposure inoculum plus 10 fold increase) after elimination of antimicrobial and the corresponding time for the non-exposed control.

Epsilometer test (Etest) technology has been extended to PAE (Bölstrom, 1994). This method involves transferring the remaining inoculum in a 3 by 3 mm square next to the Etest strip at the concentration to be tested to broth and agar (Figure 3). The appropriate controls are required to ensure that minimal antimicrobial carryover has occurred. This method is limited to commercially available Etest antimicrobials and requires further evaluation.

Figure 3. PAE determination using Etest.




Microorganisms were inoculated onto agar plates by flooding and the Etest strips applied (a). The plates were incubated for 1 h after which 3 mm squares were sampled at concentrations equivalent to 4 X MIC and a non-exposed control (a significant distance from the predicted ellipse area). The inoculum was transferred to the appropriate transfer areas (b and c) and streaked out to obtain viable counts.

Other methods used by investigators have allowed real time monitoring of growth kinetics by the mechanical measurements of spectrophotometry (Rescott *et al.*, 1988), impedance (conductance)(Baquero *et al.*, 1986) and electrical particle counting (Nadler *et al.*, 1989), the quantification of PAE is again different for each method.

Rescott and coworkers (1988) spectrophotometrically determined PAE by monitoring the time for detection of growth (a 5% decrease in transmittance) after antimicrobial exposure compared to the time for detection of growth of a control of the same inoculum. Whereas, Lowdin and coworkers (1993) calculated PAE as the difference in the time required for the exposed culture and the non-exposed control culture to reach 50% of the maximum absorbance of the control cultures.

Correlation between spectrophotometric measurements and viable counts is usually poor due to differences in the morphology of microorganisms resulting from aging or antimicrobial exposure which modifies the light scattering properties of microbial suspensions (Nadler *et al.*, 1989). Bergan and coworkers (1980) reported that spectrophotometric underestimation of the bactericidal activity of ß-lactams and aminoglycosides on Gram-negative bacilli led to PAEs being longer than those obtained by viable counts. Conversely, they noted a good correlation between the two methods for chloramphenicol and oxytetracycline PAEs on *Escherichia coli* and *Staphylococcus aureus* (Bergan *et al.*, 1980).

Impedance measurements monitor microbial metabolic activity by analysing changes in electrical conductance produced by charged metabolites in the growth media, these changes are directly proportional to the number of microorganisms present (Baquero *et al.*, 1986). A microbial growth analyzer has been used to study PAE, it continuously (every 6 minutes) measures resistance of the growth media and calculates changes in conductance during growth (Gould *et al.*, 1989). PAE was quantified by the difference in time for the test and the control culture to reach an inoculum equivalent to $10^7$ CFU/mL. The method of impedance measurement is amenable to replicates, faster, more accurate and less labour intensive than viable counting. However, impedance also generates negative PAEs with antimicrobials that cause filamentation, due to division of these filamentous forms (Hanberger *et al.*, 1991b). Viable counts and impedance measurements do not always correlate (MacKenzie and Gould, 1993).

Electrical particle counting is based on alterations in current flux density produced by microorganisms passing through an aperture which is set to detect microorganisms of predetermined diameters. PAEs determined by this method are comparable to those obtained by viable counts for *S. aureus* exposed to oxacillin (Nadler *et al.*, 1989a) and *S. aureus* exposed to dicloxacillin, *E. coli* exposed to tetracycline and *E. faecalis* exposed to erythromycin (Li *et al.*, 1996). However, this method is unreliable if the microorganisms in the test culture are smaller or more filamented than those of the control culture. In addiiton, this method is laborious, unsuitable for large numbers of determinations (MacKenzie and Gould, 1993) and unless viable counts are performed simultaneously is only a total cell count method (Li *et al.*, 1996).

Flow cytometry has also been applied to observe PAE, this method allows specific subpopulations to be defined by size and nucleic acid content and examined objectively (Gottfredsson *et al.*, 1993a).

Various different direct biochemical measurements of metabolic activity have also been utilised to determine PAE. For example; intracellular bacterial adenosine triphosphate (ATP) measured by bioluminescence (Hanberger *et al.*, 1991), the rate of $^{3}$H-thymidine incorporation (Gottfredsson *et al.*, 1989a) and $CO_2$ generation (Gottfredsson *et al.*, 1991).

PAE determination by bioluminescence assay of intracellular bacterial ATP rapidly measures the direct effect of the antimicrobial on the bacterial suspension (Isaksson *et al.*, 1993). The bioluminescence assay is based on the reaction between firefly luciferase and reduced luciferin which, when ATP is present, yields luciferin-AMP complex. Following the introduction of oxygen this complex dissociates, releasing light energy which is then measured (MacKenzie and Gould, 1993). PAE has been defined using this method as PAE = $T$ - $C$ where, $T$ is either the time required for the bacterial ATP in the exposed test culture to increase 2 or 3 $\log_{10}$ (Hanberger *et al.*, 1991) or 5 picomoles (Winstanley and Hastings, 1990) above that observed immediately after antimicrobial removal and C is the corresponding time required for the non-exposed control culture. Good (Isaksson *et al.*, 1988) and poor (Odenholt *et al.*, 1988) correlations between PAE determined by ATP and viable counts have been reported.

The bioluminescence method assumes that the ATP content of a microorganism is constant, although after antimicrobial removal, it may be smaller and contain less ATP. Therefore, before regrowth, microorganisms may manufacture ATP to achieve their normal concentrations. Thus, the increase in viable counts would be less rapid than the increase in ATP, leading to a reduction in the PAE determined by bioluminescence. Conversely, with antimicrobials that cause filamentation, one colony detected by viable counting would appear as many times the biomass of one microorganism by ATP measurement. Viable counts would increase when the filaments septate, which would not be accompanied by an equivalent increase in ATP, leading to an increase in the PAE determined by bioluminescence. Discrepancies have also been observed between ATP measurement and viable counts due to some damaged organisms producing ATP and being repaired in broth but being incapable of forming colonies on solid media (Odenholt *et al.*, 1989a). This overestimation of the initial kill by viable counts has been confirmed by Hanberger and coworkers (1991) using light microscopy. These sub-lethally damaged microorganisms are viable *in vivo* and may be responsible for the recurrence of certain infections, for instance, in lower respiratory tract infections during and shortly after therapy (MacKenzie *et al.*, 1994). Also, in some instances, ATP measurement underestimates the bactericidal activity of an antimicrobial due to dead yet intact bacteria still containing intracellular ATP and therefore leading to longer PAEs than those determined by viable counts. However, quantal analysis (proliferative units) which is in close agreement with viable counts, has demonstrated that the inability of damaged microorganisms to form colonies on solid media can be eliminated as the explanation for discrepancies between results of viable counting and those of bioluminescence (Sörén *et al.*, 1995).

Good correlation was observed between PAE determined by viable counts and $^{3}H$ thymidine incorporation (DNA synthesis) with *E. coli* and *S. aureus* for rifampin, dicloxacillin, vancomycin, gentamicin and ciprofloxacin (Gottfredsson *et al.*, 1989a). The advantage of this method over bioluminescence and spectrophotometry is that unlysed dead microorganisms do not affect the measurements. However, Guan and coworkers (1992) found quinolone PAE determined by DNA synthesis of *E. coli* did not correlate to the PAE determined by viable count but was longer.

The BACTEC blood culture system has been used to measure microbial $CO_2$ production and to follow, for example, the dicloxacillin, vancomycin, rifampin, gentamicin and ciprofloxacin PAEs on *S. aureus*. Correlation was demonstrated between the PAEs determined by this method and by viable counts (Gottfredsson *et al.*, 1991). In addition, Zhanel and coworkers (1998) determined amikacin, azithromycin, clarithromycin, clofazimine, ethambutol, rifampin and sparfloxacin PAEs on *Mycobacterium avium*.

PAE has also been determined by using interference phase contrast microscopy to follow the bacterial morphology of *E. coli* after exposure to ampicillin and ciprofloxacin (Lorian and Joyce, 1985). PAE was arbitrarily defined as the time for the population to revert to 90% normal bacilli and 10% filaments, which led to a PAE 2-3 h longer than that determined by viable counts.

Gottfredsson and coworkers (1993) followed the ultrastructural changes using transmission electron microscopy of *S. aureus* and *P. aeruginosa* and observed an increase in cross walls and intracellular electron-dense aggregates, respectively, after exposure to different antimicrobials. The persistence of these ultrastructural alterations correlated with the duration of PAE determined by viable counts. However, Guan and coworkers (1992) found quinolone PAE on *E. coli* defined by nucleoid morphology longer than that determined by viable count.

In standard MIC determinations a relatively small number of bacteria, for example, $10^4$ CFU are continuously exposed to a constant concentration of antimicrobial. This differs greatly from the *in vivo* situation where, a large number of bacteria are exposed to fluctuating levels of antimicrobial. In addition, MIC determinations are evaluated after 18-24 h incubation, therefore they simply are a measure of the ability of an antimicrobial agent to prevent the appearance of visible growth at that time without taking into account changes in bacterial growth kinetics within the considered time period (Greenwood, 1981). Usually, the determination of PAE partially follows *in vivo* kinetics, utilising only a short exposure to a constant antimicrobial concentration. Therefore, PAE yields pharmacodynamic data that is more clinically realistic than that of the other standard *in vitro* methods of antimicrobial activity determination.

Furthermore, several *in vitro* models have been developed which enable the effect of fluctuating antimicrobial concentrations on PAE to be followed; these are the dilution (Gerber *et al.*, 1982) and dialysis models (Blaser *et al.*, 1985). Both these models can mimic either intravenous (IV) bolus administration or

intramuscular (IM) adsorption. The dilution model consists of a culture flask into which fresh media is added and equal volumes are removed. The major disadvantage of this method is that the microorganism is diluted along with the antimicrobial and therefore viable counts have to be corrected (Gerber *et al.*, 1982). Alternatively, an outlet filter can be included to minimise the loss of bacteria (Shah, 1981). The dialysis model consists of a closed system containing test culture separated from fresh media by a semipermeable membrane (Blaser *et al.*, 1985), thus preventing organism dilution. In both these models, differentiation between the effects due to PAE and those due to subinhibitory antimicrobial concentrations is difficult.

### 1.1.3 *In vivo* methods of quantification of PAE.

Six animal models have been developed to determine PAE: thigh infection in mice (Gudmundsson *et al.*, 1993); pneumonia in mice (Craig *et al.*, 1991a); infected subcutaneous threads in mice (Renneberg and Walder, 1989); meningitis in rabbits (Täuber *et al.*, 1984); infected tissue-cages in rabbits (Odenholt *et al.*, 1988) and endocarditis in rats (Hessen *et al.*, 1988). All of these models allow tissue or body fluid to be sampled repeatedly to facilitate the determination of CFUs and antimicrobial concentration, therefore enabling calculation of PAE.

Craig and Gudmundsson (1996) defined the *in vivo* PAE as PAE = $T - C - M$, where $M$ represents the time antimicrobial serum levels exceed the MIC, $T$ represents the time for the test count in CFU to increase 1 $\log_{10}$ above the count at time $M$, and $C$ represents the time for the control count in CFU to increase 1 $\log_{10}$ after microorganism inoculation.

The results obtained from these models demonstrate that PAE is also an *in vivo* phenomenon. An overall examination of reports on carbapenem PAEs suggests that there is a need for caution in drawing conclusions about their *in vivo* PAEs from *in vitro* data (Majcherczyk *et al.*, 1994). For example, imipenem does not produce an *in vivo* PAE in a *P. aeruginosa* rat endocarditis model (Renneberg and Walder, 1989), although it exhibits a significant *in vitro* PAE and an adequate explanation for this discrepancy has not been determined. However, in general, *in vivo* PAEs are longer than *in vitro*, due to either the gradual removal of antimicrobial and subsequent sub-MIC effects, the action of host defences, for example leucocytes or the lower rate of bacterial growth.

### 1.1.4 Factors affecting PAE.

Both the presence and duration of PAE can be affected by many factors associated with the microorganism, the antimicrobial and the experimental conditions. Differences in the presence and duration of PAE may be dependent upon the specific microorganism-antimicrobial combination (Table 1). With staphylococci and streptococci, inhibitors of nucleic acid and protein synthesis tend to produce longer PAEs than those observed with ß-lactams.

For Gram-negative bacilli, inhibitors of nucleic acid and protein synthesis also produce much longer PAEs than the ß-lactams, which produce no significant PAE or even a negative PAE. The only exceptions in the ß-lactam group to produce significant PAEs with Gram-negative bacilli are the carbapenems and penems, this remains an unexplained phenomenon.

Table 1. *In vitro* postantibiotic, postantifungal and postantiseptic effects.

| | *staphylococci & streptococci* | *Enterobacteriaceae* | *Pseudomonas* species | *Bacteroides* species | *Legionella* species | *Candida* species | *Mycoplasma* species | *Mycobacteria* species | *Listeria* species | *Helicobacter pylori* |
|---|---|---|---|---|---|---|---|---|---|---|
| Penicillins | ++ | 0/+ | 0 | 0/+ | | | | | | |
| Cephalosporins | ++ | 0/+ | 0 | 0/+ | | | | | | |
| Imipenem | ++ | +/++ | ++ | + | | | | | | |
| Faropenem | +/++ [b] | 0/+ [b] | | | | | | | | |
| Aztreonam | | 0/+ | 0 | | | | | | | |
| Vancomycin | ++ | | | | | | | | | |
| Aminoglycosides | +/++ | +/++ | ++ | | +++ [c] | | | ++++ [d] | ++ [a] | |
| Quinolones | ++ | ++ | ++ | | ++++ [a] | | ++++ | ++++ [a] | | |
| Trovafloxacin | | | +/++ [e] | | | | | | | |
| Moxifloxacin | +/++ [f] | +/++ [f] | | | | | | | | |
| Rifampicin | +++ | +++ | +++ | | +++ [g] | | | ++++ [k] | | |
| Macrolides | ++/+++ | | | | 0/+++ [a] | | ++++ | ++++ [k] | | +++/++++ [m] |
| Azithromycin | ++ [h] | | | | | | | | | |
| Ketolides | +++ [i] | | | ++/+++ [i] | | | | | | |
| Clindamycin | +++ | | | +++ | | | | | | |
| Tetracyclines | +++ | +++ | | | | | ++++ | +++ | | |
| Chloramphenicol | ++ | ++ | | ++ | | | | | | |
| Nitrofurantoin | +++ | +++ | | | | | | | | |
| Trimethoprim | ++ | 0/+ | | | | | | | | |
| Streptogramin | +-+++ [j] | | | | | | | | | |
| Metronidazole | | | | +++ | | | | | | |
| 5-Fluorocytosine | | | | | | ++/++++ [a] | | | | |
| Amphotericin B | | | | | | ++++ [a] | | | | |
| RO-239424 | +/+++ [l] | +++ [l] | ++ [l] | | | | | | | |
| Chlorhexidine | ++ [n] | 0/+ [n] | +++ [n] | | | | | | | |
| Povidone-iodine | + [n] | + [n] | + [n] | | | | | | | |

Summary of published postantibiotic, postantifungal and postantiseptic effect data from a = Craig and Gudmundsson, 1996, b = Boswell *et al.*, 1997, c = Dubois and St-Pierre, 1989, d = Bermundez *et al.*, 1991, e = Boswell *et al.*, 1997a, f = Boswell *et al.*, 1997b, g = Dubois and St-Pierre, 1992, h = Diculencu *et al.*, 1996, i = Boswell *et al.*, 1998, j = Boswell *et al.*, 1994, k = Fuursted, 1997, l = Klein and Neu, 1992, m = Einarsson *et al.*, 1993, n = Fuursted *et al.*, 1997b, other data = Craig, 1991. 0 = <0.5 h, + = 0.5-1.5 h, ++ = 1.5-3 h, +++ = >3 h and ++++ = >10 h.

Eagle (1949) first noted that the duration of PAE increased as the antimicrobial concentration increased. Generally, PAEs are observed when the antimicrobial exposure concentrations are greater than or equal to the MIC, although sub-MIC levels of aminoglycosides can cause a PAE (Isaksson *et al.*, 1988). Maximal PAEs are usually achieved at a concentration of about 10 X MIC (Vogelman and Craig, 1985). However with *S. aureus* and rifampicin a maximal PAE was not observed even at a concentration of 200 X MIC (Bundtzen *et al.*, 1981). In addition, a paradoxical amoxycillin PAE (PAE decreased with increasing concentrations) has been noted with *E. faecalis* (Yourassowsky *et al.*, 1988).

Many investigators have demonstrated that lengthening the duration of antimicrobial exposure increases the duration of PAE (Vogelman and Craig, 1985) and maximal effects are usually observed after a 2 h exposure. PAE correlates with the *in vitro* area under the concentration curve (AUC), that is, the concentration (mg/L) multiplied by duration of exposure (h) (Bundtzen *et al.*, 1981). Furthermore, dose response relationships have shown that PAE is sigmoidally related to log AUC, such that

$$PAE = \frac{PAE_{max}.AUC^n}{E_{50}^n + AUC^n}$$

where, $PAE_{max}$ is the maximal PAE, $E_{50}$ is the AUC at which 50% of maximum PAE is reached and n is a constant determined from *in vitro* PAE determinations at varying exposure concentrations and durations and is calculated from regression analysis of the linear transformation of the equation (Turnidge, 1991).

Other factors affecting the presence and duration of PAE and thus requiring standardisation include inoculum size; growth phase; culture media and pH. Large inocula decrease PAE especially on Gram-negative bacilli (Odenholt-Tornqvist, 1989); whereas, only a slight decrease was observed with staphylococci (Craig and Gudmundsson, 1996). Most investigations into PAE have been performed with microorganisms in the logarithmic phase of growth (Craig and Gudmundsson, 1996).

Only a few investigators have stated whether cultures were shaken or stationary during antimicrobial exposure. Lee and coworkers (1982) found that the ampicillin PAE on *E. coli* decreased with shaking, whereas, Wilson and Rolinson (1979) found no significant difference in the penicillin PAE on *S. aureus* with shaking.

The use of different culture media has demonstrated variable effects on PAE, with supplemented Mueller-Hinton broth (MHB) being the most commonly employed (MacKenzie and Gould, 1993). The effect of cations on PAE has been determined by many investigators. For example, magnesium ions reduced the activity and ofloxacin PAE on *S. aureus* (Gürdal *et al.*, 1990) whereas, the presence of calcium and magnesium ions had little effect on the ciprofloxacin PAE on *S. aureus* (Gudmundsson *et al.*, 1989).

PAE requires further study in body fluids such as serum, urine and cerebrospinal fluid (CSF) to fully assess its biological significance (Zhanel *et al.*, 1992). Bundtzen and coworkers (1981) found that the PAE of rifampin and tetracycline on *E. coli* was significantly reduced in 90% heat-inactivated human serum.

The reduction of PAE observed in human serum is often attributed to protein binding. The binding of antimicrobials to plasma proteins is a reversible phenomenon, in which protein and unbound antimicrobial exist in equilibrium with the non-diffusible and inactive protein bound antimicrobial (Rolinson, 1973). When the antimicrobial concentrations were corrected to account for serum protein binding the PAEs were virtually identical in both broth and serum (Bundtzen *et al.*, 1981). Human serum significantly increased the fluoroquinolone PAEs on *S. aureus*, although a subsequent decrease was observed with heat-inactivated serum suggesting that complement or some other heat-labile component played an important role along with the other more stable component(s) (Davidson *et al.*, 1991a).

It has been demonstrated that pooled human urine dramatically reduced fluoroquinolone activity and consequently PAE on *E. coli*, although this was attributed to the reduction in pH (Zhanel *et al.*, 1991b). Consequently, Chin and Neu (1987) and Zhanel and coworkers (1991b) found fluoroquinolone PAE in human urine to be similar to that obtained in pH adjusted MHB. *In vivo* conditions have been mimicked by Choe and coworkers (1993) who used MHB pH 7.4 with 1.5 mM magnesium as a blood simulation medium and MHB pH 5.5 with 8 mM magnesium as a urine simulation medium to determine the cotrimoxazole, amoxycillin and ciprofloxacin PAEs. Zhanel and coworkers (1992) noted that pooled human CSF markedly increased the cefotaxime, ciprofloxacin and gentamicin PAEs on *E. coli* compared to MHB, and they concluded that some protein component(s) was interacting with the antimicrobial agents and increasing PAE.

Only a limited number of studies have examined the effect of pH on PAE, most have been performed in non-cation supplemented media at a physiological pH of 7.4 (Gudmundsson *et al.*, 1991). In general, acid pH has a detrimental effect on aminoglycoside and fluoroquinolone activity leading to a reduction in PAE, ß-lactam activity is much less affected by pH (Craig and Gudmundsson, 1996).

Microorganisms in PAE phase are more susceptible to the inhibitory effects of sub-MIC antimicrobial concentrations (Odenholt Tornqvist *et al.*, 1991). Postantibiotic sub-MIC effect (PA SME) is the combination of PAE and the effects of exposure to sub-MIC concentrations (Odenholt-Tornqvist, 1993).

PA SME = $T_{PA}$ - C, where $T_{PA}$ is the time for the cultures previously exposed to antimicrobials which thereafter were exposed to different sub-MICs to increase by 1 $\log_{10}$ above the counts observed immediately after initial antimicrobial removal and C is the corresponding time for the non-exposed control culture. PA SME may simulate *in vivo* antimicrobial exposure more accurately than PAE since subinhibitory concentrations will persist between antimicrobial doses. The postexposure to sub-MIC concentrations of ß-lactams as low as 0.1 X MIC increases the ß-lactam PAE (Odenholt Tornqvist *et al.*, 1991). Imipenem (0.3 X MIC) PA SMEs of 6.2 - >21.8 h on *P. aeruginosa* were reported by Odenholt-Tornqvist and coworkers (1991a). Odenholt-Tornqvist (1993) hypothesised that when microorganisms are exposed to ß-lactams binding to the active sites of PBPs occurs but their synthesis continues. On repeated challenge most PBPs will be already inactivated therefore, only a low antimicrobial concentration is needed to inhibit the newly produced PBPs. PA SMEs have also been observed with fluoroquinolones (Licatta *et al.*, 1997; Odenholt-Tornqvist *et al.*, 1992).

Several investigators have studied the influence of antimicrobial combinations on the duration of the PAE. Varying results have been observed depending on whether the combination exhibited synergy or antagonism, such that PAE either increased or decreased respectively (Gudmundsson *et al.*, 1991a). PAE synergism is defined as a PAE induced by an antimicrobial combination that is at least 1 h (Gudmundsson *et al.*, 1991a) or 2 h (Gudmundsson *et al.*, 1993) longer than sum of PAEs for individual drugs, for example, tobramycin and rifampin on *E. coli*. PAE addition is defined as a PAE induced by a combination that is approximately the mathematical sum of the individual PAEs for example, imipenem and tobramycin on *P. aeruginosa* (Gudmundsson *et al.*, 1991a). PAE indifference is defined as a PAE induced by an antimicrobial combination that is no different from longest of individual PAEs, for example, meropenem and gentamicin on *P. aeruginosa* (Ferrara *et al.*, 1993). PAE antagonism is defined as a PAE induced by an antimicrobial combination that is at least 1 h (Gudmundsson *et al.*, 1991a) or 2 h (Gudmundsson *et al.*, 1993) less than longest of individual PAEs for example, ceftazidime and tobramycin on *P. aeruginosa*.

Since most infections are treated with multiple doses of antimicrobials which result in several antimicrobial exposures it is important to consider consistency of PAE in the design of dosage regimens (McGrath *et al.*, 1993). Only a few studies have investigated the effect on PAE of antimicrobial reexposure. For example, multiple antimicrobial exposures increased the gentamicin PAE on *E. coli* (Karlowsky *et al.*, 1992), conversely, the aminoglycoside PAE on *P. aeruginosa* reduced (Karlowsky *et al.*, 1994).

However, the imipenem PAE on *P. aeruginosa* and *E. coli* remained constant after three consecutive exposures (McGrath *et al.*, 1993) and even after six successive exposures (Majcherczyk *et al.*, 1994). Hence, PAE may alter following multiple cycles of antimicrobial exposure and microbial regrowth depending on the antimicrobial-microorganism combination (McGrath *et al.*, 1993).

The correlation between the lipid solubility of the antimicrobial and its PAE has been investigated, although no correlation was observed (Klastersky, 1981).

Intracellular PAEs have been investigated by one research group, who found that the intracellular exposure to rokitamycin, josamycin (Shibutani *et al.*, 1993) and ofloxacin (Uda *et al.*, 1995) produced PAEs on *S. aureus* that were longer than non-intracellular PAEs due partially to the penetration of antimicrobial into polymorphonuclear leukocytes, that is, intracellular exposure concentration is greater than extracellular (Shibutani *et al.*, 1995).

Erlendsdottir and coworkers (1993) studied the dicloxacillin and rifampin PAEs on *S. aureus*, and the ceftazidime, imipenem, gentamicin and ciprofloxacin PAEs on *Klebsiella pneumoniae* at temperatures of 35.5, 38.5 and 41.5 °C and found they were independent of temperature. Conversely, Magnusson and coworkers (1995) found the imipenem PAE on *P. aeruginosa* was reduced at higher temperatures.

Hyperoxia (100% $O_2$) significantly enhanced the tobramycin PAE on *P. aeruginosa* compared with normoxia (21% $O_2$) (Park *et al.*, 1993). This enhancement was concluded to be related to the mechanism of aminoglycoside action. Conversely, Bayer and coworkers (1989) observed that the enhanced exopolysaccharide production by *P. aeruginosa* strains at higher oxygen tensions was associated with a slower rate of aminoglycoside-induced killing and a shorter PAE.

### 1.1.5 Other phenomena associated with PAE.

The alteration of several physiological characteristics is also associated with the postantibiotic suppression of microbial growth, these include virulence factors, antimicrobial efflux and uptake, postantibiotic leucocyte enhancement (PALE) and adaptive resistance.

Clinically, the most relevant physiological alteration is that of microbial virulence suppression. The microbial factors such as morphology, growth rate, tissue invasion, adherence, products of metabolism and death all contribute to bacterial virulence (Lorian and Gemmell, 1991). The association of these factors and the duration of PAE has been insufficiently studied. For example, the activity of haemolysin enzymes (that cause the release of haemoglobin from erythrocytes) during PAE phase of quinolones has been assessed (Guan and Burnham, 1992). In addition, reduced haemolytic activity of *Streptococcus* species in macrolide PAE has been observed (Ramadan *et al.*, 1993).

Bacterial adherence is influenced by the net surface charge and or specific binding arrangement by host factors. During the PAE phase the residual antimicrobial may cause leakage of bacterial adhesins (ß-lactams) or may suppress the formation and expression of adhesins (aminoglycosides). A decrease in adherence of treated bacteria has been observed during macrolide PAE phase (Ramadan *et al.*, 1993).

Post ß-lactamase inhibitor effect (PLIE) has also been reported, this occurs when cultures exposed to a ß-lactam and inhibitor are then regrown in the presence of a ß-lactam only. A continued suppression of ß-lactamase activity is seen which is longer than the conventional PAE (Waters and Tauber, 1993; Thorburn *et al.*, 1996).

Increased accumulation of fluoroquinolones has been demonstrated by microorganisms in PAE phase, due to the depressed activity of their active efflux system, the duration of this accumulation is longer than the suppression of growth (Davidson *et al.*, 1994). Conversely, aminoglycoside uptake is reduced during PAE phase and for 2 h after the suppression of growth (Karlowsky *et al.*, 1993).

The phagocytic capacity of leucocytes and the bactericidal activity of serum are important host defence mechanisms against invading microorganisms (Raponi *et al.*, 1989). Exposing *E. coli* and *S. aureus* to high levels of penicillin, ciprofloxacin, ampicillin and chloramphenicol enhances their susceptibility to the antibacterial activity of human polymorphonuclear leucocytes (McDonald *et al.*, 1981; Pruul and McDonald, 1990).

This *in vivo* and *in vitro* phenomenon has been termed postantibiotic leucocyte enhancement (PALE) (McDonald *et al.*, 1981). PALE is both strain and antimicrobial dependent and it is thought to be due to antibiotic induced modifications to the bacterial cell surface which thereby, enhances their susceptibility to phagocytosis or intracellular killing or both (Pruul and McDonald, 1990). The PALE produced by meropenem is significantly higher than that observed with imipenem (Novelli *et al.*, 1997). In addition, platelets secrete platelet microbicidal protein which has been shown to increase the oxacillin and vancomycin PAEs on *S. aureus* (Yeaman *et al.*, 1992).

Apparently, microorganisms during the PAE phase can also demonstrate altered susceptibility to the bactericidal activity of certain antimicrobials (Gerber and Craig, 1981). A short lived effect of reduced bactericidal activity following a second antimicrobial exposure has been described *in vitro* for aminoglycosides (adaptive resistance) and quinolones (Daikos *et al.*, 1990). The mechanism of adaptive resistance is not completely understood. It is probably related to the reversible down regulation of aminoglycoside uptake during the period of accelerated energy dependent antimicrobial transport and to the decreased proton motive force during the adaptive resistance interval (Xiong *et al.*, 1996). The susceptibility of *Streptococcus pneumoniae* to ampicillin decreases when in erythromycin PAE phase and *E. coli* in rifampicin PAE phase are less susceptible to norfloxacin, perfloxacin and ciprofloxacin (Meng *et al.*, 1994). Gudmundsson and coworkers (1994) also found that the degree of inhibition of bactericidal activity during rifampicin and erythromycin PAE was dependent on both the class of antimicrobial agent (ß-lactam > aminoglycoside) and the microorganism (Gram-

negative bacilli > *S. aureus*). No change in susceptibility is seen on reexposure to a ß-lactam during ß-lactam PAE phase (Odenholt *et al.*, 1989).

Whether microorganisms in PAE phase are more or less susceptible obviously depends on which antimicrobials are used to induce PAE, which are added in PAE phase and on the microbial species studied (Odenholt *et al.*, 1994). Further studies need to be performed *in vitro* and *in vivo*, as these observations could have important implications for the timing of doses during combination antimicrobial therapy. Prolonging the interval between doses may not only lower the cost of treatment and the risk of toxicity but may also ensure the efficacy of the subsequent doses.

### 1.1.6 Proposed mechanisms of PAE.

Although over the years a vast amount of information has been obtained on PAE, the precise mechanisms by which antimicrobials induce a PAE are still unknown. Several theories on the duration of PAE have been proposed including the prolonged persistence of an antimicrobial agent either at the cellular site of action or within the periplasmic space, or the true recovery period from non-lethal damage, that is, time to repair damaged but viable cells or the time required to synthesise new enzymes or proteins before growth (MacKenzie and Gould, 1993), or even the production of endogenous growth-suppressive factors similar to the SOS-response observed in *E. coli* (Gottfredsson *et al.*, 1993). The observed differences in the PAEs of various microorganism-antimicrobial combinations would suggest that multiple mechanisms and various metabolic pathways are involved.

The linear dose response curves observed and maximal PAE suggest an antimicrobial receptor interaction may have a role in PAE. Therefore, the PAE in this instance could be due to the persistence of the antimicrobial at the site of action. For example, erythromycin, tetracycline and chloramphenicol all reversibly bind to specific subunits of bacterial ribosomes (Weisblum and Davies, 1968). So, PAE in this case may represent the time it takes for the antimicrobial to dissociate from the receptor and diffuse from the ribosome. This theory has been challenged by Gerber and Craig (1981) who observed with *Streptococcus pneumoniae* that after incubation at 4°C for 24 hours an erythromycin PAE is still present. This incubation should have allowed the antimicrobial to diffuse from the ribosome and out of the cell. The unique PAEs observed with the carbapenems and penems were initially thought to be due to their high affinity to PBP 2. However, this was disproved by Majcherczyk and Livermore (1990) who demonstrated that an *E. coli* mutant with a thermosensitive PBP 2 (which does not bind ß-lactams at 44°C) exhibits carbapenem PAEs (of similar duration to the parent strain) at both 30 and 44°C.

Exposure to antimicrobials that inhibit protein or nucleic acid synthesis depletes the microorganism of functional proteins necessary for their intermediary metabolism and unhampered growth (Schmitt *et al.*, 1990). Therefore, the duration of the PAE in this case may represent a period of protein resynthesis. Gottfredsson and coworkers (1991a) stated that quinolone PAE duration could be the time needed to repair the damage where the quinolone was bound to the bacterial DNA gyrase. However, it is now thought that quinolones act by trapping and stabilising the gyrase-DNA complex and thus blocking DNA or RNA

polymerases (Maxwell, 1992), therefore the above hypothesis may be incorrect. ß-lactam PAE could represent either the time required for spheroplasts to resynthesise the cell wall or the time required for filament reseptation (Hanberger *et al.*, 1991b). Alternatively, PAE may be either the period of time during which microorganisms synthesis new PBPs or the effect of ß-lactam which has remained in the periplasmic space where it continues to inhibit the enzyme activities of newly formed PBPs (Bush *et al.*, 1989). The recent observations of Li and coworkers (1997) on five antimicrobials with different mechanisms of action suggested that cellular recovery from non-lethal damage following antimicrobial exposure may be the major determinant of PAE.

The repair is possibly mediated by a mechanism analogous to the SOS-response in *E. coli* and *S. aureus* (Gottfredsson *et al.*, 1991a). The SOS-response is a regulatory network that is induced by DNA damage and the subsequent physiological reaction is due to the induction of more than 17 damage-inducible genes (*din*) controlled by regulatory proteins which are products of *lexA* and *recA* genes (Walker, 1987). Responses include inhibition of cell division and stimulation of error prone repair. The RecA protein has a proteolytic activity that cleaves LecA protein which subsequently derepresses the *recA* gene leading to further RecA production (Little and Mount, 1982).

### 1.1.7 Clinical significance of PAE.

Curative antimicrobial therapy relies on the susceptibility of the infecting microorganism to the antimicrobial and the availability of antimicrobial at the site of infection. The purpose of an antimicrobial dosing regimen is to deliver effective antimicrobial concentrations to the site of infection for adequate periods of time.

The theoretical basis for the design of antimicrobial dosing regimens has not changed appreciably for nearly 50 years, that is, to maintain active levels (serum concentration above MIC/MBC) for most of the dosing interval which is calculated by matching the pharmacokinetics obtained in normal volunteers to the antimicrobial *in vitro* activity (Ebert *et al.*, 1988). Although this method is generally effective without dose response studies it is difficult to determine the dosing regimen that will achieve maximal efficacy with least amount of antimicrobial and thus minimise cost and toxicity (Vogelman *et al.*, 1988). With penicillin G, early clinical experience suggested that many infections could be treated adequately with intermittent antimicrobial therapy even though serum and tissue levels fell below the MIC for many hours (Tompsett *et al.*, 1949).

The outcome of a clinical microbial infection depends on the complex interaction of three factors; firstly, the pharmacodynamics of the antimicrobial agent (the interaction between antimicrobial and microorganism), for example, rate of bactericidal killing, PAE, ERT, PALE and PA SME. Secondly, pharmacokinetics (correlation of antimicrobial and clinical effect), for example, antimicrobial penetration at site of infection, protein binding, metabolism, elimination and excretion.

Thirdly, the interaction between the host and the microorganism for example, opsonisation, phagocytosis and the bactericidal effect of serum (Hanberger, 1992). Figure 4 demonstrates the pharmacokinetic and pharmacodynamic parameters of an antimicrobial administered by IV bolus. These parameters can vary markedly for different antimicrobial classes and hence the schedule of antimicrobial administration is important in the outcome of serious infections.

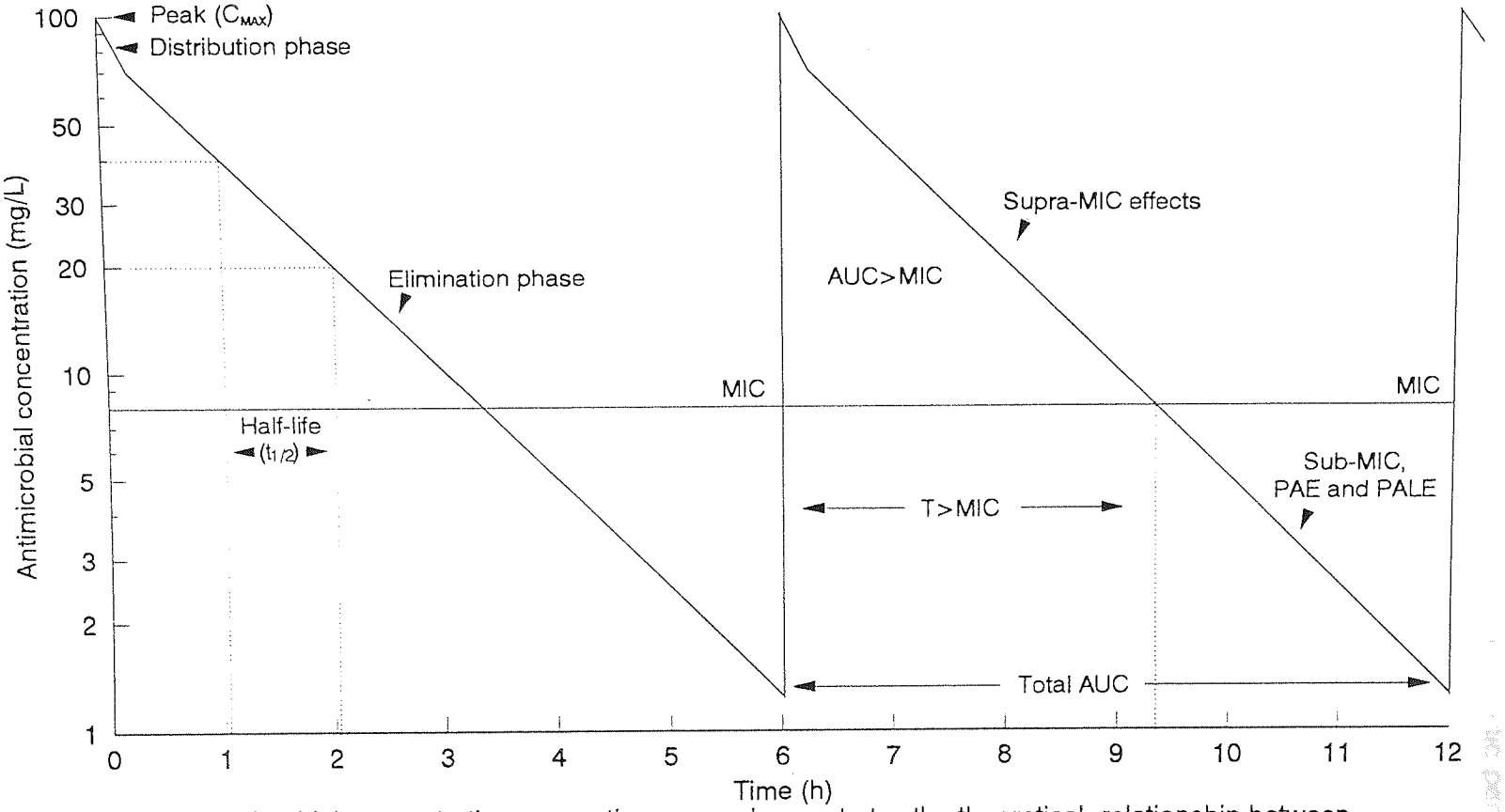
A rational process for using both pharmacokinetics and pharmacodynamics to optimise antimicrobial therapy has yet to be thoroughly established. However, data obtained from PAE determinations on novel antimicrobials could provide the theoretical rationale for either intermittent or continuous dosing depending on the infecting microorganism. Intermittent dosing regimens would primarily apply to antimicrobial-microorganism combinations that exhibit a prolonged PAE. Whereas, more continuous dosing would apply to antimicrobial-microorganism combinations lacking a PAE. Although, the link between the demonstration of PAE and the efficacies of a more prolonged dosage interval has not been proven (McGrath *et al.*, 1993).

In general, ß-lactam action on Gram-negative bacilli is not highly concentration dependent and due to the lack of PAE the most rational course for administration is either a frequent schedule (determined by elimination half-life) or a constant infusion. However, it has been suggested that the activity of ß-lactams against slow growing microorganisms might be enhanced by an antimicrobial-free period during which optimal growth would occur prior to the next dose (Craig and Ebert, 1992).

The duration of time that the serum levels exceed the MIC/MBC appears to be the important pharmacokinetic parameter for efficacy against Gram-negative bacilli, exceptions may be the carbapenems and penems, which possess a PAE (Drusano, 1988). In fact, the prolonged PAE might be the reason why imipenem is effective in the treatment of *P. aeruginosa* infections with only thrice daily dosing instead of continuous infusion. However, the precise dosing regimen can only be determined by human clinical studies.

The presence of an *in vivo* PAE should permit antimicrobial levels to fall such that antimicrobial activity could be absent for a portion of the dosing interval without loss of efficacy (Hessen *et al.*, 1989). The relevance of PAE to therapy is difficult to assess unequivocally. Gengo and coworkers (1984) stated that the ideal dosing interval is the sum of duration of time above the MBC, the duration of PAE and time for the microorganisms to enter a sensitive logarithmic growth phase.

Figure 4. Pharmacokinetic and pharmacodynamic parameters of an antimicrobial administered by IV bolus every 6 hours.




The antimicrobial concentration versus time curve demonstrates the theoretical relationship between pharmacokinetics and pharmacodynamics. In this example, the microorganism MIC of 8 mg/L is exceeded for approximately half of the dosing interval.

The knowledge of the pharmacodynamics of an antimicrobial has been used to speculate which pharmacodynamic/pharmacokinetic parameter best predicts *in vivo* efficacy. By knowing the pharmacodynamic/pharmacokinetic parameter that best determines efficacy for the different antimicrobial microorganism combinations one can alter the dosage of currently available antimicrobials and design future dosing regimens in an effort to reduce toxicity and cost while maintaining efficacy (Vogelman *et al.*, 1988)(Table 2).

Craig and co-workers (1993) stated that for optimum efficacy, against both Gram-positive and Gram-negative microorganisms, serum concentrations should be greater than the MIC for 30%, 40% and 50% of the dosing interval for carbapenems, penicillins and cephalosporins, respectively. Whereas, Nightingale (1997) stated that for time dependent antimicrobial agents the goal of therapy is that concentrations should be greater than the MIC for at least 50% of the dosing interval.

**Table 2. Rationale for dosing.**

| Antimicrobial class | Pharmacodynamic characteristics | Pharmacodynamic/pharmacokinetic parameter which correlates with efficacy |
|---|---|---|
| Penicillins | Time dependent kill and PAE with Gram-positive microorganisms | T > MIC + PAE |
| Cephalosporins | Time dependent kill and PAE with Gram-positive microorganisms | T > MIC + PAE |
| Carbapenems | Concentration dependent kill and PAE | T > MIC + PAE |
| Quinolones | Concentration dependent kill and PAE | AUC/MIC |
| Aminoglycosides | Concentration dependent kill and PAE | $C_{max}$/MIC |
| Streptogramins | Some concentration dependent kill and PAE | 24 h AUC/MIC |
| Ketolides | Time dependent kill and PAE | AUC/MIC |
| Macrolides | Time dependent kill and PAE | ?T > MIC, ?$C_{max}$/MIC |

The knowledge of which pharmacodynamic/pharmacokinetic parameter best determines efficacy for the different antimicrobial microorganism combinations can be used to provide an rationale for dosing. Data from MacGowan and Bowker, 1997 and Vesga *et al.*, 1997 and 1997a.

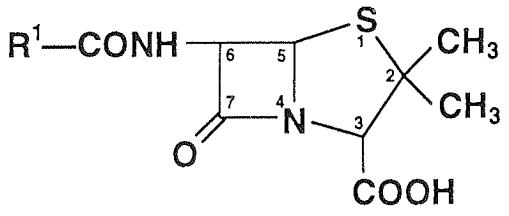
Antimicrobial combinations are frequently required for the successful treatment of serious infections, generally the same dosing schedules are used whether the antimicrobials are used alone or in combination. Combination therapy provides better antimicrobial coverage in empiric therapy prior to the identification of pathogens, can enhance bactericidal activity with synergy, is used for the treatment of polymicrobial infections and aids prevention of emergence of resistance (Gudmundsson *et al.*, 1991a).

Optimal dosage schedules for antimicrobial combinations remain an unsolved clinical problem (Isaksson *et al.*, 1991a), for example, whether the optimal dose schedule will be constant infusion of ß-lactams with intermittent administration of an aminoglycoside or staggered schedule administration of each antimicrobial remains to be seen.

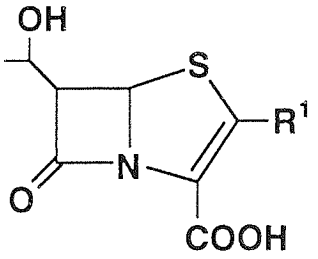
**1.2 ß-lactam antimicrobial agents.**

The ß-lactam antimicrobial agents are the most varied and widely used of all classes, accounting for half of all the systemic antimicrobials in use (Livermore and Williams 1996). ß-lactams are potent broad-spectrum bactericidal agents with low eukaryotic toxicity (Georgopapadakou, 1993). The activity of a ß-lactam is influenced by the type of substitutions (R-groups) attached to the basic nucleus (Bryan and Godfrey, 1991). They encompass an enormous number of mostly semi-synthetic compounds which can be conveniently divided into three groups: bicyclic penicillins (namely, penams, penems, carbapenems, oxapenams); cephalosporins (namely, cephems, cephamycins, oxacephems, carbacephems) and monocyclic (isolated ß-lactam ring) monobactams (Figure 5).

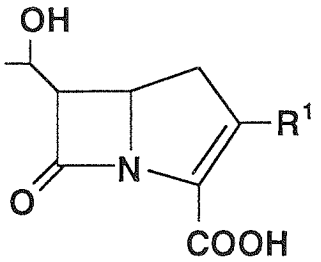
## *Bicyclic Penicillins*



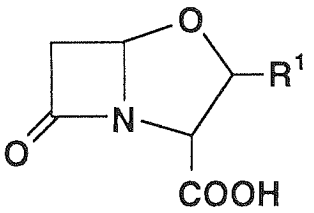

Penam




Penem

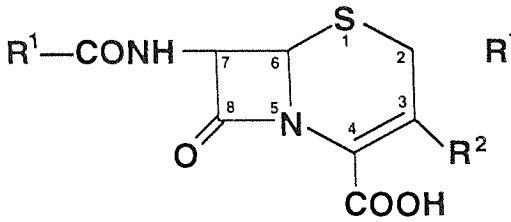



Carbapenem

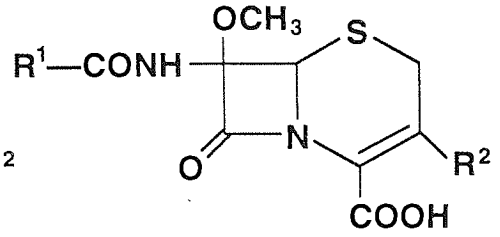



Oxapenam

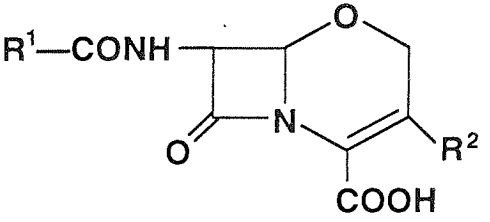
## *Cephalosporins*



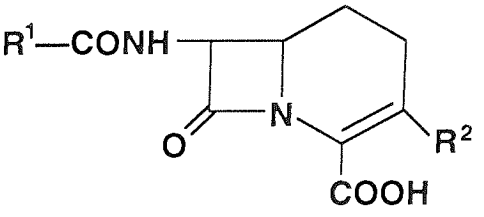

Cephem




Cephamycin




Oxacephem

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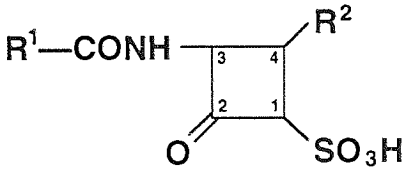



Monobactam

Figure 5 Classes of β-lactam antimicrobial agents

In addition, a novel tricyclic ß-lactam (tribactam), sanfetrinem, has been investigated (Di Modugno *et al.*, 1994).

The antibacterial activity of ß-lactams on Gram-negative bacteria depends essentially on their ability to cross the outer membrane permeability barrier (via water-filled transmembrane pores composed of porin proteins - hydrophilic pathway), their stability to any periplasmically located ß-lactamase(s) and their affinity for essential PBPs (Hayes and Ward, 1991). Resistance to ß-lactams reflects an alteration in any of these three factors (Georgopapadakou, 1993).

ß-lactams bind covalently to one or more essential PBPs thereby inhibiting the final stages of biosynthesis of peptidoglycan which is the major polymer of the Gram-negative bacterial cell wall, maintaining cell shape and protecting against osmotic forces (Livermore and Williams, 1996).

The Gram-negative bacterial cell wall contains approximately 10% peptidoglycan (murein) (Figure 6) which is a meshwork of glycan strands that are cross linked by short peptide bridges (Höltje, 1997). The glycan moiety is a chain of alternating *ß*-1,4-glycoside linked units of *N*-acetyl-D-glucosamine and *N*-acetyl muramic acid (Bryan and Godfrey, 1991). The carboxy group of muramic acid is substituted with a peptide chain made up of alternating L- and D-amino acids.

Assembly of uncross-linked glycan chains is performed by a transglycosylation reaction. In the uncross-linked form the pentapeptide substituents on the muramic acid residues terminate in D-alanyl-D-alanine. Cross-linkage between glycan chains occurs between the residue at position 3 of the peptide chain (usually diaminopimelic acid or lysine) and the D-alanine at position 4 in the peptide subunit of an adjacent glycan chain (transpeptidase reaction)(Figure 7). A separate carboxypeptidase reaction results in cleavage of the terminal D-alanine without cross-linkage of the peptides. This reaction limits the amount of cross-linkage by denying the transpeptidases D-alanyl-D-alanine substrates.

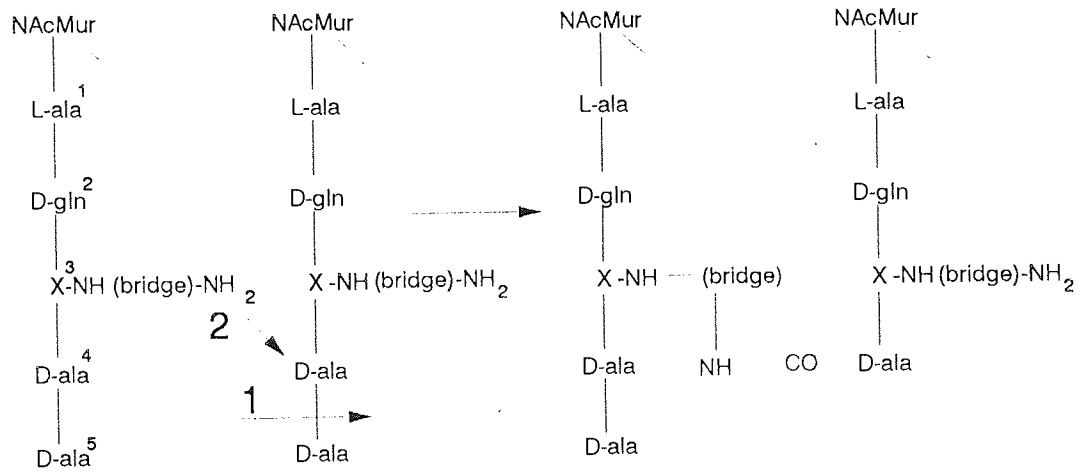



**Figure 7. Details of cross-link formation.**

1. Action of D-alanine-D-alanine carboxypeptidases.

2. Action of D-alanine-D-alanine transpeptidases.

The peptidoglycan layer forms a covalently closed string-bag-like structure, called sacculus. Thus growth of a bacterium depends on the simultaneous enlargement of its peptidoglycan sacculus. In order to enlarge the peptidoglycan netting the concerted activities of enzyme cleaving meshes and enzymes inserting new subunits, that is peptidoglycan hydrolases and synthases are needed (Höltje, 1997). However the peptidoglycan hydrolases must not act separately to avoid dissolution of the sacculus and hence bacteriolysis. Recent experimental results indicate that the potentially autolytic peptidoglycan hydrolases seem to be integrated within a multienzyme complex, a peptidoglycan synthesising machinery combining both hydrolases and syntheses (Höltje, 1997). Accordingly, any pause in the supply of activated peptidoglycan precursors will result in idling of the machinery with the peptidoglycan hydrolases degrading the peptidoglycan (Höltje, 1997). The tendency of D-alanyl-D-alanine trans- and carboxy-peptidases to form acyl esters with ß-lactams provides these enzymes with their collective name, PBPs. The acylation of the active-site serine of a PBP by a ß-lactam is shown in Figure 8. There is a wide variation in the amount, number and possibly nature of PBPs found in different bacteria. The PBPs of Gram-negative bacteria are located on the outer face of the cytoplasmic membrane and they have defined morphological roles in cell growth and division. However, half the total amount of PBPs of *E. coli* may be sacculus located proteins linked to the outer membrane probably through peptidoglycan bridges, except PBP 3 which is almost exclusively located on the cytoplasmic membrane (Rodriguez-Tébar *et al.*, 1985). The inner and outer membranes fuse at various points along their length and it has been suggested that these regions contain the most active PBPs, in addition it has been suggested that PBPs concentrate along the cell's equator (Livermore, 1988).

In *E. coli*, they are responsible for maintenance of rod shape (PBP 2), cell elongation (PBP 1) and septum formation (PBP 3) (Livermore and Williams, 1996). Strains of *P. aeruginosa* exhibit similar but not identical PBP patterns to those of *E. coli* and the individual proteins perform similar functions in bacterial morphogenesis in both organisms (Curtis *et al.*, 1980). Therefore, differing effects of ß-lactams on the morphology of Gram-negative bacilli are governed by their relative binding affinities for different PBPs.

Almost all Gram-negative bacilli have an identical pattern of seven PBPs, designated in order of descending molecular weight; 1a, 1b, 2, 3, 4, 5 and 6 (Table 3) (Livermore and Williams, 1996). Inhibition of any or all of the essential PBPs results eventually in cell death with the microorganism first undergoing a characteristic morphological change. Selective inactivation of PBPs 1a and 1b (for example, by cephaloridine) causes spheroplast formation, rapid lysis and death, whereas, selective inactivation of PBP 1a or 1b singly has no effect. Selective inactivation of PBP 2 (for example, by mecillinam) causes spherical cell formation and slow lysis. Finally, selective inactivation of PBP 3 (for example, by aztreonam) causes long non-septate filaments which lyse slowly and release endotoxin, which in turn causes septic shock *in vivo* (Livermore and Williams, 1996).

Table 3. PBPs of Gram-negative bacilli.

| PBP | Molecular mass (kDa) | Activity | Molecules per cell (*E. coli*)[b] | Essential |
|---|---|---|---|---|
| 1a | 88-118 | transglycosylase/D-alanyl-D-alanine transpeptidase | } 230 | Yes |
| 1b | 81-103 | transglycosylase/D-alanyl-D-alanine transpeptidase | } | Yes |
| 2 | 63-78 | transglycosylase/D-alanyl-D-alanine transpeptidase | 20 | Yes |
| 3 | 59-66 | transglycosylase/D-alanyl-D-alanine transpeptidase | 50 | Yes |
| 4 | 44-51 | D-alanyl-D-alanine carboxypeptidase | 110 | No |
| 5 | 40-44 | D-alanyl-D-alanine carboxypeptidase | 1800 | No |
| 6 | 38-41 | D-alanyl-D-alanine carboxypeptidase | 570 | No |

The molecular masses (ranges indicate values found by different workers), enzymatic activity, molecules per *E. coli* cell and the necessity of the PBPs of Gram-negative bacilli. Data from Livermore and Williams, 1996 and Spratt, 1977[b].

The low molecular weight PBPs 4, 5 and 6 are thought to be 'non-essential' since their inhibition has no adverse effect on cell shape. The basis of the bactericidal effect of ß-lactams is unclear, the binding of PBPs does not necessarily result in death but indirectly triggers the mechanism(s) responsible for the lethal effect (Odenholt *et al.*, 1989). Bacterial cells exposed to ß-lactams can either lyse, die then lyse, die and not lyse, or survive; the outcome being affected by their autolytic system, growth rate and nutritional state (Bryan and Godfrey, 1991). The target PBPs of *E. coli* are present in an excess of the quantity required for normal growth under *in vitro* conditions, that is, no lysis was observed when 1a and b were 90% saturated (Gutmann *et al.*, 1986). The balance between ß-lactam uptake and clearance determines the fate, of the cell rather than either factor alone (Livermore, 1988).

### 1.3 Carbapenem antimicrobial agents.

Carbapenems isolated from fermentation products of various *Streptomycetes* are a relatively new class of ß-lactam antimicrobial agents containing more than 40 members. The carbapenem class includes thienamycins, olivanic acids, carpetimycins, asparenomycins and pluracidomycins (Wise, 1986)(Table 4). They have the broadest spectra of activity of all the ß-lactams and were first discovered in the mid 1970s.

**Table 4. Carbapenem antimicrobial agents.** (Bryskier, 1995)

| Carbapenems | | | |
|---|---|---|---|
| Group A<br>$CH_2$ | Group B<br>1-ß-methyl | Group C<br>Polycyclic | Group D<br>Others |
| Imipenem | Meropenem | GV 118819 | BMS 181139 |
| L 695 256 | Biapenem | S 903012 | Ro 403485 |
| BMY 25174 | BO 2727 | | |
| Panipenem | S 4661 | | |
| SYN-513 | CL 190294 | | |
| | CL 188624 | | |
| | CL 191121 | | |
| | B 2502 A | | |
| | DX-8739 | | |
| | CS 834 | | |

The term carbapenem is derived from the substitution of **carb**on for sulphur, the similarity in the 4:5 fused lactam ring of the **peni**cillins, and an unsaturated five membered ring (**em**, double bond)(Duenas and Barriere, 1989). They all contain a hydroxyethyl side chain instead of the amino-acyl group of the penicillins and cephalosporins and differ from each other in the configuration of their side chains at the C2 and C6 position. Carbapenems are stable to most clinically significant ß-lactamases including those with an extended spectrum of activity, for example TEM and SHV enzymes, due to the *trans* configuration of the hydroxyethyl side chain (Figure 9).

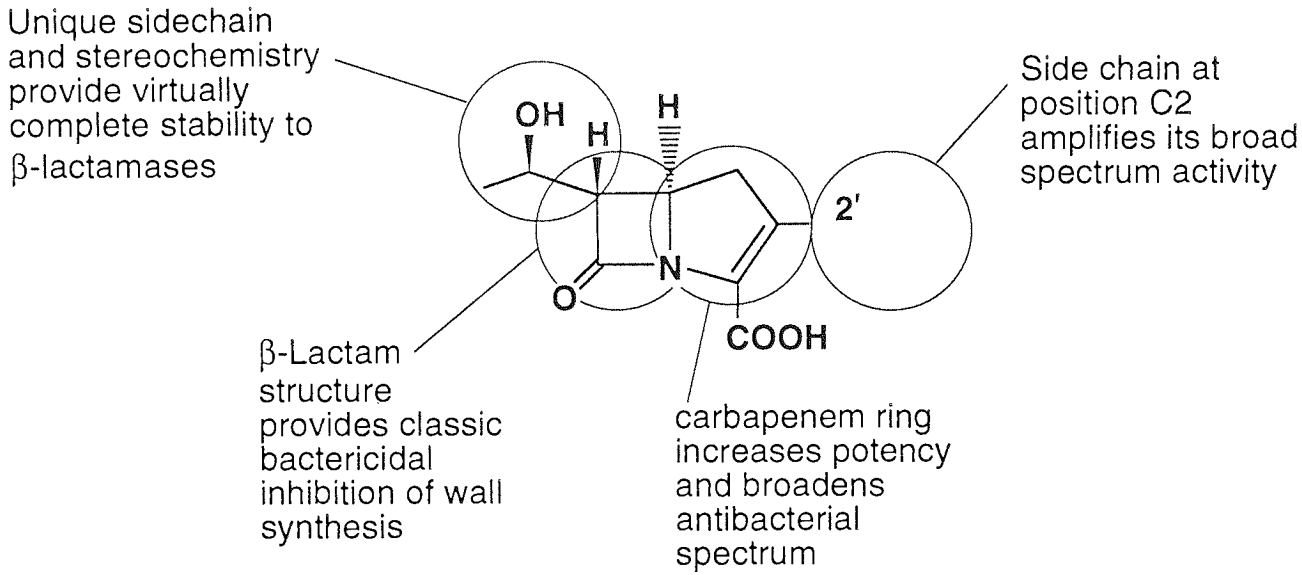



**Figure 9 Structure activity relationships of carbapenems**

*Enterobacter cloacae*, *Serratia marcescens* and *P. aeruginosa* are common producers of serine (type I) ß-lactamases which are weak carbapenemases that hydrolyse 1 or 2 molecules of carbapenem per minute (Iaconis *et al.*, 1994). However, carbapenem therapy is usually not affected by serine ß-lactamases unless enzyme production is associated with a second resistance mechanism, for example, reduced uptake (Iaconis *et al.*, 1994; Quinn *et al.*, 1997). However, they are hydrolysed by chromosomal metallo-ß-lactamases (zinc-dependent carbapenemases, molecular class B) that are present in *Flavobacterium* species, *Stenotrophomonas maltophilia*, *S. marcescens*, some *Burkholderia cepacia*, the occasional *Aeromonas* species, *Bacteroides* species, *Legionella gormanii* and *Bacillus cereus* (Livermore, 1997). Unlike serine ß-lactamases they use an active site zinc ion to hydrolyse ß-lactam ring (Livermore, 1993).

New modes of carbapenem resistance are emerging and may present future problems (Table 5). For example, IMP-1 a plasmid-mediated zinc ß-lactamase which hydrolyses carbapenems and extended spectrum ß-lactams has been isolated in Japan (Livermore, 1997). Other acquired serine carbapenemases for example, IMI-1, NMC-A and Sme-1 have also been identified in a few *E. cloacae* and *S. marcescens*, respectively (6 clinical isolates worldwide) (Rasmussen and Bush, 1997). One approach to the problem of metallo-ß-lactamases might be the administration of metallo-ß-lactamase inhibitiors such as, mercaptoacetic acid thiol ester derivatives (Payne *et al.*, 1997). In Italy 19.3% and 9% of *P. aeruginosa* strains are imipenem and meropenem resistant, respectively (Bonfiglio *et al.*, 1998). This is greater than in the UK where frequencies of resistance are 2.5% and 1.1%, respectively (Chen *et al.*, 1995).

Table 5. Summary of mechanisms of resistance to imipenem.

| Mechanism | Microorganism | Incidence | Details |
|---|---|---|---|
| Alterations in permeability | *P. aeruginosa* | Common | Deficiency in OprD |
| | | Rare | Modification to OprF hydrophobicity and Opr D deficiency |
| | | Rare | Overproduction of OprM and OprD deficiency |
| | *Proteus mirabilis* | Rare | Deficiency in outer membrane protein |
| | *Enterobacter aerogenes* | | |
| | *Enterobacter cloacae* | | |
| Carbapenemases chromosomal | *S. maltophilia* | Universal | Chromosomal metalloenzyme L1 |
| | *Aeromonas* spp. | Universal | Chromosomal metalloenzyme A2 |
| | *Bacillus cereus* | Universal | Chromosomal ß-lactamase II (metalloenzyme) |
| | *Bacteroides fragilis* | Unknown | Chromosomal metalloenzymes |
| | *Serratia marçescens* | Rare | Serine-based ß-lactamase or metalloenzyme |
| | *E. cloacae* | Rare | Chromosomal non-metallo-ß-lactamase |
| plasmid-mediated | *P. aeruginosa* | Rare | Plasmid mediated metalloenzyme |
| | *B. fragilis* | Rare | Plasmid mediated metalloenzyme |
| Binding site alterations | MRSA | Common | Modification of PBP 2a |
| | *Acinetobacter baumannii* | Rare | Modification of PBP |
| | *Listeria monocytogenes* | | |
| | *Rhodococcus equi* | | |
| Mixed mechanisms | *P. aeruginosa* | Occasional | Chromosomal ß-lactamase and OprD deficiency |
| | | Rare | Modification of PBP 4 and OprD deficiency |
| | *Enterobacter* spp. | Unknown | Chromosomal ß-lactamase and permeability alteration |

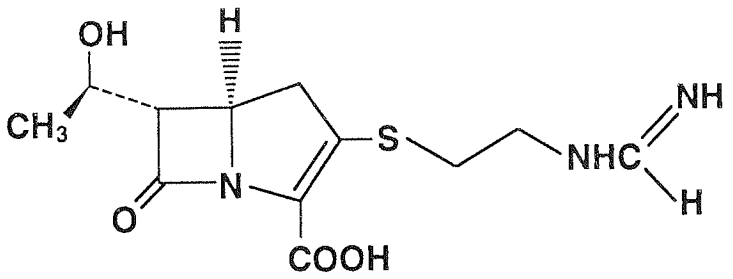
Mechanisms and incidence of imipenem resistance in clinical isolates. Data from Balfour *et al.*, 1996.

Imipenem, meropenem and biapenem (Figure 10) all bind equally to PBP 2 of *E. coli* and *P. aeruginosa*. The primary target of both imipenem and meropenem in *E. coli* is PBP 2, although in *P. aeruginosa* (depending on the strain) either PBP 2 and 3, or PBP 3 alone, are primary targets for meropenem, due to its broader PBP binding profile (Edwards, 1995)(Table 6). Due to these differences in binding affinities meropenem tends to cause filamentation and imipenem tends to cause spherical cell formation (Sumita *et al.*, 1990).

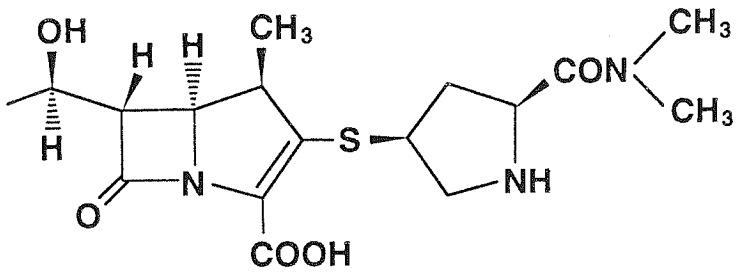
**Table 6. Affinities of carbapenems for PBPs**

| | | $IC_{50}$ (µg/mL) | | |
|---|---|---|---|---|
| | PBP | Imipenem | Meropenem | Biapenem |
| *E. coli* | 1a | 0.07 | 0.3 | 0.06 |
| | 1b | 0.2 | 0.2 | 0.8 |
| | 2 | ≤0.03 | ≤0.01 | ≤0.01 |
| | 3 | 5 | 0.2 | 3 |
| | 4 | 0.02 | 0.02 | 0.03 |
| | 5/6 | 2 | 10 | 50 |
| *P. aeruginosa* | 1a | 0.37 | 0.5 | 0.06 |
| | 1b | 0.29 | 0.5 | 0.4 |
| | 2 | 0.43 | 0.03 | 0.18 |
| | 3 | 0.42 | 0.04 | 0.3 |
| | 4 | 0.43 | 0.03 | 0.4 |
| | 5/6 | 10 | >100 | 90 |

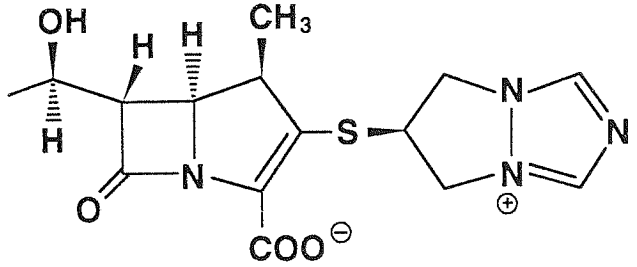
$IC_{50}$ is the concentration of unlabelled carbapenem which reduced the binding of radiolabelled benzyl penicillin to the PBPs by 50%. The *E. coli* carbapenem MICs were ≤0.06 mg/L. The *P. aeruginosa* carbapenem MICs were 0.5, 0.12 and 0.25 mg/L for imipenem, meropenem and biapenem, respectively. Data from Yang *et al.*, 1995.




Imipenem




Meropenem




Biapenem

Figure 10 Structures of imipenem, meropenem and biapenem

Imipenem a semi-synthetic N-formimidoyl thienamycin (*Primaxin*, Merck Sharp Dohme Ltd) was the first carbapenem to be registered for clinical use in 1985. It has potent activity against a very wide range of both Gram-positive and Gram-negative aerobes and anaerobes (Table 7), being bactericidal at 2-4 X MIC for most species (Bush, 1997). The activity of imipenem is usually not affected by either inoculum size (up to $10^7$ CFU/mL), pH (up to 8) or the presence of serum (Bush, 1997). It is generally well tolerated and has been used successfully in the treatment of severe infections such as septicaemia, lower respiratory tract infections including hospital acquired pneumonia, urinary tract infections, intraabdominal infections, gynaecological infections, skin and skin structure infections, granulocytopenia, cancer febrile neutropenia, mixed infections, empirical therapy, peritonitis and infections caused by organisms resistant to other antimicrobials, however it is not indicated for use with CNS infections (Bush, 1997).

Imipenem undergoes extensive metabolism by the mammalian renal tubular brush border dehydropeptidase I (DHP I)(Kropp *et al.*, 1982). A specific inhibitor of this enzyme, cilastatin was developed and is coadministered with imipenem in a 1:1 ratio. This improves the plasma concentration and urinary excretion of unmetabolised imipenem and therefore maintains an adequate and active concentration in the urinary tract (MacGregor *et al.*, 1986).

As an alternative to using enzyme inhibitors of renal dehydropeptidase carbapenems that are stable to DHP I have been developed, for example, meropenem and biapenem. Both owe their stability to the presence of a methyl group at C1 of the carbapenem ring.

Meropenem (*Meronem*, Zeneca), discovered in 1987 and registered for clinical use in 1995, is indicated for the treatment of the same medical conditions as imipenem and in addition, meningitis (because unlike imipenem, high levels are not associated with seizures) (Moellering *et al.*, 1989). Meropenem is four to eight times more active than imipenem against Gram-negative organisms due to the modification on the C2 side chain, but is marginally less active against Gram-positive organisms (Livermore and Williams, 1996) (Table 7). The activity of meropenem is little affected by inoculum size, pH or the presence of serum and it is bactericidal at concentrations close to the MIC (Bush, 1997). Meropenem is more stable to serine ß-lactamase than imipenem, a poor ß-lactamase inducer and it diffuses through D2 and a non-specific pathway used by chloramphenicol, tetracycline and cephalosporins therefore, two mutations are required for resistance to occur.

Biapenem (Lederle) is a novel investigational carbapenem that has not progressed from phase III clinical trials (for economic reasons). Biapenem has a 2-substituted triazolium moiety and has a broad-spectrum of activity against most aerobic and anaerobic Gram-positive and Gram-negative organisms

(Bush, 1997)(Table 7). Its activity is equivalent to or slightly greater than that of imipenem against Gram-negative aerobic bacteria and slightly less against Gram-positive organisms (Bush, 1997). The activity of biapenem is unaltered by 50% serum or urine (Gu *et al.*, 1991) and is bactericidal (Sasaki *et al.*, 1991).

Generally, the carbapenems exhibit rapid concentration dependent killing and uniquely among the ß-lactams, they exhibit a significant PAE on Gram-negative bacilli (Tables 8 and 9).

Imipenem, meropenem and biapenem are all administered parenterally. Imipenem and meropenem (0.5 g) are administered by IV bolus injection over 5 minutes or IV infusion over 15-30 minutes, with a 6 and 8 h dosing interval, respectively (Sanford *et al.*, 1997). Imipenem, meropenem, and biapenem have the typical pharmacokinetic characteristics of ß-lactams, that is, a short elimination half-life (approximately 1 h) and low serum protein binding ($\leq$20%) (Table 10). Both imipenem and meropenem undergo predominant renal excretion, glomerular filtration and tubular secretion (Dreetz *et al.*, 1994).

Imipenem and meropenem are currently the only carbapenems in clinical use in the UK. Another carbapenem which is marketed only in Japan, panipenem, is coadministered with betamipron a DHP I inhibitor (Edwards, 1995).

**Table 7. The *in vitro* activities (mg/L) of the carbapenems** (Wise, 1990).

| Microorganism | Imipenem | | | Meropenem | | | Biapenem | | |
|---|---|---|---|---|---|---|---|---|---|
| | $MIC_{50}$ | $MIC_{90}$ | Range | $MIC_{50}$ | $MIC_{90}$ | Range | $MIC_{50}$ | $MIC_{90}$ | Range |
| *E. coli* | 0.12 | 0.25 | 0.03-0.25 | 0.03 | 0.03 | 0.008-0.06 | 0.05 | 0.1 | 0.02-0.1 |
| *Klebsiella* spp. | 0.12 | 0.5 | 0.12-0.5 | 0.03 | 0.06 | 0.015-0.12 | 0.2 | 0.8 | 0.05-0.8 |
| *Enterobacter cloacae* | 0.5 | 1 | 0.12-1 | 0.03 | 0.06 | 0.015-0.25 | 0.1 | 0.4 | 0.05-0.8 |
| *P. mirabilis* | 2 | 4 | 0.5-4 | 0.12 | 0.12 | 0.06-0.25 | 0.8 | 3 | 0.1-6 |
| *S. marcescens* | 0.5 | 1 | 0.25-1 | 0.06 | 0.25 | 0.03-1 | 3 | 25 | 0.4-50 |
| *Providencia stuartii* | 1 | 2 | 0.25-4 | 0.06 | 0.12 | 0.03-0.5 | ND | ND | ND |
| *P. aeruginosa* | 1 | 2 | 0.12->8 | 0.5 | 2 | 0.06-16 | 0.8 | 3 | 0.2-25 |
| *Acinetobacter* spp. | 0.12 | 0.25 | 0.12-0.5 | 0.5 | 1 | 0.25-2 | 0.1 | 0.2 | 0.05-0.2 |
| *S. aureus*[+] | 0.03 | 0.03 | ≤0.015-0.03 | 0.12 | 0.12 | 0.06-0.5 | 0.05 | 0.1 | 0.02-0.8 |
| *S. epidermidis* | ≤0.015 | ≤0.015 | ≤0.015-0.12 | 0.06 | 0.12 | 0.06-0.5 | 0.05 | 0.4 | 0.02-2 |
| *S. pneumoniae*[#] | ≤0.015 | ≤0.015 | ≤0.015 | 0.015 | 0.015 | 0.008-0.015 | 0.01 | 0.01 | ≤0.006-0.02 |
| *S. pyogenes* | ≤0.015 | ≤0.015 | ≤0.015 | 0.008 | 0.008 | 0.008 | ≤0.006 | ≤0.006 | ≤0.006-0.05 |
| *E. faecalis* | 1 | 1 | 0.5-2 | 4 | 4 | 2-8 | 3 | 3 | 0.8-25 |
| *H. influenzae*[*] | 0.12 | 0.25 | 0.06-0.25 | 0.015 | 0.03 | 0.015-0.06 | 0.08 | 3 | 0.08-3 |
| *M. catarrhalis* | ≤0.015 | 0.03 | ≤0.015-0.03 | ≤0.03 | ≤0.03 | ≤0.03 | 0.05 | 0.1 | 0.01-0.1 |
| *Bacteroides fragilis* | 0.25 | 0.5 | 0.06-1 | 0.12 | 0.25 | 0.12-0.25 | 0.25 | 0.5 | 0.06-1 |

[+] methicillin sensitive, [#] penicillin sensitive, [*] ß-lactamase producers, ND = not determined.

Table 8. *In vitro* carbapenem postantibiotic effects.

| | Carbapenem | Microorganism | Removal method | Quantitation method | Exposure concentration | Duration of exposure (h) | Inoculum CFU/mL X $10^6$ | Growth phase | Media | PAE (h) |
|---|---|---|---|---|---|---|---|---|---|---|
| a | Imipenem | *P. aeruginosa* | D | VC | 4 X MIC | 1 | 10 | Log | MHB | 2.1 |
| b | Imipenem | *P. aeruginosa* | NS | VC | 8 mg/L | 2 | NS | Log | B | 1.5 |
| b | Imipenem | *P. aeruginosa* | NS | VC | 9 mg/L | 2 | NS | Log | PHS | 3.7 |
| c | Imipenem | *P. aeruginosa* | D/I | BL | 4 X MIC | 2 | 10 | Log | MHB | 4.2 |
| d | Imipenem | *P. aeruginosa* | F | I | 16 ug | 1 | 1 | Log | BHI | 1.1 |
| e | Imipenem | *P. aeruginosa* | NS | I | NS | 1.5 | NS | NS | NS | -1.4 - 1 |
| f | Imipenem | *E. coli* | W | VC | 10 X MIC | 2 | 200 | Log | MHB | 2 |
| c | Imipenem | *E. coli* | D/I | BL | 4 X MIC | 2 | 10 | Log | MHB | 2.6 |
| d | Imipenem | *E. coli* | F | I | 16 ug | 1 | 1 | Log | BHI | 1.6 |
| g | Meropenem | *P. aeruginosa* | D/I | VC | 2 X MIC | 2 | 10 | NS | MHB | 0.7 |
| h | Meropenem | *P. aeruginosa* | D | VC | 10 X MIC | 1 | NS | NS | MHB | -0.3 |
| g | Meropenem | *P. aeruginosa* | D/I | BL | 2 X MIC | 2 | 10 | NS | MHB | 3.7 |
| g | Meropenem | *E. coli* | D/I | VC | 4 X MIC | 2 | 10 | NS | MHB | 0.5 |
| g | Meropenem | *E. coli* | D/I | BL | 4 X MIC | 2 | 10 | NS | MHB | 4.8 |
| i | Meropenem | *E. coli* | D/I | I/VC | 10 X MIC | 2 | 1 | NS | MCB | 0.3 |
| j | Biapenem | *P. aeruginosa* | NS | NS | NS | NS | NS | NS | NS | 0.4 - 0.6 |
| k | Imipenem | *P. aeruginosa* | D | BL | 4-16 mg/L | 2 | NS | LOG | MHB | 3.5-4.6 |
| k | Imipenem | *P. aeruginosa* | D | BL | 4-16 mg/L | 2 | NS | NG | MHB | 0.2-0.3 |
| l | Imipenem | *P. cepacia* | D | VC | 1 X MIC | 2 | 1 | NS | HI | 0.6-4.5 |

Summary of published *in vitro* carbapenem postantibiotic effect data from a - Gudmundsson *et al.*, 1991, b - Manek *et al.*, 1986, c - Hanberger, 1992, d - Baquero *et al.*, 1986, e - Majcherczyk and Livermore, 1991, f - Renneberg and Walder, 1989, g - Hanberger *et al.*, 1995, h - Martin Caminero *et al.*, 1993, i - MacKenzie *et al.*, 1994 j - Houston *et al.*, 1993, k - Svensson *et al.*, 1993 and l - Kumar *et al.*, 1992.

NS = Not stated, D = Dilution, I = Inactivation, F = Filtration, W = Washing, VC = Viable counts, BL = Bioluminesence, I = Impedence, NG = non growing. MHB = Muller Hinton broth, B = Broth, PHS = Pooled human serum, BHI = Brain heart infusion broth, MCB = Malthus columbia broth, HI = heart infusion broth.

Table 9. *In vivo* carbapenem postantibiotic effects.

| | Carbapenem | Microorganism | Exposure concentration mg/kg | Time above MIC (h) | Inoculum $CFU/mL \times 10^6$ | Growth phase | Model phase | In vitro PAE (h) | In vivo PAE (h) |
|---|---|---|---|---|---|---|---|---|---|
| a | Imipenem | *P. aeruginosa* | 200 | 2 | 2 | Log | NMTI | | -1.8-1.6 |
| b | Imipenem | *P. aeruginosa* | 50-200 | 1.7-2.4 | 1 | Log | NMTI | | 0.9-4.6 |
| c | Imipenem | *E. coli* | 80 | 1.5 | 20 | Log | NMCTI | 2.0 | 2.9 |
| c | Imipenem | *P. aeruginosa* | 80 | 1.1 | 20 | Log | NMCTI | 1.8 | 2.4 |
| d | Imipenem | *P. aeruginosa* | 120 | 4 | 1 | NS | REI | 2.6-3 | 0 |
| e | Imipenem | *E. coli* | 25 | 1.8 | NS | NS | NMTI | | -0.1 |
| f | Imipenem | *P. aeruginosa* | 3 | NS | 12 | NS | NMLI | | >5.15 |
| f | Meropenem | *P. aeruginosa* | 3 | NS | 12 | NS | NMLI | | 4.55 |
| g | Meropenem | *P. aeruginosa* | 20 | 0.92 | 10 | NS | NMTI | -0.3 | 0.1 |
| g | Meropenem | *E.coli* | 20 | 0.90 | 10 | NS | NMTI | 0.57 | 0.3 |

Summary of published *in vivo* carbapenem postantibiotic effect data from
a - Majcherczyk *et al*., 1994, b - Gudmundsson *et al*., 1986, c - Renneberg and Walder, 1989, e - Craig and Gudmundsson, 1996, f - Miwa *et al*., 1994 and g - Martin Caminero *et al*., 1993.
NS = Not stated, NMTI = neutropenic murine thigh infection, NMCTI = normal murine cotton thread infection, REI = rat endocarditis infection and NMLI = neutropenic murine lung infection.

**Table 10. Carbapenem pharmacokinetics.**

| Carbapenem | Dose mg | $C_{max}$ µg/mL | AUC µg.mL.h | Apparent volume of distribution L | Protein binding % | $t_{1/2}$ h |
|---|---|---|---|---|---|---|
| Imipenem | 500-1000 | 52.1-69.9 | 34.7-74.1 | 12.6 - 24.5 | 9-20 | 0.9-1.25 |
| Meropenem | 500-1000 | 24.8-61.6 | 70.1 | 10.5 - 26.6 | 2-8 | 0.8-1.1 |
| Biapenem | 150-1000 | 32.4 | 15.0-55.0 | No data available | 15 | 0.95-1 |

Summary of available carbapenem pharmacokinetic data from Balfour *et al.*, 1996, Biapenem investigators brochure, 1992, Dreetz *et al.*, 1994, Drusano *et al.*, 1984, Meropenem Technical Monograph, 1997, Nakashima *et al.*, 1991, Wise *et al.*, 1986, and Wiseman *et al.*, 1995.

## 1.4 Gram-negative bacteria.

*E. coli* is a Gram-negative bacillus and a member of the *Enterobacteriaceae*. The *Enterobacteriaceae* are widely distributed in soil, water and human and animal intestines. They account for 50% of all clinically significant isolates in medical microbiology laboratories (Farmer, 1995). *E. coli* is predominantly a pathogen of the gut and urinary tract but also causes neonatal meningitis, septicaemia, sepsis in operation wounds and abscesses in a variety of organs.

*P. aeruginosa* is an environmental Gram-negative bacillus that is found in soil and water, it causes endocarditis, wound infections, septicemia amongst debilitated immunocompromised individuals and immunosuppressed granulocytopenic patients (Hancock, 1986). *P. aeruginosa* is also one of the most prevalent and clinically significant opportunistic pathogens involved in nosocomial infections, accounting for about 10% of all infections caused by Gram-negative bacilli. In hospitalised patients, *P. aeruginosa* can be a cause of catheter related urinary tract infections, bed sores, burns or eye infections and can infect almost any external site (Govan, 1992).

Sufferers of cystic fibrosis, the most common lethal genetic disorder in caucasians, are very susceptible to *P. aeruginosa* infection. Once colonised by *P. aeruginosa* there is a relentless decline in pulmonary function leading to respiratory failure and death (Grimwood *et al.*, 1989). The expression of a number of physiological processes, for example, virulence factor (extracellular cytotoxic substances)(Table 11) production, is considered more essential than replication in the pathogenesis of *P. aeruginosa* (Molinari *et al.*, 1993).

**Table 11. *P. aeruginosa* virulence factors** (Bodey *et al.*, 1983)

| Virulence factor | Biological effect(s) |
|---|---|
| Extracellular | |
| Proteases, for example elastase. | Tissue invasion, cellular damage responsible for destruction of arterial, elastic lamina, decreased complement-mediated defence mechanisms (degrade plasma proteins, immunoglobulins coagulation and complement factors), liquefy gelatin, dissolve elastin, casein, haemoglobin, fibrin and destroy collagen. |
| Exotoxin A | Cellular damage, toxicity to macrophages |
| Pigments | Suppress growth of other bacteria thus facilitating colonisation by *P. aeruginosa* |
| Phospholipase C | Destruction of pulmonary surfactant |
| Cellular | |
| Pili | Adherence to epithelial cells |
| Slime polysaccharide | Toxicity for neutrophils, endotoxin like effects |
| Mucoid polysaccharide | Antiphagocytic effects, decreased pulmonary clearance |
| Lipopolysaccharide | Possible antiphagocytic effects |
| O antigen | |
| Lipid A | Endotoxic effects |

Subinhibitory concentrations of ciprofloxacin, tobramycin and ceftazidime reduced the exoenzyme concentrations *in vitro* and in an *in vivo* rat model where no significant histological injury was observed. Therefore, suppression of these exoenzymes may limit the progressive lung damage experienced by patients with cystic fibrosis (Grimwood *et al.*, 1989).

Infections caused by *P. aeruginosa* are difficult to treat due to this microorganism's intrinsic resistance to many antimicrobials and its ability to develop resistance during therapy. For example, the permeability of the outer membrane of *P. aeruginosa* to ß-lactams in general is 100 to 500-fold lower than for *E. coli* (Trias *et al.*, 1989). Therefore, combination antimicrobial therapy has been advocated for serious *P. aeruginosa* infections (Korvick *et al.*, 1992). Combined therapy of an aminoglycoside with either an antipseudomonal ß-lactam (generally a carboxy- or ureido penicillin) or third generation cephalosporin or monobactam is used. Alternatively, a carbapenem can be used as empirical monotherapy. Nevertheless, the mortality rates for bacteraemia due to *P. aeruginosa* continue to be consistently higher than for other bacterial pathogens (Korvick *et al.*, 1992).

## 1.5 Aims and Objectives.

Many PAE investigations have been performed in numerous institutions, but on inspection of data, no consistency can be observed in the methodology used and consequently in the factors which influence the duration of PAE. This may be due to the fact that at present, there is neither an accepted standard method for *in vitro* PAE determination nor defined criteria for its reproducibility. The initial aim of this thesis was the development of a method to evaluate the PAE of three carbapenems, namely imipenem, meropenem and biapenem on Gram-negative bacilli under reproducible laboratory conditions which partially mimicked those occurring *in vivo*. This method was developed by investigating the various factors that effect the carbapenem PAE, for example, the carbapenem removal method used, pooled human serum, concentration, exposure duration, culture agitation, inoculum size and growth phase. Further investigations to determine the reproducibility of PAE, the consistency of MICs before and after PAE determination, the effect of multiple successive exposures and the effect of inter-strain variation were also performed.

At present, carbapenems and penems are the only members of the ß-lactam group of antimicrobial agents that exhibit a significant PAE on Gram-negative bacilli. The PAEs of imipenem and meropenem (the only carbapenems clinically available in the UK) and biapenem (a novel investigational carbapenem) on two genera of clinically important Gram-negative bacilli were investigated, in order to determine whether all three carbapenems exhibit tantamount PAEs and if all Gram-negative bacilli display the same carbapenem PAE.

Although a vast amount of information has been obtained on PAE, the precise mechanisms by which antimicrobials induce a PAE are still unknown. Several theories interpreting the duration of PAE have been proposed including the prolonged persistence of an antimicrobial agent either at the cellular site of action or within the periplasmic space, or the true recovery period from non-lethal damage, that is, time to repair damaged but viable cells or the time required to synthesise new enzymes or proteins before growth (MacKenzie and Gould, 1993), or even the production of endogenous growth-suppressive factors similar to the SOS-response observed in *E. coli* (Gottfredsson *et al.*, 1993).

Further investigations were performed to study the as yet undetermined mechanism underlying carbapenem PAE. Including, the effects of differing L-lysine concentrations and increasing salinity during recovery on the carbapenem PAE of *P. aeruginosa* as information on the nature of recovery may elucidate the mechanism behind PAE. *P. aeruginosa* virulence factor production was also monitored throughout PAE determination as microorganisms may still be physiologically abnormal after the classical PAE duration. In addition, the reversion of *P. aeruginosa* cell morphology was studied by epi-fluorescent and scanning electron microscopy during PAE determination.

# 2. MATERIALS AND METHODS

## 2.1 Gram-negative bacteria studied, their identification and storage.

Five clinically significant strains of *E. coli* and twenty six of *P. aeruginosa* recently isolated at the Microbiology Department, City Hospital NHS Trust (Table 12) and the *P. aeruginosa* type culture PAO1 (American Type Culture Collection (ATCC) 15692) were studied.

After identification using the API 20 E and 20 NE systems (BioMerieux, Marcy l'Etoile, France), these microorganisms were maintained throughout the duration of these investigations incorporated in lyophilized gelatin discs (Stamp, 1947). They were stored desiccated at 4°C and subcultured as required onto 5% defibrinated horse blood (Bradsure Biologicals Ltd., Loughborough, UK) agar (Columbia agar base No. 2. Difco Laboratories, Detroit, USA) plates and incubated for 18-24 h in air at 35-37°C.

## Table 12. Clinical isolates studied and their isolation site.

| Microorganism and identification code | Isolation site |
|---|---|
| *E. coli* I425 | Sputum |
| *E. coli* I428 | Mid stream urine |
| *E. coli* I431 | Mid stream urine |
| *E. coli* I633 | Mid stream urine |
| *E. coli* I635 | Mid stream urine |
| *P. aeruginosa* G284 | Mid stream urine |
| *P. aeruginosa* G291 | Sputum |
| *P. aeruginosa* G304 | Ear swab |
| *P. aeruginosa* G306 | Catheter specimen urine |
| *P. aeruginosa* G314 | Mid stream urine |
| *P. aeruginosa* G315 | Sputum |
| *P. aeruginosa* G318 | Catheter specimen urine |
| *P. aeruginosa* G319 | Sputum |
| *P. aeruginosa* G341 | Mid stream urine |
| *P. aeruginosa* G354 | Mid stream urine |
| *P. aeruginosa* G361 | Wound swab |
| *P. aeruginosa* G363 | Catheter specimen urine |
| *P. aeruginosa* G364 | Wound swab |
| *P. aeruginosa* G372 | Mid stream urine |
| *P. aeruginosa* G377 | Catheter specimen urine |
| *P. aeruginosa* G379 | Mid stream urine |
| *P. aeruginosa* G388 | Catheter specimen urine |
| *P. aeruginosa* G404 | Catheter specimen urine |
| *P. aeruginosa* G405 | Mid stream urine |
| *P. aeruginosa* G406 | Ear swab |
| *P. aeruginosa* G407 | Wound swab |
| *P. aeruginosa* G408 | Mid stream urine |
| *P. aeruginosa* G409 | Sputum |
| *P. aeruginosa* G411 | Catheter specimen urine |
| *P. aeruginosa* G412 | Catheter specimen urine |
| *P. aeruginosa* G414 | Wound swab |

## 2.2 Carbapenems studied, storage and stock solution preparation.

The three carbapenems studied were imipenem, meropenem and biapenem, these were supplied by Merck Sharp and Dohme (Hoddesdon, UK), Zeneca Pharmaceuticals (Macclesfield, UK) and Lederle (Gosport, UK), respectively. The carbapenem powders were stored at 4°C over desiccant and were allowed to reach room temperature prior to weighing. The potencies (µg of active carbapenem per mg of powder) of the batches of imipenem, meropenem and biapenem used were 936, 877 and 979 µg/mg, respectively. Stock solutions (1000 mg/L) of imipenem, meropenem and biapenem were prepared as required (prior to use) by weighing (MC1 Analytic balance, Sartorius AG, Gottingen, Germany) 21.4, 22.8 and 20.4 mg, respectively, into 20 mL glass volumetric flasks (Grade A). The carbapenem powders were initially dissolved and made up to 20 mL with sterile 0.02 M 3-(*N*-Morpholino) propane-sulphonic acid (MOPS) pH 7 buffer (Sigma-Aldrich Company Limited, Poole, UK. Appendix 1). These stock solutions were diluted further in MOPS to concentrations of 100, 10 and 1 mg/L, as required. Before use all stock solutions were sterilised using 0.2 µm pore-size filters (Sartorius AG).

## 2.3 Media used and culture conditions.

Pre-dried Iso-Sensitest agar (Oxoid, Basingstoke, UK. Appendix 3) plates and pre-warmed Iso-Sensitest broth (Oxoid. Appendix 3) were used predominantly throughout these investigations. In some experiments minimal media broth (Appendix 4) or 90% heat-inactivated (56°C for 30 minutes) pooled human serum/10% Iso-Sensitest broth (volume/volume) were used.

The pooled human serum used throughout was commercially purchased and screened for Hepatitis B and human immunodeficiency virus (Bradsure Biologicals Ltd). All media were prepared according to the manufacturer's instructions (Appendices 3 & 4). The agar plates were incubated in air for 18-24 h at 35-37°C and the broths were shaken aerobically for the required duration at 35-37°C in a water bath. Eighteen hour broth cultures (approximately $10^9$ CFU/mL) were obtained by inoculating an Iso-Sensitest broth with several colonies from a purity plate followed by incubation in air for 18 h at 35-37°C.

**2.4 MIC and MBC determination by broth dilution.**

The minimum inhibitory concentrations and minimum bactericidal concentrations (MBCs) of the carbapenems were determined by a broth microdilution method (adapted from Working Party of the British Society of Antimicrobial Chemotherapy, 1991). The broth dilution method was chosen as opposed to an agar incorporation method because all PAE determinations were performed in a broth milieu.

The microorganism inocula were prepared by diluting 18 h Iso-Sensitest broth cultures one hundred-fold into sterile phosphate-buffered saline (PBS. Appendix 2) (pH 7.3, Oxoid). Then these were diluted one hundred-fold further into pre-warmed Iso-Sensitest broth to give approximately 5 x $10^5$ CFU/mL, the recommended inoculum (Working Party of the British Society of Antimicrobial Chemotherapy, 1991). Carbapenem MICs were determined at this inoculum for all the microorganisms studied. This method of MIC determination was also used to determine the imipenem MIC for *P. aeruginosa* PAO1 in minimal media broth.

Additionally, imipenem and meropenem MICs and MBCs for *P. aeruginosa* PAO1 were determined using inocula of approximately $10^6$, $10^7$ and $10^8$ CFU/mL, in this instance the 18 h Iso-Sensitest broth cultures were diluted ten-, one hundred-and one thousand-fold, respectively. All these diluted cultures were incubated for 1 h to allow them to achieve logarithmic growth phase (Section 2.6).

A doubling dilution step range (up or down from 1 mg/L) of carbapenem concentrations (at twice the final concentration required due to a final two-fold dilution) was prepared in Iso-Sensitest broth from carbapenem stock solutions (Table 13). Sterile U bottom microtitre trays (96 well, 8 horizontal rows and 12 vertical rows. Bibby Sterilin Limited, Stone, UK) were labelled, one vertical row per concentration and one horizontal row per strain (Appendix 5). A 100 µL aliquot of each carbapenem concentration was pipetted into the appropriate well and 100 µL of carbapenem-free Iso-Sensitest broth was pipetted into the last vertical row for each microorganism as a growth control. A 100 µL aliquot of the diluted microorganism culture was then pipetted into each appropriate well including the carbapenem-free growth control well. The tray was sealed with a plastic sealer and incubated in air for 18-24 h at 35-37°C. Bacterial viable counts (Section 2.5) were performed on the diluted microorganism cultures to verify that the inocula used were correct. The carbapenem concentrations made in broth, the carbapenem-free broth and diluted microorganism cultures were also incubated. After incubation, the diluted microorganism cultures were examined for growth and the other broths were examined for sterility. The viable counts were calculated to verify the correct inocula had been used.

The MICs were then determined by placing the microtitre tray on a tray viewer and examining each well for visible growth. The MIC was defined as the lowest concentration of carbapenem that inhibited the visible growth of a microorganism after 18-24 h incubation.

**Table 13. Preparation of carbapenem concentrations for MIC and MBC determination by broth dilution.**

| Volume of broth (mL) | Volume of carbapenem stock (mL) | Carbapenem stock (mg/L) | Concentration in broth (mg/L) | Final plate concentration (mg/L) |
|---|---|---|---|---|
| 4.925 | 0.075 | 1 | 0.015 | 0.008 |
| 4.850 | 0.15 | 1 | 0.03 | 0.015 |
| 4.970 | 0.03 | 10 | 0.06 | 0.03 |
| 4.940 | 0.06 | 10 | 0.12 | 0.06 |
| 4.875 | 0.125 | 10 | 0.25 | 0.12 |
| 4.975 | 0.025 | 100 | 0.5 | 0.25 |
| 4.950 | 0.05 | 100 | 1 | 0.5 |
| 4.900 | 0.10 | 100 | 2 | 1 |
| 4.980 | 0.02 | 1000 | 4 | 2 |
| 4.960 | 0.04 | 1000 | 8 | 4 |
| 4.920 | 0.08 | 1000 | 16 | 8 |

Volume (mL) of stock to be added to broth =

$$\frac{\text{concentration required (mg/L) X volume required (mL)}}{\text{stock solution (mg/L)}}$$

For example, 0.02 mL = $\frac{4 \times 5}{1000}$

0.02 mL of 1000 mg/L was added to 4.98 mL broth to give a concentration of 4 mg/L.

The MBC determinations were then performed by subculturing 100 µL from each well onto carbapenem-free Iso-Sensitest agar plates and incubating these in air for 18-24 h at 35-37°C. After incubation, the plates were examined for growth and the MBC was defined as the lowest concentration of carbapenem that reduced the initial inoculum by 99.9%. When a standard inoculum of 5 X $10^5$ CFU/mL was used the MBC was defined as less than 10 colonies. To use this definition for higher inocula, for example, $10^6$, $10^7$ and $10^8$ CFU/mL a ten-, hundred- and thousand-fold dilution, respectively, in PBS of broth from the microtitre tray was necessary prior to subculture.

**2.5 Bacterial viable counts.**

Viable counts were used throughout these investigations to follow bacterial growth kinetics. Viable counts were determined on cultures (after dilution in sterile PBS, if necessary, except in Section 2.9.2) using a spiral plater (Model B. Don Whitley Scientific Limited, Skipton, UK). The spiral plater (Appendix 6) is a specialised, mechanical dispenser which permits the determination of microbial numbers over a range of three orders of magnitude (for example, $10^3$-$10^6$ CFU/mL) on one agar plate, without serial dilution of the sample. Therefore, fewer dilutions and agar plates were required for each sample than with spread or pour plate viable count techniques, thus reducing both labour and material costs. The spiral plater system employed has a lower limit of microbial detection of 3 X $10^2$ CFU/mL and coefficient of variance (CV) of 15% (Spiral Systems Inc.).

After sterilisation with Sanichlor (2500 parts per million. Henkel Ecolab Limited, Swindon, UK) and washing with sterile distilled water the sample was drawn into the hollow stylus (using a vacuum of 500-700 mm mercury). On starting the plater, the variable cam-activated syringe dispensed, through the stylus, a standard volume (36 µL) of the culture sample onto the surface of a rotating Iso-Sensitest agar plate. The stylus moved from the centre to the edge of the plate and dispensed the sample in the form of an Archimedes spiral. Thus, the amount of sample delivered to the plate surface decreased logarithmically and therefore, the quantity of sample deposited on any given area of the plate was known and constant. After incubation in air for 18-24 h at 35-37°C, colonies grew on the lines of the spiral. The bacterial count in CFU/mL was determined by manually enumerating the discrete colonies (10-200) using the illuminated colony viewer and grid (Appendix 7). Every area marked on the grid corresponds to a known and constant volume of sample deposited on the plate. In order to balance any irregularities in sample deposition or plate quality the same segments in opposite sectors were counted. The bacterial density was then estimated by dividing the count obtained by the volume contained in all the segments counted and if necessary multiplying by any dilution factor (Appendix 7). Plates of 1-2 microorganism dilutions were enumerated and the mean CFU/mL were calculated.

## 2.6 Bacterial growth curves.

PAE determinations throughout these investigations were performed on microorganisms in logarithmic growth phase (except Section 2.9.6). Therefore, it was initially necessary to perform bacterial growth curves in both Iso-Sensitest broth and minimal media broth to ascertain the time taken for the cultures to reach logarithmic growth phase.

18 h Iso-Sensitest and minimal media broth cultures of *P. aeruginosa* PAO1 were diluted one thousand-fold in pre-warmed Iso-Sensitest or minimal media broths as appropriate, to give approximately $10^6$ CFU/mL. They were then shaken aerobically in a water bath at 35-37°C for 4 h. Viable counts were determined at 0.5, 1, 1.5, 2, 3 and 4 h. After incubation in air for 18-24 h at 35-37°C bacteria were enumerated and the counts of $\log_{10}$ CFU/mL were plotted against time.

## 2.7 Effect of exposure to pooled human serum on the growth of *P. aeruginosa* strains.

For the determination of carbapenem PAE in 90% heat-inactivated pooled human serum it was necessary to employ microorganisms whose growth was least affected by exposure to serum. Therefore, the inhibitory effect on the growth of 22 clinical strains of *P. aeruginosa* after exposure to 90% pooled human serum was determined. Any inhibitory effect was ascertained by comparing growth after exposure to 90% pooled human serum with growth in Iso-Sensitest broth.

An 18 h Iso-Sensitest broth culture of each *P. aeruginosa* strain was diluted one hundred-fold into pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. These cultures were then diluted one hundred-fold further into both pre-warmed 90% pooled human serum and Iso-Sensitest broth. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h, diluted twenty-fold into pre-warmed Iso-Sensitest broth and then incubated with continuous shaking at 35-37°C for 6 h. Viable counts were performed on Iso-Sensitest agar plates prior to exposure (to verify original inocula) and hourly after exposure for 6 h. After incubation in air for 18-24 h at 35-37°C the bacteria were enumerated and the counts of $\log_{10}$ CFU/mL were plotted against time. The effect of a 1 h exposure to heat-inactivated pooled human serum (effect of serum (EOS)) was determined by calculating the difference between the time for the microorganism to increase 1 $\log_{10}$ after exposure to pooled human serum and the time for the microorganism to increase 1 $\log_{10}$ in Iso-Sensitest broth.

### 2.8 Time-kill kinetics of three carbapenems on *P. aeruginosa* PAO1.

Time-kill curves are pharmacodynamic examples of bactericidal activity expressed as rate of killing by a fixed concentration of antimicrobial and are one of the most reliable methods of determining tolerance (NCCLS, 1992). The time-kill kinetics of imipenem, meropenem and biapenem on *P. aeruginosa* PAO1 at an inoculum of approximately 3.5 X $10^6$ CFU/mL were evaluated at 4 X MIC in Iso-Sensitest broth to determine their bactericidal activity.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted thousand-fold in pre-warmed Iso-Sensitest broths and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to the logarithmic phase cultures of approximately $10^6$ CFU/mL to give a final carbapenem concentration equivalent to 4 X MIC. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 5 h.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and at 0.5, 1, 2, 3, 4, and 5 h after carbapenem addition. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time.

## 2.9 *In vitro* PAE method development.

### 2.9.1 Comparison of different rapid methods of carbapenem removal.

The imipenem PAEs on *P. aeruginosa* PAO1 were evaluated and compared in Iso-Sensitest broth using three different methods of rapid carbapenem removal.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broths and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures of approximately 2 X $10^6$ CFU/mL. The imipenem stock solutions (prepared according to Section 2.2) were added to the diluted logarithmic phase cultures to give a final imipenem concentration equivalent to 4 X MIC. Imipenem-free broth growth controls were also included. The cultures were shaken aerobically in a

water bath at 35-37°C for 1 h. The imipenem concentrations were then reduced by either; a) one thousand-fold dilution into pre-warmed imipenem-free Iso-Sensitest broth, or b) incubating with equal volumes of ß-lactamase (Appendix 8. Genzyme Biochemicals Limited, Maidstone, UK) for 10 minutes at 35-37°C, followed by a ten-fold dilution into pre-warmed imipenem-free Iso-Sensitest broth, or c) washing the broths twice by centrifugation at 5,000 *g* (MSE High Speed 18. Fisons, Crawley, UK) for 5 minutes, completely removing the supernatant and resuspending the pellet in PBS. After the third spin the pellet was resuspended in pre-warmed imipenem-free Iso-Sensitest broth.

After imipenem removal, the cultures were shaken aerobically in a water bath at 35-37°C for 6 h. The three growth control cultures (one for each method of removal) were treated in exactly the same manner as the test cultures. Viable counts were performed on imipenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after imipenem removal. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time.

The PAE was calculated using PAE = $T - C$ where, $T$ is the time required for the count in CFU in the exposed test culture to increase by 1 $\log_{10}$ above the count observed immediately after imipenem removal and $C$ is the time required for the count in CFU in the non-exposed control to increase by 1 $\log_{10}$ above the count observed immediately after completion of the same procedure used on the test culture for imipenem removal (Bundtzen *et al.*, 1981).

### 2.9.2 Comparison of different viable count diluents on carbapenem PAE.

MacKenzie and Gould (1993) stated that viable counts are erroneous due to the use of inappropriate diluents, such as phosphate buffered saline or water. To compare the effect of different viable count diluents on carbapenem PAE on *P. aeruginosa* PAO1, viable counts were performed on carbapenem-free Iso-Sensitest plates using either distilled water, 0.9% sodium chloride or PBS.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to logarithmic phase cultures to give final carbapenem concentrations equivalent to 4 X MIC. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control culture was treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates using either distilled water, 0.9% sodium chloride or PBS prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.9.3 Effect of exposure concentration on carbapenem PAE.

#### 2.9.3.1 Effect of exposure concentration on imipenem PAE.

The imipenem PAEs on *P. aeruginosa* PAO1 were evaluated in duplicate at various concentrations in Iso-Sensitest broth to determine if imipenem PAE was concentration dependent.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one hundred-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The imipenem stock solutions (prepared according to Section 2.2) were added to pre-warmed Iso-Sensitest broth to give final imipenem concentrations equivalent to 0.5, 1, 4, 16 and 64 X MIC. Logarithmic phase culture was then added to these broths to give final inocula of approximately 2 X $10^6$ CFU/mL. An imipenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The imipenem concentrations were then reduced by one thousand-fold dilution into pre-warmed imipenem-free Iso-Sensitest broths which were then incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control culture was treated in exactly the same way. Viable counts were performed on imipenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T - C$, as described previously.

### 2.9.3.2 Effect of exposure concentration on imipenem and meropenem PAE.

The imipenem and meropenem PAEs on *P. aeruginosa* PAO1 at an inoculum of approximately 3 X $10^7$ CFU/mL were evaluated at various concentrations in Iso-Sensitest broth to determine whether their PAEs were concentration dependent.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted fifty-fold in pre-warmed Iso-Sensitest broths and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to the diluted logarithmic phase cultures of approximately $10^7$ CFU/mL to give final carbapenem concentrations equivalent to 0.5, 1, 2, 4, 8 and 16 X MIC. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 2 h. The carbapenem concentrations were then reduced by washing twice in PBS, by centrifugation at 5,000 *g* for 5 minutes and resuspending in pre-warmed carbapenem-free Iso-Sensitest broths and the cultures were then incubated aerobically in a shaking water bath at 35-37°C for 24 h. The control culture was treated in exactly the same way. Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and at 0, 2, 4, 6, 8 and 24 h after neutralisation. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T - C$. Control-related effective regrowth time (CERT) was also determined as CERT = $T - C$ where, $T$ is the time required for the microbial population in the test culture to return to preexposure inoculum (Nilsson *et al.*, 1995) after elimination of the antimicrobial and $C$ is the corresponding time for the non-exposed control culture.

### 2.9.4 Effect of duration of exposure on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 were evaluated after durations of exposure of either 1 or 2 h in Iso-Sensitest broth to determine if carbapenem PAE was exposure time dependent.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broths and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to the diluted logarithmic phase cultures of approximately 2 X $10^6$ CFU/mL to give a final carbapenem concentration equivalent to 4 X MIC. Carbapenem-free broth growth controls were included. The cultures were shaken aerobically in a water bath at 35-37°C for either 1 or 2 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.9.5 Effect of inoculum size on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 were evaluated at various inocula in Iso-Sensitest broth to determine if carbapenem PAE was inoculum dependent.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted ten-fold, one hundred-fold and one thousand-fold in pre-warmed Iso-Sensitest broths and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to the diluted logarithmic phase cultures of approximately $10^6$, $10^7$ and $10^8$ CFU/mL to give a final carbapenem concentration equivalent to 4 X MIC. Carbapenem-free broth growth controls were included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using $PAE = T - C$.

### 2.9.6 Effect of growth phase on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 in both logarithmic and stationary growth phase were evaluated in Iso-Sensitest broth to determine if carbapenem PAE was growth phase dependent.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broths and either shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture or diluted prior to carbapenem addition for stationary phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to logarithmic and stationary phase cultures to give final carbapenem concentration equivalent to 4 X MIC. Carbapenem-free broth growth controls were included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.9.7 Effect of culture agitation on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 in Iso-Sensitest broth either shaken or stationary during exposure were evaluated to determine the effect of culture agitation on carbapenem PAE.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to logarithmic phase cultures to give final carbapenem concentration equivalent to 4 X MIC. Carbapenem-free broth growth controls were included. The cultures were either shaken aerobically or incubated without agitation at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.9.8 Effect of pooled human serum on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *E. coli* I431 and *P. aeruginosa* G341 were evaluated in duplicate at various concentrations in 90% pooled human serum and Iso-Sensitest broth, to determine the effect of serum on PAE.

An 18 h Iso-Sensitest broth culture of each of the microorganisms was diluted one hundred-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to both pre-warmed 90% pooled human serum and Iso-Sensitest broth to give final carbapenem concentrations equivalent to 0.5, 1, 4, 8, 16, 32, and 64 X MIC. Logarithmic phase cultures were then added to these broths to give final inocula of approximately 5 X $10^6$ CFU/mL. Carbapenem-free serum and broth growth controls were included for each microorganism. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way. Residual carbapenem controls were also included to assess the effect of constant exposure to one thousandth of the highest exposure concentration (64 X MIC). Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.9.9 Reproducibility of carbapenem PAE.

The intra and inter run reproducibilities of the imipenem, meropenem and biapenem PAEs in Iso-Sensitest broth on *P. aeruginosa* strain PAO1 were determined. The following method was performed six times on one day to ascertain the intra run reproducibility and on eight separate days to ascertain the inter run reproducibility.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one hundred-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to pre-warmed Iso-Sensitest broths to give final carbapenem concentrations equivalent to 16 X MIC. Logarithmic phase culture was then added to these broths to give final inocula of approximately 1.5 X $10^6$ CFU/mL. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control culture was treated in exactly the same way.

Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$. The mean PAEs and standard deviations (SDs) were calculated for both intra and inter run reproducibility.

### 2.9.10 Susceptibility prior to and after PAE determination.

To verify that carbapenem PAE is not due to the selection of a resistant slow growing subpopulation, MICs were performed (as in Section 2.4) on cultures prior to and after carbapenem PAE determination performed in Section 2.9.4 (1 h exposure to 4 X MIC concentration).

### 2.9.11 Effect of multiple successive carbapenem exposures on susceptibility and PAE.

Since most infections are treated with multiple doses of antimicrobials which result in several exposures the effect of three successive carbapenem exposures on the susceptibility and PAE of *P. aeruginosa* PAO1 was evaluated. The following method was performed on three separate days using the previously exposed culture (after 22 h incubation) in place of the 18 h broth culture.

Initially an 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. In addition, to verify that selection of resistant slow growing subpopulations does not occur, MICs were performed (as in Section 2.4) on cultures prior to and after carbapenem PAE determinations. The carbapenem stock solutions (prepared according to Section 2.2) were added to logarithmic phase cultures to give final carbapenem concentration equivalent to 4 X MIC. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h.

The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control culture was treated in exactly the same way. Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using

PAE = $T$ - $C$.

## 2.10 Comparison of carbapenem PAEs on *E. coli* and *P. aeruginosa*.

The imipenem, meropenem and biapenem PAEs in 90% pooled human serum were evaluated on five strains each of *E. coli* and *P. aeruginosa* to determine whether these carbapenems exhibit similar PAEs to each other and are similar in both genera.

An 18 h Iso-Sensitest broth culture of each of the microorganisms was diluted one hundred-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The carbapenem stock solutions (prepared according to Section 2.2) were added to pre-warmed pooled human sera to give final carbapenem concentrations equivalent to 4 X MIC. Logarithmic phase cultures were then added to these sera to give final inocula of approximately 5 X $10^6$ CFU/mL. Carbapenem-free serum and broth controls were included for each microorganism. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-

free Iso-Sensitest broth and then these cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way. Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.11 Strain variation of biapenem PAE.

The biapenem PAEs in pooled human serum were evaluated on eleven clinical strains of *P. aeruginosa*, to determine whether this novel carbapenem consistently produced a PAE.

An 18 h Iso-Sensitest broth culture of each of the microorganisms was diluted one hundred-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase cultures. The biapenem stock solutions (prepared according to Section 2.2) were added to pre-warmed pooled human sera to give final biapenem concentrations of 1 and 5 mg/L. Logarithmic phase cultures were then added to these sera to give final inocula of approximately 5 X $10^6$ CFU/mL. A biapenem-free serum control was included for each microorganism. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The biapenem concentrations were reduced by incubating with equal volumes of ß-lactamase for 10 minutes at 35-37°C, followed by a ten-fold dilution into pre-warmed biapenem-free Iso-Sensitest broth. These cultures were incubated aerobically in a shaking water bath at 35-37°C for 24 h.

The control cultures were treated in exactly the same way. Viable counts were performed on biapenem-free Iso-Sensitest agar plates prior to exposure, hourly for 6 h and at 24 h after inactivation of the carbapenem. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

## 2.12 Mechanism of PAE.

### 2.12.1 PAE of three carbapenems on *P. aeruginosa* PAO1 in the presence of lysine.

The carbapenem susceptibility of *P. aeruginosa* increases in low amino acid media (Fukuoka *et al.*, 1991). This is due to basic amino acids, such as L-lysine, competitively inhibiting carbapenem permeation through the D2 porin channel to the periplasm by binding to a site in the D2 channel (Trias and Nikaido, 1990a). To determine whether the presence of lysine would affect the imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1, differing L-lysine monohydrochloride (Sigma-Aldrich Company Limited) concentrations were added during carbapenem exposure and microorganism recovery.

An 18 h minimal media broth culture of the microorganism was diluted one hundred-fold in pre-warmed minimal media and shaken aerobically in a water bath at 35-37°C for 2 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to pre-warmed minimal media broths with L-lysine concentrations of 0, 20, 50, 70 and 100 mM to give a final carbapenem concentration of 2 mg/L. Logarithmic phase culture was added to these broths to give final inocula of approximately 6 X $10^5$ CFU/mL.

Carbapenem-free minimal media broth growth controls with the same L-lysine concentrations were included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h.

The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free minimal media broth without L-lysine and in addition for imipenem with 1, 1.5, 2, 4, 6, 8, 10, 20, 30, 50, 70 and 100 mM added L-lysine. The cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way. Viable counts were performed on carbapenem free Iso-Sensitest agar plates containing no additional lysine prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

**2.12.2 Effect on carbapenem PAE of increasing salinity during recovery.**

Information on the nature of the recovery process may help to elucidate the mechanism behind PAE. Determining the effect on the carbapenem PAE of *P. aeruginosa* PAO1 of increasing salinity during recovery may indicate the loss of salt tolerance thus suggesting some sub-lethal damage of the components of the cell wall (Fuursted, 1997a).

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to

logarithmic cultures to give final carbapenem concentrations equivalent to 4 X MIC. A carbapenem-free broth growth control was included. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were then reduced by one thousand-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth containing 0, 1 and 3% added sodium chloride (w/v)(BDH Chemicals Limited, Poole, UK) and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The control cultures were treated in exactly the same way. Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by dilution. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

### 2.12.3 *P. aeruginosa* virulence factors during PAE phase.

*P. aeruginosa* produces several virulence factors, the association of which with the duration of PAE has been insufficiently studied. It is thought that the classical PAE duration is proportionally shorter than the actual recovery time, that is, the treated microorganisms may still be physiologically abnormal (Bergeron, 1992). Therefore several virulence factors were evaluated during carbapenem PAE phase.

An 18 h Iso-Sensitest broth culture of the microorganism was diluted one thousand-fold in pre-warmed Iso-Sensitest broth and shaken aerobically in a water bath at 35-37°C for 1 h to obtain logarithmic phase culture. The carbapenem stock solutions (prepared according to Section 2.2) were added to

logarithmic cultures to give a final carbapenem concentration equivalent to 4 X MIC. The cultures were shaken aerobically in a water bath at 35-37°C for 1 h. The carbapenem concentrations were reduced by incubating with equal volumes of ß-lactamase for 10 minutes at 35-37°C, followed by a ten-fold dilution into pre-warmed carbapenem-free Iso-Sensitest broth and the cultures were incubated aerobically in a shaking water bath at 35-37°C for 6 h. The carbapenem-free growth control was treated in exactly the same way. Viable counts were performed on carbapenem-free Iso-Sensitest agar plates prior to exposure and hourly for 6 h after neutralisation by inactivation. After incubation in air for 18-24 h at 35-37°C the counts of $\log_{10}$ CFU/mL were plotted against time and PAE was calculated using PAE = $T$ - $C$.

The following virulence factors were semi-quantitatively evaluated by pipetting 10 µl onto the appropriate plates (Molinari *et al.*, 1993) in triplicate prior to exposure and hourly for 6 h after neutralisation by inactivation. Control samples were serially diluted to give five different inocula so comparison with the lower inocula of test cultures could be performed. A negative control consisting of ß-lactamase in Iso-Sensitest broth was also evaluated. The following media were prepared in 100 mL volumes and dispensed 30 mL per plate. Nutrient agar (Oxoid) plates containing 1% elastin (w/v)(Sigma-Aldrich Company Limited) were incubated at 35-37°C for 48 h and then transferred to room temperature for three days. Clearing of the opaque medium around the inoculum was taken to indicate elastolytic activity. Tryptone soya agar (Oxoid) plates containing 10% egg yolk enrichment (v/v)(Difco Laboratories). A white precipitate around or beneath inoculum after incubation at 35-37°C for 48 h indicated lecithinase activity.

DNase agar (Oxoid) plates were incubated at 35-37°C for 72 h then flooded with 1 N hydrochloric acid and a clear zone around the growth area manifested DNase activity. Mueller-Hinton agar (Difco Laboratories) plates containing 5% horse red cells were incubated for 72 h at room temperature and the extent of the haemolytic (haemolysin) zones were evaluated. Mueller-Hinton agar plates containing 0.4% gelatin (w/v)(Sigma-Aldrich Company Limited) were incubated for 48 h at 35-37°C and the plates were flooded with saturated ammonium sulphate and examined for zones of clearing around the inoculum to determine gelatinase production.

Nutrient agar plates supplemented with 1% Tween 80 (v/v)(Sigma-Aldrich Company Limited) and 0.01% calcium chloride (w/v)(Sigma-Aldrich Company Limited) were incubated at 35-37°C for 48 h and at room temperature for 5 additional days. Lipolytic (lipase) activity was indicated by the appearance of a turbid zone around the inoculum. Motility media (Appendix 9) were also used to monitor microorganism motility. Pigment production was evaluated on the viable count plates after 48 h incubation at 35-37°C.

### 2.12.4 Spheroplast recovery in PAE phase using *N*-acetyl-D-glucosamine.

Spheroplasts have previously been cultured by using media containing *N*-acetyl-D-glucosamine (Roberts *et al.*, 1984). Therefore, carbapenem PAE was performed (in duplicate) on logarithmic phase cultures as in Section 2.9.6 and viable counts were performed using carbapenem-free Iso-Sensitest plates with or without 1 g/L *N*-acetyl-D-glucosamine (Sigma-Aldrich Company Limited).

### 2.12.5 Morphology during PAE phase.

Microscopic examination of cellular morphology allows indirect study of the activity of the PBPs. The reversion of the altered morphology of exposed cells to normal rod-shaped cells over time was evaluated by both epi-fluorescent and scanning electron microscopy.

The morphology of *P. aeruginosa* PAO1 was examined by epi-fluorescence microscopy during imipenem, meropenem and biapenem PAE phase. The PAE was performed as in Section 2.12.3 and 20 µL samples of both exposed and non-exposed control cultures were examined after staining with acridine orange (Appendix 10) using an epi-fluorescence light microscope (Zeiss) at 1250 X magnification during exposure and hourly for 6 h after carbapenem removal. In addition, imipenem and meropenem PAEs were performed as in Section 2.12.3 and both exposed and non-exposed control cultures were examined by scanning electron microscopy (Jeol 120CXII) in order to observe any differences in cell morphology during exposure and hourly for 3 h after carbapenem removal. Culture samples were prepared (as performed by P. Stanley of Birmingham University) by initially fixing in plastic eppendorf tubes with 2.5% (v/v) glutaraldehyde (Agar Scientific Limited) in phosphate buffer (0.2 M, BDH Chemicals Limited) followed by centrifugation for 2 minutes. The tip of the eppendorf was then sliced off and processed through a graded acetone series, 2 X 15 minutes in each of 50%, 70%, 90%, 100% and 100% dried acetone (BDH Chemicals Limited). The tip of the eppendorf was then critical point dried (Polaron E3000), mounted on a Jeol copper stub with conducting carbon tape and then sputter coated with platinum (Emscope SC500).

# 3. RESULTS

## 3.1 MICs and MBCs.

With inocula of 5 X $10^5$ CFU/mL, the $MIC_{90}$s (range) for imipenem, meropenem and biapenem were 0.12 (0.12), 0.015 (0.015) and 0.03 (0.03) mg/L, respectively, for the *E. coli* strains studied and 2 (0.5-4), 0.5 (0.06-2) and 0.5 (0.12-1) mg/L, respectively, for the *P. aeruginosa* strains studied (Table 14). Meropenem and biapenem exhibited activities 4 times greater than that of imipenem against *P. aeruginosa*. For *E. coli*, meropenem and biapenem had activities 8 and 4 times greater than that of imipenem, respectively. All three carbapenems demonstrated greater activity against *E. coli* than *P. aeruginosa*; the $MIC_{90}$s of *P. aeruginosa* being 16, 16 and 32 times greater than those of *E. coli* for imipenem, biapenem and meropenem, respectively.

For *P. aeruginosa* PAO1, the imipenem MICs in minimal media broth and Iso-Sensitest broth were 1 and 4 mg/L, respectively.

For *P. aeruginosa* PAO1, the imipenem and meropenem MIC/MBCs were 4/8, 4/4 and 4/8 mg/L and 2/2, 2/2 and 4/4 mg/L at inocula of $10^6$, $10^7$ and $10^8$ CFU/mL, respectively, demonstrating a lack of inoculum effect. The MIC and MBC values for both carbapenems were the same or within one dilution step of each other.

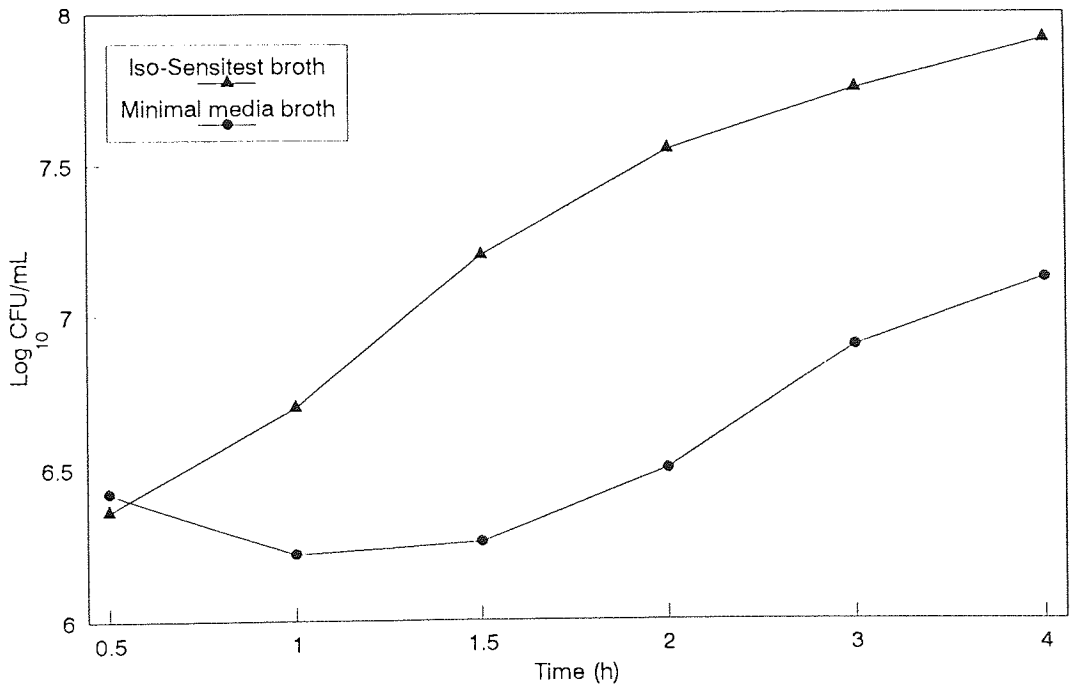
## Table 14. Carbapenem MIC results.

| Microorganism and identification code | Imipenem mg/L | Meropenem mg/L | Biapenem mg/L |
|---|---|---|---|
| *P. aeruginosa* G315 | 1 | 0.5 | 0.5 |
| *P. aeruginosa* G284 | 2 | 0.12 | 0.5 |
| *P. aeruginosa* G372 | 1 | 0.25 | 0.25 |
| *P. aeruginosa* G414 | 4 | 0.5 | 0.25 |
| *P. aeruginosa* G407 | 1 | 0.12 | 0.12 |
| *P. aeruginosa* G412 | 0.5 | 0.25 | 0.12 |
| *P. aeruginosa* G404 | 1 | 0.25 | 0.25 |
| *P. aeruginosa* G377 | 1 | 0.5 | 0.25 |
| *P. aeruginosa* G409 | 2 | 0.06 | 0.25 |
| *P. aeruginosa* G406 | 0.5 | 0.06 | 0.25 |
| *P. aeruginosa* G388 | 1 | 0.12 | 0.25 |
| *P. aeruginosa* G318 | 2 | 0.5 | 0.5 |
| *P. aeruginosa* G379 | 1 | 0.5 | 0.25 |
| *P. aeruginosa* G354 | 0.5 | 0.25 | 0.12 |
| *P. aeruginosa* G306 | 1 | 0.5 | 0.5 |
| *P. aeruginosa* G411 | 2 | 0.06 | 0.5 |
| *P. aeruginosa* G314 | 1 | 0.12 | 0.25 |
| *P. aeruginosa* G408 | 1 | 0.06 | 0.25 |
| *P. aeruginosa* G405 | 1 | 0.25 | 0.25 |
| *P. aeruginosa* G291 | 1 | 0.25 | 0.5 |
| *P. aeruginosa* G361 | 1 | 0.25 | 0.5 |
| *P. aeruginosa* G363 | 2 | 0.12 | 0.25 |
| *P. aeruginosa* G364 | 1 | 0.25 | 0.25 |
| *P. aeruginosa* G304 | 0.5 | 0.06 | 0.12 |
| *P. aeruginosa* G319 | 1 | 0.5 | 0.5 |
| *P. aeruginosa* G341 | 1 | 0.25 | 0.5 |
| *P. aeruginosa* PAO1 | 4 | 2 | 1 |
| *E. coli* I425 | 0.12 | 0.015 | 0.03 |
| *E. coli* I428 | 0.12 | 0.015 | 0.03 |
| *E. coli* I431 | 0.12 | 0.015 | 0.03 |
| *E. coli* I633 | 0.12 | 0.015 | 0.03 |
| *E. coli* I635 | 0.12 | 0.015 | 0.03 |

### 3.2 Bacterial growth curves.

Growth curves of *P. aeruginosa* PAO1 in Iso-Sensitest broth and minimal media broth were performed to ascertain the time taken for the cultures to reach logarithmic growth phase. These growth curves demonstrated that *P. aeruginosa* PAO1 reached logarithmic growth phase after approximately 1 and 2 h incubation in Iso-Sensitest broth and minimal media broth, respectively (Figure 11).

Figure 11. Bacterial growth curves in Iso-Sensitest broth and minimal medium broth.




Growth curves in Iso-Sensitest broth and minimal media broth of *P. aeruginosa* PAO1 at 37 °C.

### 3.3 Effect of exposure to pooled human serum on the growth of *P. aeruginosa* strains.

The effect on growth of a 1 h exposure to 90% heat-inactivated pooled human serum (EOS) on 22 clinical strains of *P. aeruginosa* was determined by calculating the difference between the time for the number of microorganisms exposed to serum to increase by 1 $\log_{10}$ (A) and the time for the non-serum exposed microorganisms to increase by 1 $\log_{10}$ (B) (Table 15).

Considerable variation in the inhibitory effect of pooled human serum was observed between the 22 strains of *P. aeruginosa* (EOS range of -0.3 to 4.03 h). The least affected strains were used in further PAE studies involving serum. Considerable variation in the growth rate in Iso-Sensitest broth between the 22 strains studied was also observed, for example, the time taken for counts in CFU/mL to increase 1 $\log_{10}$ ranged from 1 to 3 h.

**Table 15. The effect of exposure to pooled human serum on the growth of *P. aeruginosa* strains.**

| Microorganism identification code | A | B | Effect of serum (h) EOS = A - B |
|---|---|---|---|
| G414 | 1.87 | 1.00 | 0.87 |
| G407 | 1.88 | 2.08 | -0.20 |
| G412 | 1.90 | 2.20 | -0.30 |
| G404 | 2.00 | 1.75 | 0.25 |
| G377 | 2.00 | 2.00 | 0.00 |
| G409 | 2.03 | 2.00 | 0.03 |
| G406 | 2.23 | 2.12 | 0.11 |
| G388 | 2.30 | 2.10 | 0.20 |
| G318 | 2.37 | 1.00 | 1.37 |
| G379 | 2.57 | 2.25 | 0.32 |
| G354 | 2.57 | 1.50 | 1.07 |
| G306 | 2.57 | 1.00 | 1.57 |
| G411 | 2.70 | 3.00 | -0.30 |
| G314 | 2.93 | 1.00 | 1.93 |
| G408 | 3.03 | 2.10 | 0.93 |
| G405 | 3.05 | 2.00 | 1.05 |
| G291 | 3.53 | 1.00 | 2.53 |
| G361 | 3.63 | 1.00 | 2.63 |
| G363 | 4.13 | 1.70 | 2.43 |
| G364 | 4.23 | 1.58 | 2.65 |
| G304 | 4.27 | 1.00 | 3.27 |
| G319 | 5.03 | 1.00 | 4.03 |

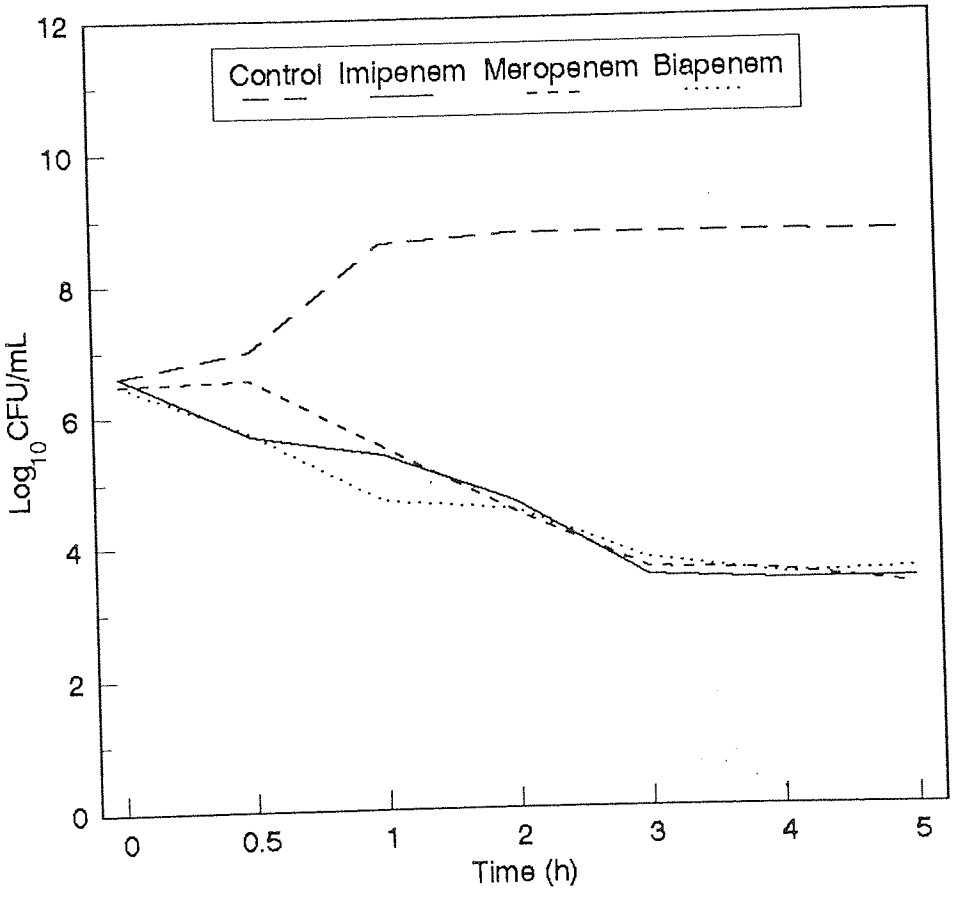
A. Time for count (CFU/mL) to increase 1 $\log_{10}$ after exposure to pooled human serum (h).

B. Time for count (CFU/mL) to increase 1 $\log_{10}$ in Iso-Sensitest broth (h).

### 3.4 Time-kill kinetics of three carbapenems on *P. aeruginosa* PAO1.

The time-kill kinetics of imipenem, meropenem and biapenem on *P. aeruginosa* PAO1 were evaluated at 4 X MIC in Iso-Sensitest broth to determine their bactericidal activity. Bactericidal activity defined as a 3 $\log_{10}$ decrease (99.9% kill) in CFU/mL (NCCLS, 1992) was observed at 2.9, 4.2 and 4 h for imipenem, meropenem and biapenem, respectively (Figure 12).

Figure 12. Time-kill kinetics of three carbapenems on *P. aeruginosa* PAO1




Bactericidal activity of imipenem, meropenem and biapenem in Iso-Sensitest broth at concentration of 4 X MIC on *P. aeruginosa* PAO1.

### 3.5 *In vitro* PAE method development.

#### 3.5.1 Comparison of different rapid methods of carbapenem removal.

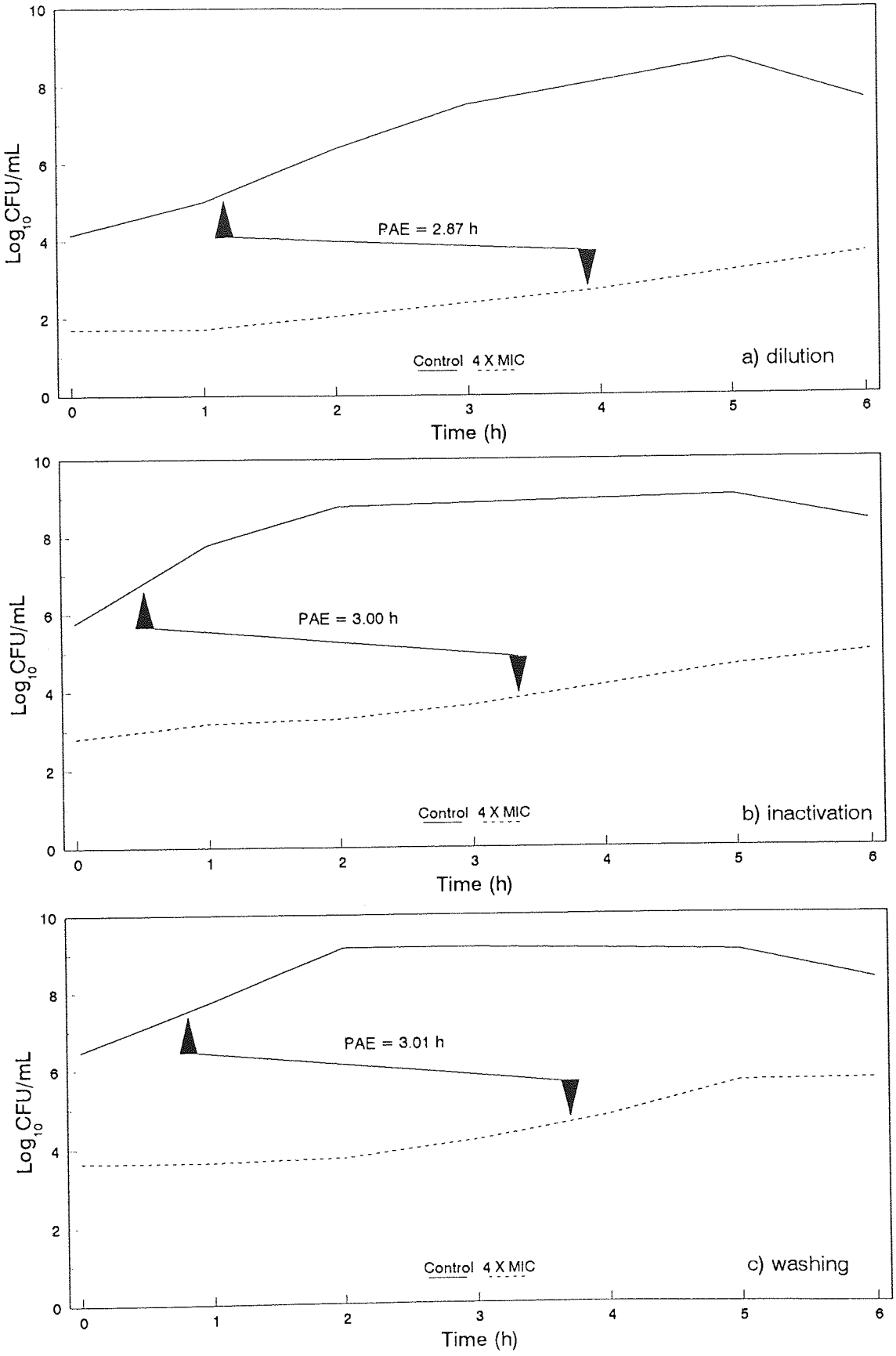
The imipenem (4 X MIC, 1 h exposure) PAEs on *P. aeruginosa* PAO1 were evaluated in Iso-Sensitest broth using either a one thousand-fold dilution, inactivation with ß-lactamase, or washing twice as methods of rapid imipenem removal. The PAEs ranged from 2.87 to 3.01 h and were within approximately 5% of each other (Table 16 and Figure 13). Comparison of the time required for the count (CFU/mL) of the controls to increase 1 $\log_{10}$, demonstrates that the washing and dilution controls were 1.5 and 2 times longer respectively, than the inactivation control.

**Table 16. Comparison of different rapid methods of imipenem removal.**

| | Time for CFU/mL to increase 1 $\log_{10}$ (h) | | PAE (h) |
|---|---|---|---|
| | Control | 4 X MIC | |
| Dilution | 1.13 | 4.00 | 2.87 |
| Inactivation | 0.53 | 3.53 | 3.00 |
| Washing | 0.82 | 3.83 | 3.01 |

The differences in time for the imipenem exposed *P.aeruginosa* PAO1 culture (exposure for 1 h at concentrations of 4 X MIC) and the non-exposed control to increase 1 $\log_{10}$ in Iso-Sensitest broth and the PAE (mean of duplicate determinations). Imipenem was removed either by 1000-fold dilution, inactivation with ß-lactamase or washing twice.

Figure 13. Comparison of different rapid methods of imipenem removal.




Growth curves of *P. aeruginosa* PAO1 following a 1 h exposure to imipenem at a concentration of four times the MIC. Imipenem was removed by either 1000-fold dilution (a), inactivation with β-lactamase (b) or washing twice (c).

### 3.5.2 Comparison of different viable count diluents on carbapenem PAE.

The effect of different viable count diluents on carbapenem PAE on *P. aeruginosa* PAO1 was determined using either distilled water, 0.9% sodium chloride or PBS (Table 17). The different viable count diluents made no significant differences to the observed imipenem, meropenem and biapenem PAEs, mean (SD) 2.7 (0.2), 1.4 (0.26) and 1.98 (0.04) h, respectively.

**Table 17. Effect of different viable count diluents on carbapenem PAE.**

| | Viable count diluent | | |
|---|---|---|---|
| | PBS | 0.9% sodium chloride | $H_2O$ |
| Imipenem PAE (h) | 2.9 | 2.5 | 2.8 |
| Meropenem PAE (h) | 1.1 | 1.55 | 1.55 |
| Biapenem PAE (h) | 2.0 | 1.93 | 2.0 |

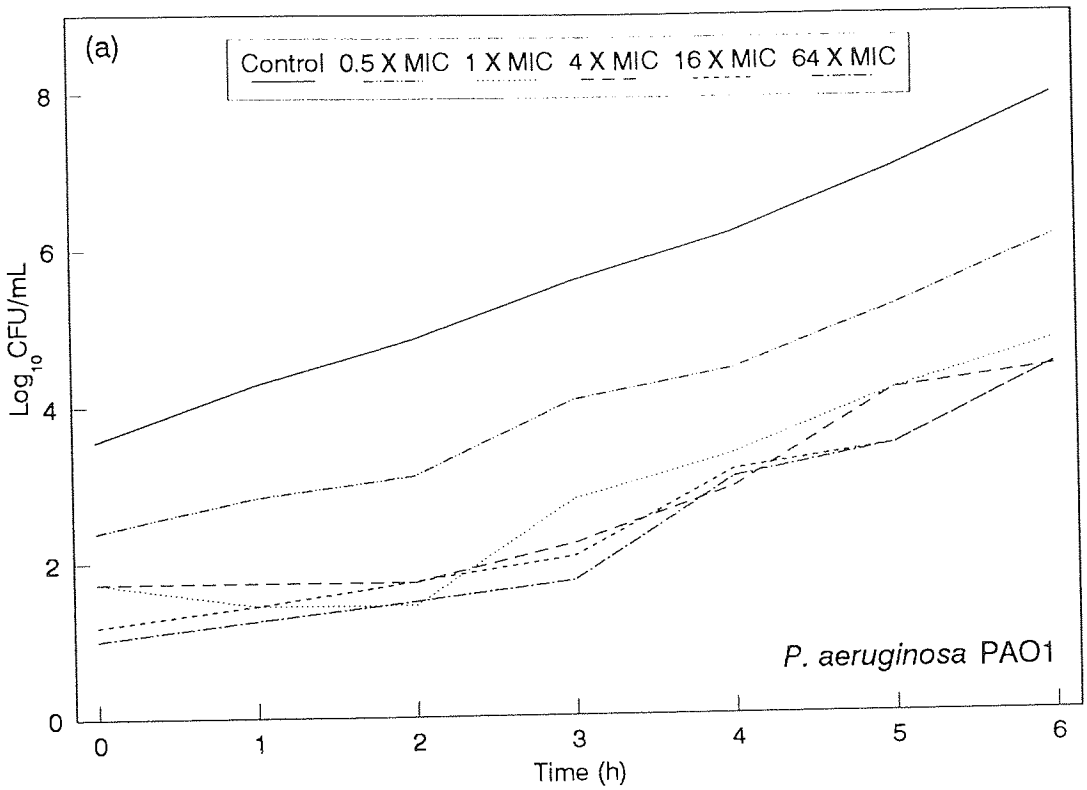
The carbapenem PAEs (mean of duplicate determinations) on *P. aeruginosa* PAO1 (following exposure for 1 h at concentrations of 4 X MIC in Iso-Sensitest broth) determined by viable counts using either PBS, 0.9% sodium chloride or distilled water as diluents.

### 3.5.3. Effect of exposure concentration on carbapenem PAE.

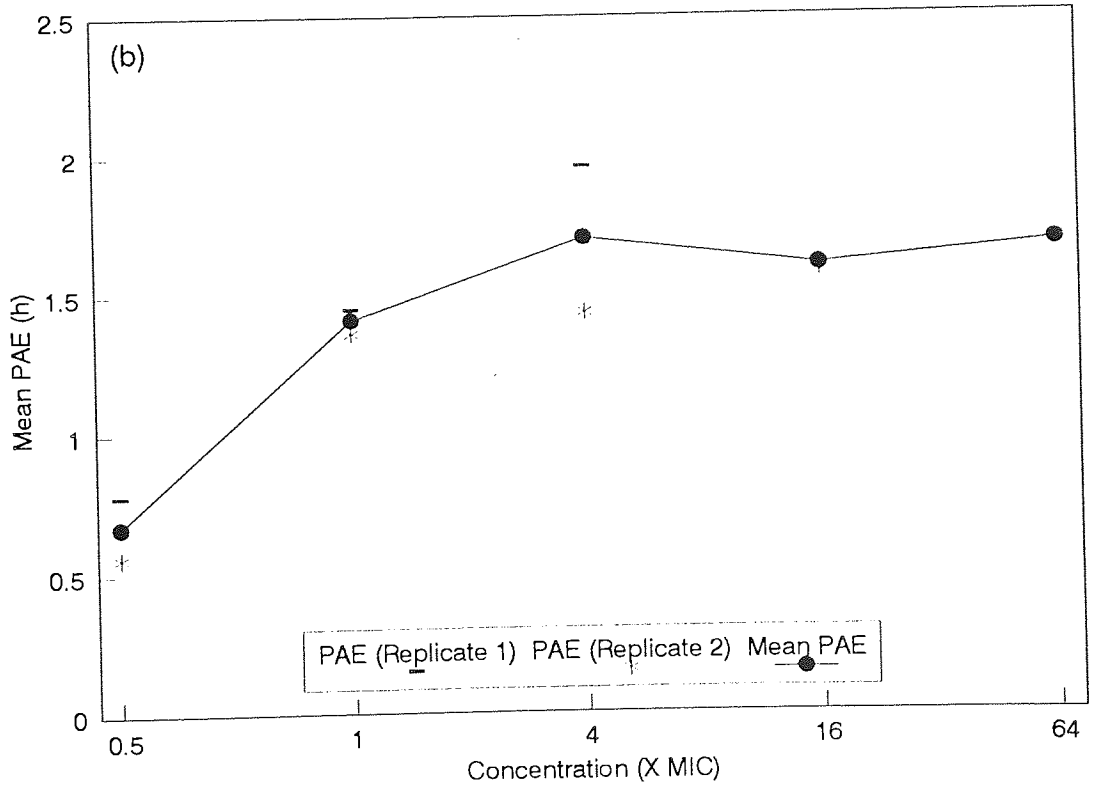
The effect of exposure concentration on carbapenem PAE was investigated using two methods of removal, different inocula and durations of exposure. Firstly, the imipenem PAEs on *P. aeruginosa* PAO1 cultures of approximately 2 X $10^6$ CFU/mL were evaluated (in duplicate) at various concentrations for 1 h and the concentrations were then reduced by dilution. Figure 14a is the plot of count ($log_{10}$ CFU/mL) against time (for 1 replicate) for each concentration of imipenem studied. This plot demonstrates that as imipenem concentration increased so did both the initial kill and PAE. The plot of mean PAE against concentration, also demonstrates that as concentration increased so did the PAE up to 4 X MIC, above this a maximal effect appears to be achieved (Figure 14b). Imipenem PAEs at 0.5 X MIC and above are significant (>0.5 h)

Secondly, the imipenem and meropenem PAEs on *P. aeruginosa* PAO1 cultures of approximately 3 X $10^7$ CFU/mL were evaluated at various concentrations for 2 h and the concentrations were then reduced by washing. Figure 15 is the plot of count ($log_{10}$ CFU/mL) against time for each concentration of meropenem studied. This plot demonstrates that as the meropenem concentration increased both the initial kill and PAE increased.

Figure 14. Effect of exposure concentration of imipenem on recovery and PAE.

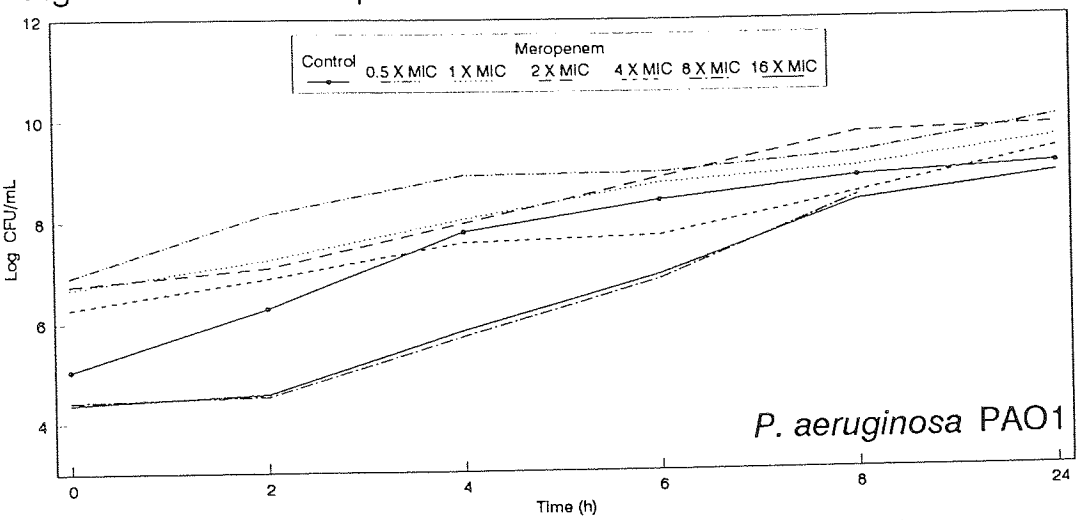



a) Growth curves of *P. aeruginosa* PAO1 following a 1 h exposure to imipenem at concentrations of 0.5, 1, 4, 16 and 64 X MIC (1 replicate). Imipenem was removed by 1000-fold dilution.




b) Mean and replicate imipenem PAE data of *P. aeruginosa* PAO1 following a 1 h exposure to concentrations of 0.5, 1, 4, 16 and 64 X MIC. Imipenem was removed by 1000-fold dilution.

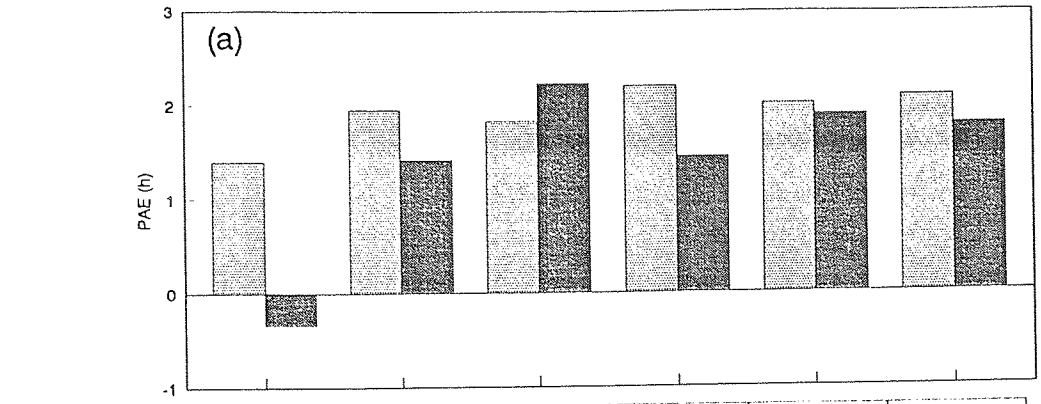
Figure 15. Effect of exposure concentration of meropenem on recovery.




Growth curves of *P. aeruginosa* PAO1following a 2 h exposure to meropenem at concentrations of 0.5, 1, 2, 4, 8 and 16 X MIC. Meropenem was removed by washing.

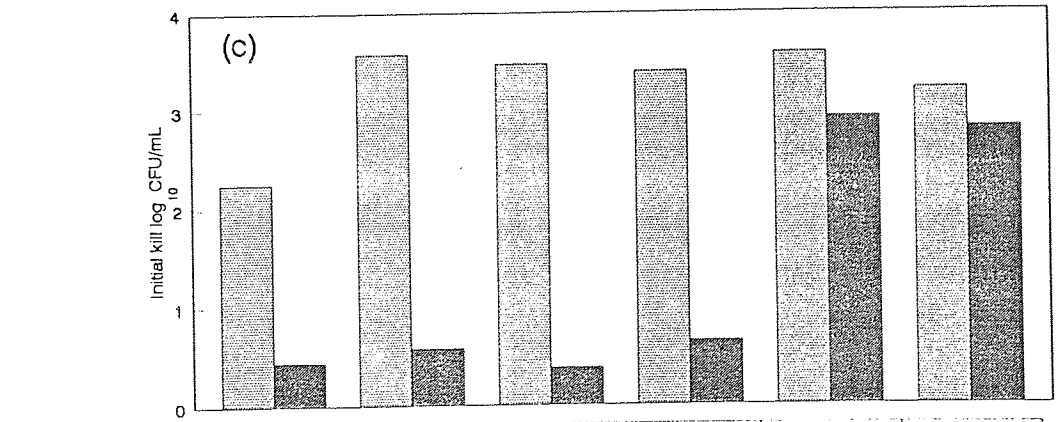
The plot of imipenem and meropenem PAE against concentration, demonstrates that as the concentration of both carbapenems increased so did the PAE up to 4 X MIC for imipenem and 2 X MIC for meropenem, above these concentrations a maximal effect appears to be achieved (Figure 16a). Figure 16b demonstrates that CERT shows a similar trend to PAE. Figure 16c demonstrates that initial kill (reduction in $\log_{10}$ CFU/mL during exposure) does not correlate with the duration of PAE, correlation coefficients ($r^2$) were 0.6 and 0.14 for imipenem and meropenem, respectively. A negative meropenem PAE was observed at 0.5 X MIC, but at higher concentrations the imipenem and meropenem PAEs were both positive, significant and similar. The increase in duration of exposure may explain the increase in PAE, for example, imipenem (0.5 X MIC) PAEs on *P. aeruginosa* PAO1 culture ($10^6$ CFU/mL) after 1 h exposure and ($10^7$ CFU/mL) after 2 h exposure were 0.67 and 1.4 h, respectively. However, this is not a direct comparison due to differences in inocula and carbapenem removal methods.

Figure 16. Effect of exposure concentration on imipenem and meropenem PAE, CERT and initial kill.




| Concentration (X MIC) | 0.5 | 1 | 2 | 4 | 8 | 16 |
|---|---|---|---|---|---|---|
| Imipenem PAE | 1.40 | 1.95 | 1.83 | 2.20 | 2.02 | 2.10 |
| Meropenem PAE | -0.35 | 1.42 | 2.23 | 1.45 | 1.90 | 1.80 |


| Concentration (X MIC) | 0.5 | 1 | 2 | 4 | 8 | 16 |
|---|---|---|---|---|---|---|
| Imipenem CERT | 2.10 | 2.12 | 2.05 | 2.42 | 2.90 | 2.15 |
| Meropenem CERT | -0.60 | 0.70 | 0.83 | 1.88 | 1.57 | 1.30 |




| Concentration (X MIC) | 0.5 | 1 | 2 | 4 | 8 | 16 |
|---|---|---|---|---|---|---|
| Imipenem Initial kill | 2.25 | 3.58 | 3.47 | 3.39 | 3.58 | 3.20 |
| Meropenem Initial kill | 0.44 | 0.57 | 0.38 | 0.64 | 2.92 | 2.81 |

Imipenem and meropenem PAE (a), CERT (b) and initial kill (c) of *P. aeruginosa* PAO1 following a 2 h exposure to concentrations of 0.5, 1, 2, 4, 8 and 16 X MIC. Imipenem and meropenem were removed by washing.

### 3.5.4 Effect of duration of exposure on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 were evaluated after durations of exposure of 1 or 2 h. The data in Table 18 demonstrate that carbapenem (4 X MIC) PAE is exposure duration dependent. As exposure duration increased from 1 to 2 h the imipenem, meropenem and biapenem PAEs increased by 1.3, 2.5 and 1.4 times, respectively.

**Table 18. Effect of duration of exposure on carbapenem PAE.**

| | PAE (h) | | |
|---|---|---|---|
| Exposure duration | Imipenem | Meropenem | Biapenem |
| 1 h | 1.70 | 0.28 | 0.73 |
| 2 h | 2.15 | 0.70 | 1.05 |

The carbapenem PAEs on *P. aeruginosa* PAO1 following exposure for 1 h or 2 h at concentrations of 4 X MIC in Iso-Sensitest broth.

### 3.5.5 Effect of inoculum size on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 were evaluated at inocula of 2 X $10^6$, $10^7$ and $10^8$ CFU/mL in Iso-Sensitest broth. The data in Table 19 demonstrate that with all three carbapenems (4 X MIC) as the inoculum increased the PAE decreased and is therefore inoculum dependent.

**Table 19. Effect of inoculum size on carbapenem PAE.**

| | Inocula (CFU/mL) | | |
|---|---|---|---|
| | $10^6$ | $10^7$ | $10^8$ |
| Imipenem PAE (h) | 2.16 | 0.45 | 0.15 |
| Meropenem PAE (h) | 0.60 | -0.50 | -0.15 |
| Biapenem PAE (h) | 1.38 | -0.52 | -0.45 |

The carbapenem PAEs on *P. aeruginosa* PAO1 logarithmic phase inocula of $10^6$, $10^7$ and $10^8$ CFU/mL following 1 h exposure at concentrations of 4 X MIC in Iso-Sensitest broth.

### 3.5.6 Effect of growth phase on carbapenem PAE.

The growth phase dependency of imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 was evaluated in Iso-Sensitest broth. After dilution the non-exposed control culture that was in stationary phase initially grew more slowly than the logarithmic phase culture with the time to increase 1 $\log_{10}$ being 2.2 and 1.6 h, respectively. All three carbapenems produced a negative PAE on *P. aeruginosa* PAO1 stationary phase cultures (Table 20).

**Table 20. Effect of growth phase on carbapenem PAE.**

| | Growth phase | |
|---|---|---|
| | Logarithmic | Stationary |
| Imipenem PAE (h) | 2.5 | -0.48 |
| Meropenem PAE (h) | 0.60 | -0.88 |
| Biapenem PAE (h) | 1.8 | -0.23 |

The carbapenem PAEs on *P. aeruginosa* PAO1 (following exposure for 1 h at concentrations of 4 X MIC in Iso-Sensitest broth) with either logarithmic or stationary phase inocula.

### 3.5.7 Effect of culture agitation on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs on *P.aeruginosa* PAO1 in Iso-Sensitest broth were evaluated to determine the effect of culture agitation on carbapenem PAE. Time for control to increase 1 $\log_{10}$ with and without agitation was 1.6 and 1.65 h, respectively. Agitation during exposure had little effect on carbapenem PAE (Table 21).

**Table 21. Effect of culture agitation on carbapenem PAE.**

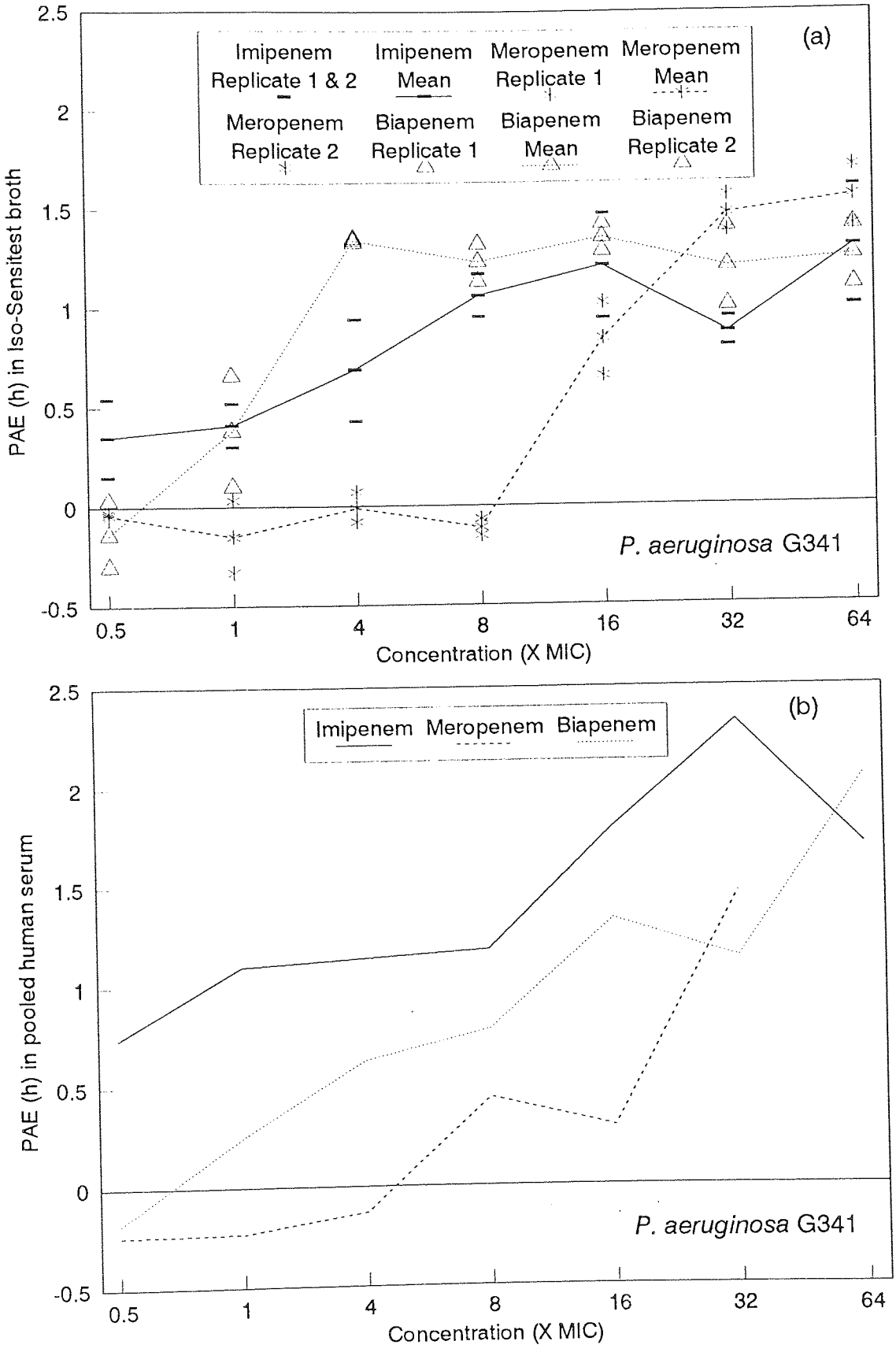
| | Agitation conditions during exposure | |
|---|---|---|
| | Shaken | Non-shaken |
| Imipenem PAE (h) | 2.5 | 2.5 |
| Meropenem PAE (h) | 0.60 | 0.70 |
| Biapenem PAE (h) | 1.7 | 1.3 |

The carbapenem PAEs on *P. aeruginosa* PAO1 with either shaken or stationary culture during exposure for 1 h at concentrations of 4 X MIC in Iso-Sensitest broth.

### 3.5.8 Effect of pooled human serum on carbapenem PAE.

The imipenem, meropenem and biapenem PAEs were evaluated in duplicate on *E. coli* I431 and *P. aeruginosa* G341 at various concentrations in both pooled human serum and Iso-Sensitest broth. Serum appeared to enhance the imipenem PAE on *P. aeruginosa* G341 (Figure 17), the meropenem and biapenem PAEs appear to be similar in both Iso-Sensitest broth and serum. The biapenem PAE at 64 X MIC on *P. aeruginosa* PAO1 was the only exception to this, the PAE in serum being almost twice that observed in Iso-Sensitest broth. The imipenem, meropenem and biapenem PAEs on *E. coli* I431 were negligible and appeared to be similar in both serum and Iso-Sensitest (Table 22). A significant PAE on G341 was observed in Iso-Sensitest broth at $\geq$4, $\geq$16 and $\geq$4 X MIC for imipenem, meropenem and biapenem respectively (Table 22). In serum, a significant PAE was observed for imipenem, meropenem and biapenem at concentrations of $\leq$0.5, $\geq$32 and $\geq$4 X MIC, respectively (Table 22). The growth of the RAC was identical to the growth control, indicating that the method of antimicrobial removal was adequate. In Iso-Sensitest broth the biapenem PAEs were greater than those of imipenem which were greater than those of meropenem. In serum the order of PAE was different, the imipenem PAEs were greater than those of biapenem which were greater than those of meropenem. With *P. aeruginosa* G341 maximal PAEs were achieved for imipenem, meropenem and biapenem in broth and serum at concentrations of >64, 32 and 4 X MIC and 32, >32 and >64 X MIC, respectively. A negative meropenem PAE was observed and a higher concentration of meropenem than imipenem and biapenem was required to produce a PAE.

Figure 17. Effect of pooled human serum on carbapenem PAE.




Imipenem, meropenem and biapenem PAEs of *P. aeruginosa* G341 in Iso-Sensitest broth (mean and replicates) (a) and pooled human serum (b). PAEs were determined at several exposure concentrations.

Table 22. Effect of pooled human serum on carbapenem PAE

| | Imipenem PAE in Iso-Sensitest broth | Imipenem PAE in pooled human serum | Meropenem PAE in Iso-Sensitest broth | Meropenem PAE in pooled human serum | Biapenem PAE in Iso-Sensitest broth | Biapenem PAE in pooled human serum |
|---|---|---|---|---|---|---|
| P. aeruginosa G341 | | | | | | |
| 0.5 X MIC | 0.35 | 0.74 | -0.04 | -0.24 | -0.14 | -0.18 |
| 1 X MIC | 0.41 | 1.10 | -0.15 | -0.23 | 0.38 | 0.25 |
| 4 X MIC | 0.69 | ND | -0.01 | -0.13 | 1.34 | 0.63 |
| 8 X MIC | 1.06 | 1.18 | -0.11 | 0.44 | 1.23 | 0.78 |
| 16 X MIC | 1.21 | 1.79 | 0.84 | 0.29 | 1.35 | 1.33 |
| 32 X MIC | 0.87 | 2.33 | 1.47 | 1.46 | 1.20 | 1.13 |
| 64 X MIC | 1.30 | 1.70 | 1.55 | ND | 1.25 | 2.05 |
| E. coli I431 | | | | | | |
| 0.5 X MIC | 0.06 | -0.08 | 0.26 | -0.03 | -0.05 | -0.11 |
| 1 X MIC | 0.07 | 0.04 | 0.10 | 0.09 | -0.12 | 0.13 |
| 4 X MIC | 0.33 | 0.25 | 0.40 | 0.32 | 0.25 | 0.12 |
| 8 X MIC | 0.06 | 0.15 | 0.18 | -0.13 | -0.11 | 0.12 |
| 16 X MIC | 0.17 | 0.18 | 0.08 | 0.19 | 0.14 | -0.17 |
| 32 X MIC | ND | 0.72 | 0.03 | -0.13 | -0.18 | -0.32 |
| 64 X MIC | ND | ND | 0.10 | -0.13 | -0.41 | -0.18 |

Imipenem, meropenem and biapenem PAEs (mean of duplicate determinations) of *P. aeruginosa* G341 and *E. coli* I431 following a 1 h exposure to concentrations of 0.5, 1, 4, 8, 16, 32 and 64 X MIC in Iso-Sensitest broth and pooled human serum.

### 3.5.9 Reproducibility of carbapenem PAE.

Reproducibility of the carbapenem PAEs on *P. aeruginosa* PAO1 were determined during an experiment (intra run) and over several experiments performed on different days (inter run). The mean (standard deviation) of imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 of six replicates performed on the same day were 1.75 h (0.28), 0.80 h (0.15) and 1.15 h (0.25), respectively (Table 23). The mean (standard deviation) of imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 of eight experiments performed on different days were 2.03 h (0.26), 1.28 h (0.36) and 1.28 h (0.22), respectively.

### 3.5.10 Susceptibility prior to and after PAE determination.

The carbapenem MICs prior to and after the determination of carbapenem PAE (1 h exposure to 4 X MIC) on *P. aeruginosa* PAO1 were compared and found to be identical or within one dilution step of each other.

### 3.5.11 Effect of multiple successive carbapenem exposures on susceptibility and PAE.

Since most infections are treated with multiple doses the effects of three successive carbapenem exposures on susceptibility and PAE of *P. aeruginosa* PAO1 were evaluated. The imipenem, meropenem and biapenem PAEs remained constant after three successive exposures, mean (SD) 2.3 (0.4), 1.0 (0.5) and 1.4 (0.2) h, respectively (Figure 18). The carbapenem MICs prior to and after three successive PAE determinations were found to be identical or within one dilution step of each other.

## Table 23. Reproducibility of carbapenem PAE.

### Intra run reproducibility

| | Imipenem PAE (h) | Meropenem PAE (h) | Biapenem PAE (h) |
|---|---|---|---|
| Replicate 1 | 1.45 | 1.02 | 1.20 |
| Replicate 2 | 1.74 | 0.60 | 0.90 |
| Replicate 3 | 1.55 | 0.75 | 0.95 |
| Replicate 4 | 2.05 | 0.80 | 1.13 |
| Replicate 5 | 2.13 | 0.70 | 1.10 |
| Replicate 6 | 1.60 | 0.90 | 1.60 |
| Range | 1.45-2.13 | 0.60-1.02 | 0.90-1.60 |
| Mean | 1.75 | 0.80 | 1.15 |
| SD | 0.28 | 0.15 | 0.25 |

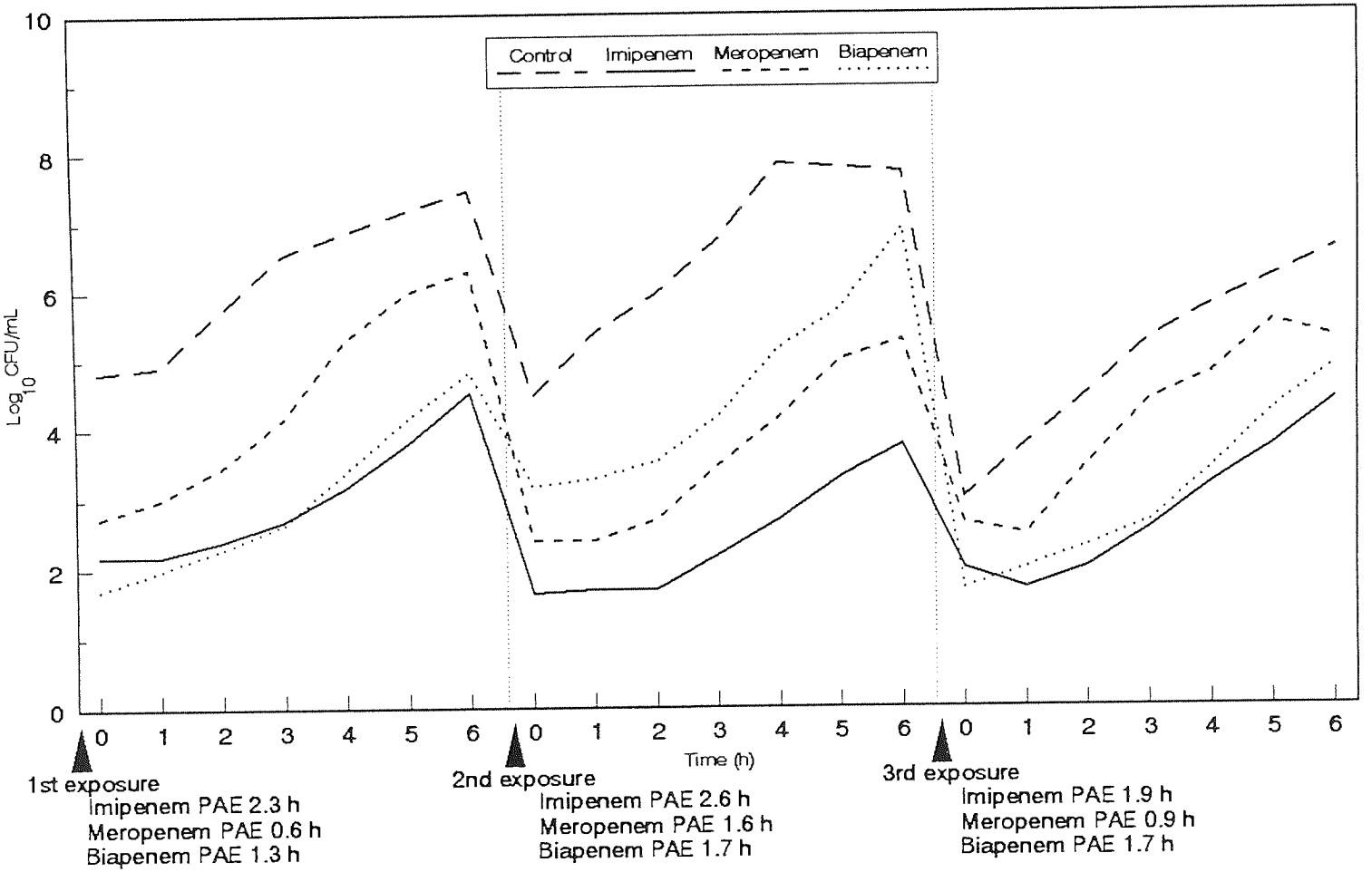
Imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 (6 replicates performed on same day) in Iso-Sensitest broth following a 1 h exposure to concentrations of 16 X MIC. Carbapenems removed by 1000-fold dilution.

### Inter run reproducibility

| | Imipenem PAE (h) | Meropenem PAE (h) | Biapenem PAE (h) |
|---|---|---|---|
| Run 1 | 2.15 | 1.65 | 1.65 |
| Run 2 | 1.70 | 1.50 | 1.60 |
| Run 3 | 2.43 | 1.60 | 1.14 |
| Run 4 | 2.15 | 0.90 | 1.13 |
| Run 5 | 2.17 | 0.90 | 1.25 |
| Run 6 | 2.10 | 1.02 | 1.05 |
| Run 7 | 1.73 | 1.00 | 1.18 |
| Run 8 | 1.78 | 1.70 | 1.20 |
| Range | 1.70-2.43 | 0.90-1.70 | 1.05-1.65 |
| Mean | 2.03 | 1.28 | 1.28 |
| SD | 0.26 | 0.36 | 0.22 |

Imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 (8 replicates performed on different days) in Iso-Sensitest broth following a 1 h exposure to concentrations of 16 X MIC. Carbapenems removed by 1000-fold dilution.

Figure 18. Effect of multiple successive carbapenem exposures on PAE.



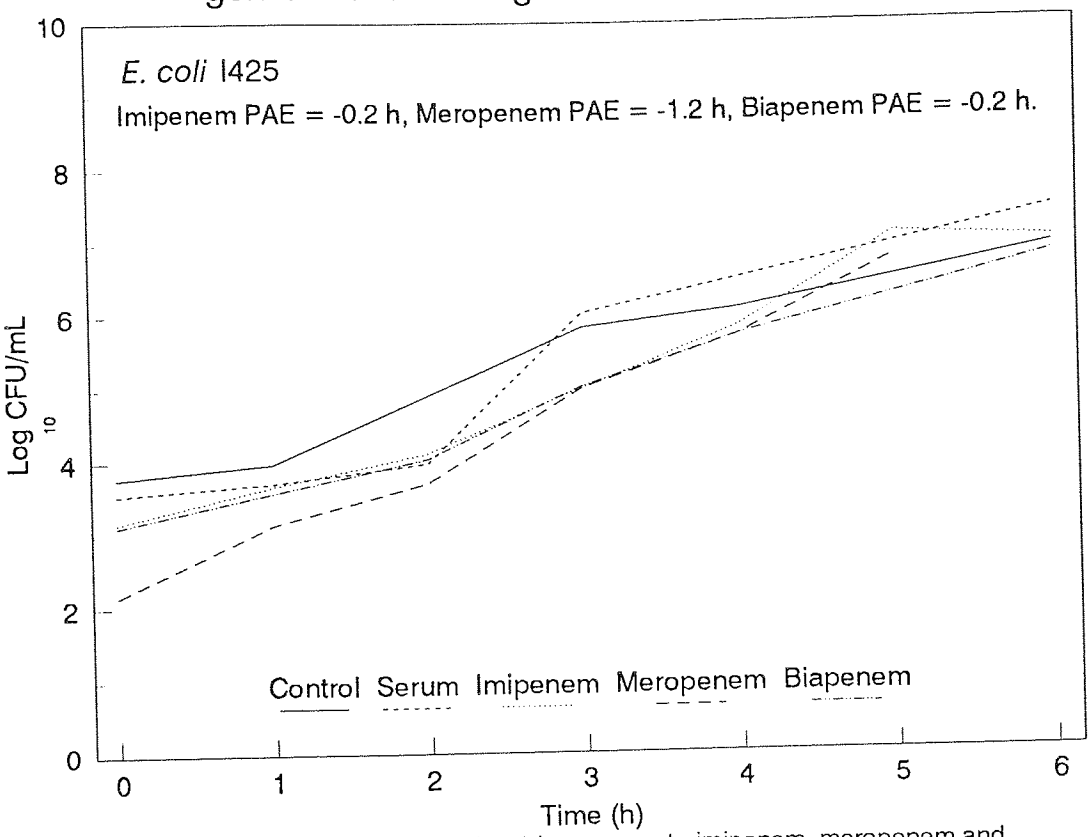

Imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 (3 successive exposures) in Iso-Sensitest broth following a 1 h exposure to concentratons of 4 X MIC. Carbapenems removed by 1000-fold dilution.

### 3.6 Comparison of carbapenem PAEs on *E. coli* and *P. aeruginosa*.

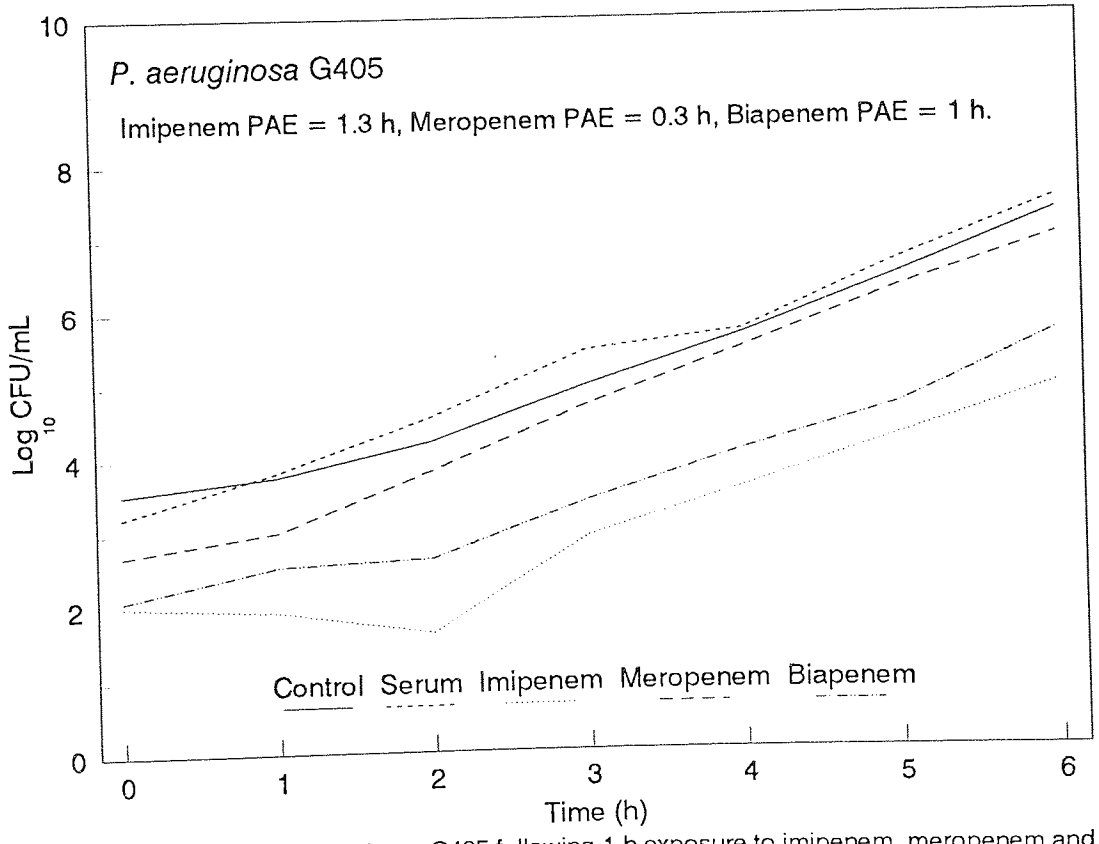
The imipenem, meropenem and biapenem (4 X MIC) PAEs in pooled human serum were evaluated on five strains each of *E. coli* and *P. aeruginosa*, to determine whether these carbapenems exhibit similar PAEs to each other and if their PAEs are similar in both genera. The observed imipenem, meropenem and biapenem PAEs on *E. coli* and *P. aeruginosa* demonstrate that the three carbapenems do not exhibit the same PAE as each other (Figure 19).

The mean imipenem, meropenem and biapenem PAEs (range) on the five *E. coli* strains studied were -0.3 (-0.9 to 0.2), -0.5 (-1.2 to 0.2) and -0.4 (-1.3 to 0.1) h, respectively. The mean imipenem, meropenem and biapenem PAEs (range) on the five *P. aeruginosa* strains studied were 1.1 (0.6 to 1.6), -0.1 (-0.9 to 0.4) and 0.7 (0.2 to 1) h, respectively. Imipenem produced a longer PAE than biapenem which in turn was longer than that of meropenem. All three carbapenems gave mean negative PAEs for *E. coli*. Amongst the five strains of each genera strain variation was observed. The mean EOS for the strains studied was 0.2 h.

Figure 19. Recovery following exposure to carbapenems of two genera of Gram-negative bacteria.




Growth curves of *E. coli* I425 following 1 h exposure to imipenem, meropenem and biapenem at concentrations of 4 X MIC. Carbapenems were removed by 1000-fold dilution.



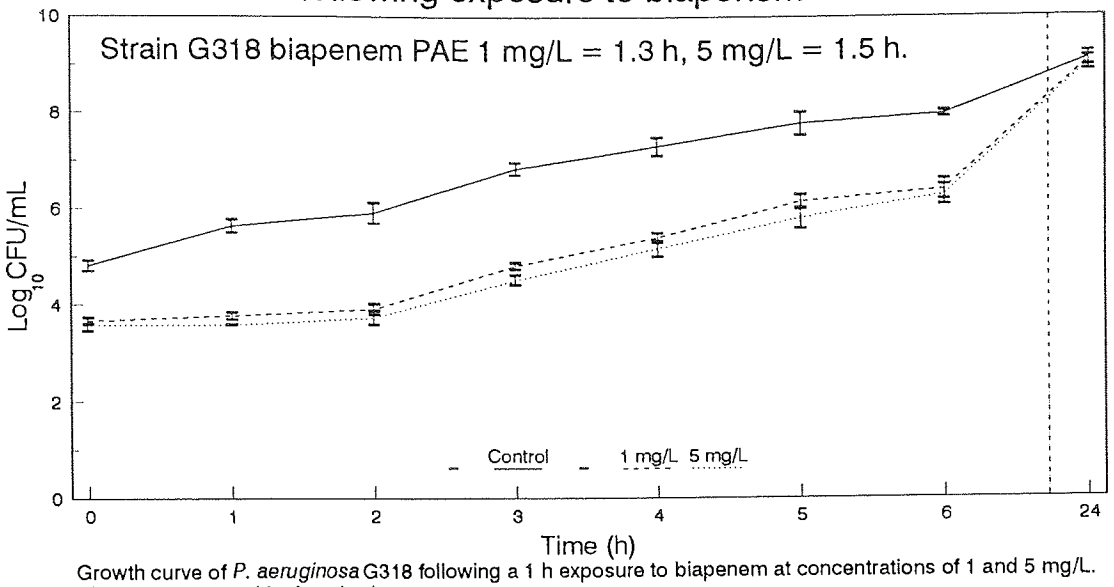

Growth curves of *P. aeruginosa* G405 following 1 h exposure to imipenem, meropenem and biapenem at concentrations of 4 X MIC. Carbapenems were removed by 1000-fold dilution.

### 3.7 Strain variation of biapenem PAE.

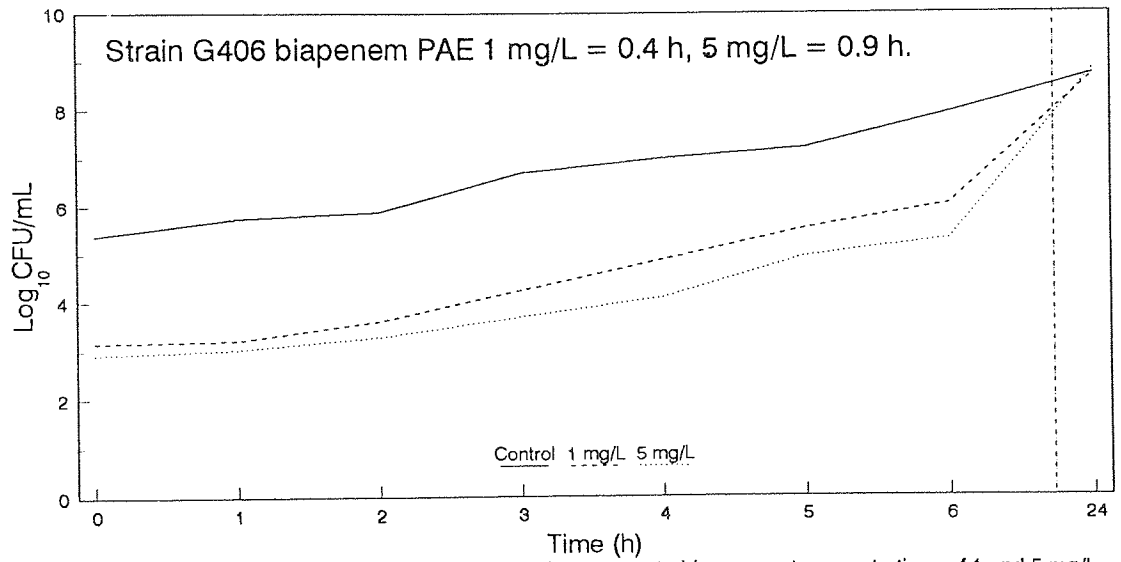
The biapenem PAEs in pooled human serum were evaluated on eleven clinical strains of *P. aeruginosa*, the mean PAEs (range) for the 1 and 5 mg/L exposures were 0.9 (0.4 to 1.4) and 1.4 (0.6 to 1.9) h, respectively (Table 24). As the wide ranges demonstrate, the biapenem PAE after exposure to 1 and 5 mg/L for 1 h for eleven strains of *P. aeruginosa* exhibited considerable strain variation. Plots of counts ($log_{10}$ CFU/mL) against time for strains G318, G406 and G388 are shown in Figure 20. The data and calculations of the viable counts of *P. aeruginosa* G318 are shown in Appendix 11.

The concentrations of biapenem investigated were equivalent to 2-8 X MIC (1 mg/L) and 10-40 X MIC (5 mg/L) depending on the microorganism. However, the duration of PAE did not correlate with MIC, that is, the microorganism with the highest MIC did not exhibit the shortest PAE, correlation coefficients ($r^2$) were 0.07 and 0.02 for 1 and 5 mg/L exposure concentrations, respectively. In addition, biapenem PAE did not correlate with initial kill (decrease in CFU/mL/h), correlation coefficients ($r^2$) were 0.41 and 0.69 for 1 and 5 mg/L exposure concentrations, respectively (Figure 21).

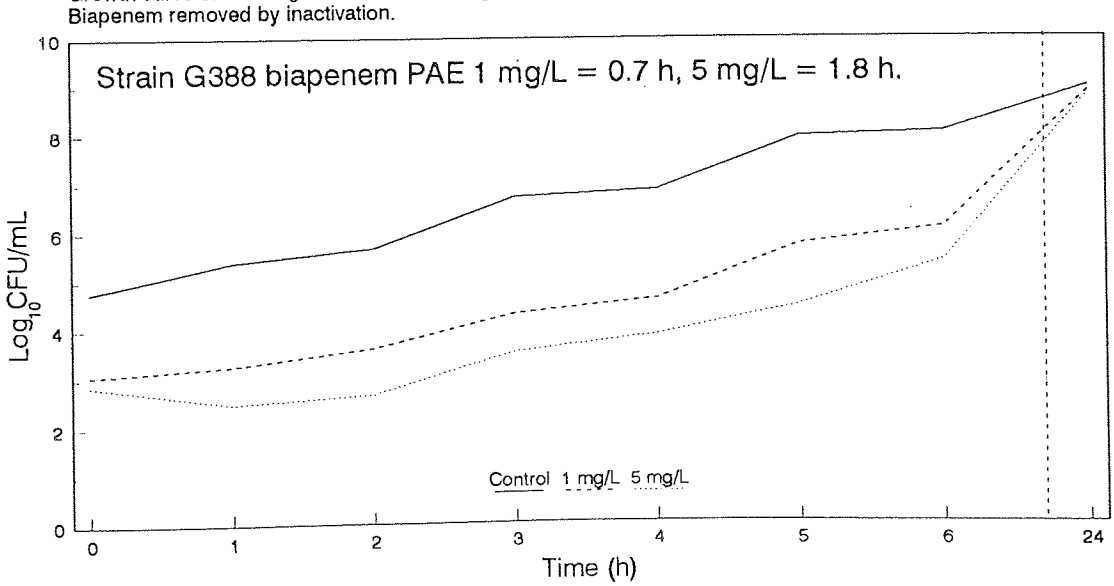
## Figure 20. Recovery of three strains of *P. aeruginosa* following exposure to biapenem




Growth curve of *P. aeruginosa* G318 following a 1 h exposure to biapenem at concentrations of 1 and 5 mg/L. Biapenem removed by inactivation.




Growth curve of *P. aeruginosa* G406 following a 1 h exposure to biapenem at concentrations of 1 and 5 mg/L. Biapenem removed by inactivation.

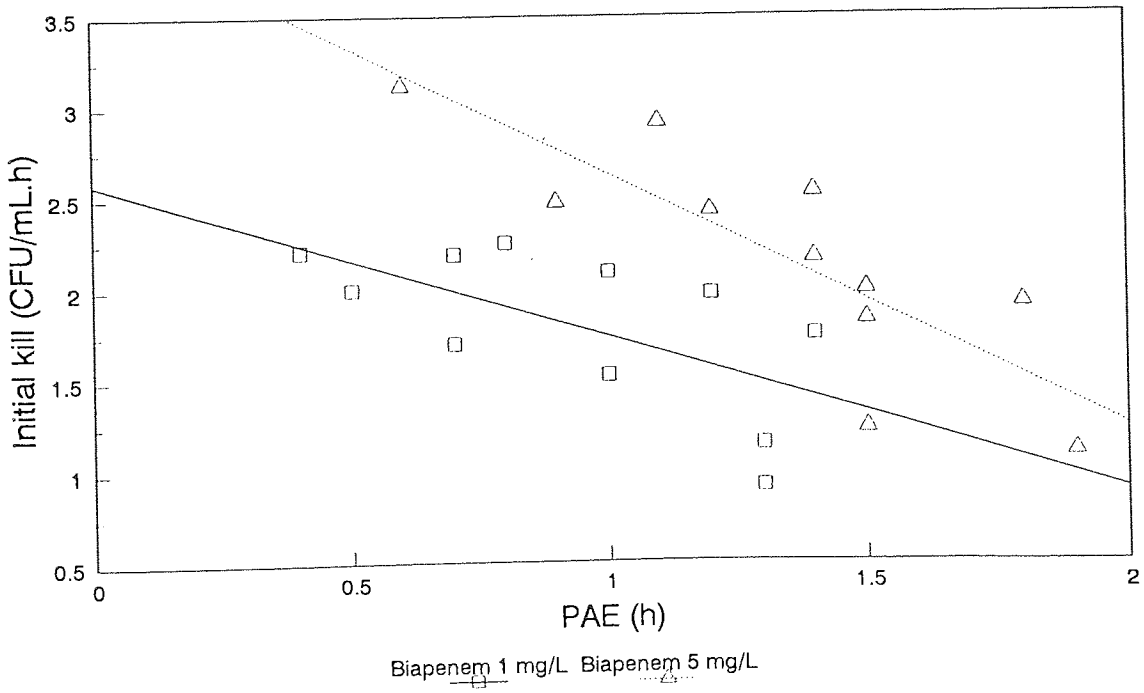



Growth curve of *P. aeruginosa* G388 following a 1 h exposure to biapenem at concentrations of 1 and 5 mg/L. Biapenem removed by inactivation.

**Table 24. The PAE of biapenem in pooled human serum on *P. aeruginosa* strains.**

| Microorganism | MIC (mg/L) | PAE (h) after exposure to 1 mg/L biapenem. | PAE (h) after exposure to 5 mg/L biapenem. |
|---|---|---|---|
| *P. aeruginosa* G414 | 0.25 | 1.0 | 1.1 |
| *P. aeruginosa* G407 | 0.12 | 0.7 | 0.6 |
| *P. aeruginosa* G377 | 0.25 | 1.2 | 1.4 |
| *P. aeruginosa* G406 | 0.25 | 0.4 | 0.9 |
| *P. aeruginosa* G379 | 0.25 | 0.5 | 1.5 |
| *P. aeruginosa* G404 | 0.25 | 1.4 | 1.5 |
| *P. aeruginosa* G409 | 0.25 | 1.0 | 1.2 |
| *P. aeruginosa* G388 | 0.25 | 0.7 | 1.8 |
| *P. aeruginosa* G354 | 0.12 | 0.8 | 1.4 |
| *P. aeruginosa* G318 | 0.5 | 1.3 | 1.5 |
| *P. aeruginosa* G412 | 0.12 | 1.3 | 1.9 |
| $PAE_{50}$ | | 1.0 | 1.4 |
| $PAE_{90}$ | | 1.3 | 1.8 |

Figure 21. Plot of biapenem initial kill and PAE.



### 3.8 Mechanism of PAE.

#### 3.8.1 PAE of three carbapenems on *P. aeruginosa* PAO1 in the presence of L-lysine.

The effect of the presence of different L-lysine monohydrochloride concentrations during exposure and recovery on carbapenem PAE on *P. aeruginosa* PAO1 was evaluated because basic amino acids can competitively inhibit carbapenem permeation through the D2 porin channel thus decreasing carbapenem susceptibility. Increasing the lysine concentration present during carbapenem (2 mg/L) exposure decreased the imipenem, meropenem and biapenem PAEs (Table 25). Increasing the concentration of L-lysine present during the recovery of the culture had no significant effect on the duration of the imipenem PAE, mean imipenem PAE (SD) 2.03 h (0.21) (meropenem and biapenem not studied) (Figure 22). Negative biapenem and meropenem PAEs were observed.

#### 3.8.2 Effect on carbapenem PAE of increasing salinity during recovery.

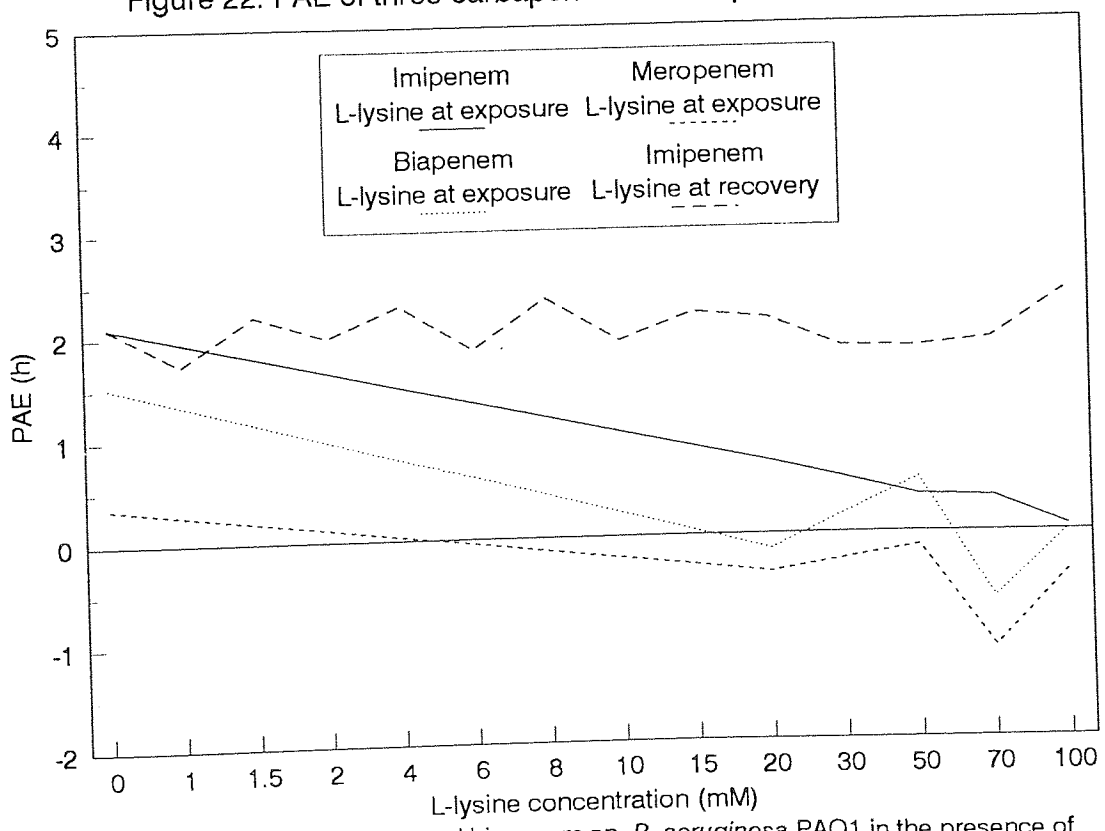
The mechanism behind PAE may be elucidated with information on the nature of the recovery process. The increase in salinity during recovery significantly increased the imipenem PAE, whereas the meropenem and biapenem PAEs remained constant, with means (SD) of 0.5 (0.06) and 2.5 (0.23), respectively (Table 26). Microorganisms in imipenem but not meropenem and biapenem PAE phase exhibit reduced salt tolerance, possibly due to sub-lethal damage of the compenents of the cell wall.

**Table 25. PAE (h) of three carbapenems in the presence of L-lysine.**

| | L-lysine concentration (mM) at exposure | | | | |
|---|---|---|---|---|---|
| | 0 | 20 | 50 | 70 | 100 |
| Imipenem 2 mg/L | 2.10 | 0.70 | 0.35 | 0.33 | 0.05 |
| Meropenem 2 mg/L | 0.35 | -0.37 | -0.14 | -1.13 | -0.39 |
| Biapenem 2 mg/L | 1.52 | -0.15 | 0.51 | -0.65 | 0.01 |

The carbapenem PAEs on *P aeruginosa* PAO1 with differing L-lysine concentrations during exposure of 1 h at concentrations of 2 mg/L in minimal media broth.

Figure 22. PAE of three carbapenems in the presence of L-lysine




PAE of imipenem, meropenem and biapenem on *P. aeruginosa* PAO1 in the presence of differing concentrations of L-lysine during exposure and recovery following a 1 h exposure to a concentration of 2 mg/L.

**Table 26. Effect on carbapenem PAE of increasing salinity during recovery.**

| | Salinity | | |
|---|---|---|---|
| | 0% | 1% | 3% |
| Imipenem PAE (h) | 2.5 | 3.6 | 4.1 |
| Meropenem PAE (h) | 0.5 | 0.6 | 0.5 |
| Biapenem PAE (h) | 2.2 | 2.6 | 2.6 |

The carbapenem PAEs (mean of duplicate determinations) on *P. aeruginosa* PAO1 following exposure of 1 h at concentrations of 4 X MIC with recovery in differing concentrations of sodium chloride in Iso-Senitest broth.

### 3.8.3 *P. aeruginosa* virulence factors during PAE phase.

Classical PAE duration is thought to be proportionally shorter than the actual recovery time, that is, the treated microorganisms may still be physiologically abnormal (Bergeron, 1992). Hence, the following virulence factors were semi-quantitatively evaluated during imipenem, meropenem and biapenem PAE 2.6, 1.2 and 2.6 h, respectively; elastase, lecithinase, DNase, gelatinase, lipase, haemolysins, motility and pigments. The virulence factors of control cultures of five different inocula were also examined so comparison with the lower inocula of test cultures could be performed. All zones around the peripheral area of growth were comparable with the controls of similar inocula and no visible apparent change in virulence factor production during carbapenem PAE phase was observed.

### 3.8.4 Spheroplast recovery in PAE phase using *N*-acetyl-D-glucosamine.

Carbapenem PAE was determined by viable counts on carbapenem-free Iso-Sensitest plates with and without 1 g/L *N*-acetyl-D-glucosamine in an attempt to culture any spheroplasts present. The presence of *N*-acetyl-D-glucosamine in the culture media had little effect on carbapenem PAE (viable counts on both media differed by no more than 0.35 $\log_{10}$ CFU/mL (Table 27)).

**Table 27. Effect on carbapenem PAE of using *N*-acetyl-D-glucosamine in recovery.**

| | Iso-Sensitest | Iso-Sensitest + 1 g/L *N*-acetyl-D-glucosamine |
|---|---|---|
| Imipenem PAE (h) | 2.25 | 2.00 |
| Meropenem PAE (h) | 0.85 | 0.80 |
| Biapenem PAE (h) | 2.25 | 1.80 |

The carbapenem PAEs (mean of duplicate determinations) on *P.aeruginosa* PAO1 following exposure for 1 h at concentrations of 4 X MIC in Iso-Sensitest broth determined by viable counts on Iso-Sensitest agar plates and Iso-Sensitest agar plates supplemented with 1 mg/L *N*-acetyl-D glucosamine.

### 3.8.5 Morphology during PAE phase.

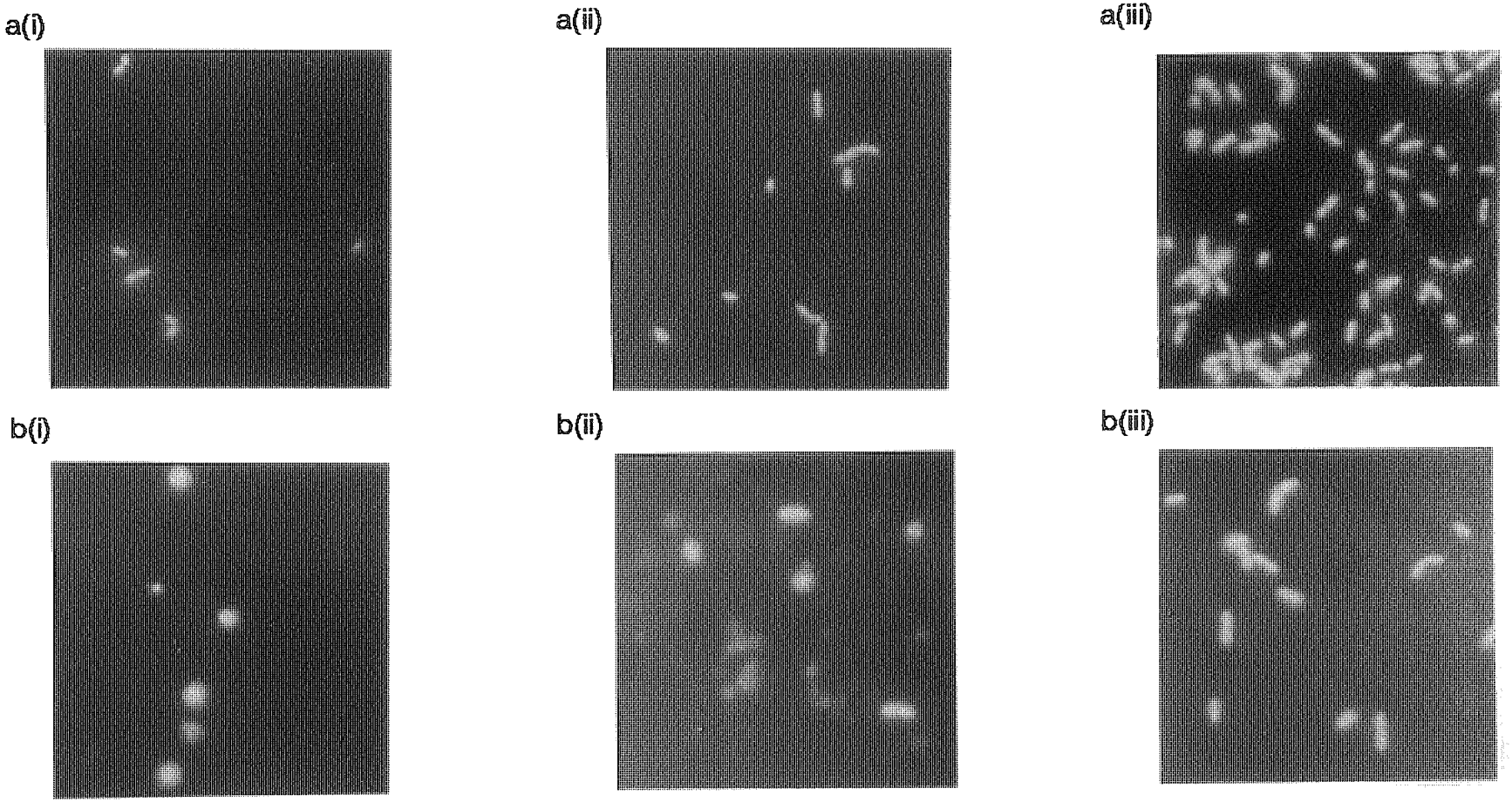
Epi-fluorescent microscopic examination of acridine-stained *P. aeruginosa* PAO1 in carbapenem PAE phase allowed cell morphology to be assessed (Table 28 and Figure 23). In the non-exposed control rod-shaped cells were present throughout. The cells in imipenem and biapenem PAE phase were spherical, whereas in meropenem PAE phase cells were filamentous in shape together with some bulged (central swelling) cells. Under epi-fluorescent microscopy cells exposed to imipenem, meropenem and biapenem exhibited altered morphology during the conventional PAE duration (determined by viable count) after which the cells became similar to those of the control.

The morphology of *P. aeruginosa* PAO1 during imipenem and meropenem PAE phase as determined by scanning electron microscopy demonstrated that imipenem and meropenem exhibited morphology alterations equivalent to the conventional PAE durations (as determined by viable count) of 2.4 and 1.2 h, respectively, after which the cells became similar to those of the control (Figure 24 and 25). During imipenem PAE phase spherical cells and short rods were present. Figure 24a demonstrates a spherical cell emerging from the middle of a rod. During meropenem PAE phase cells had swollen regions present in the middle of filaments at the point where the first division would normally have taken place (Figure 25a). These swollen regions may be due to the inhibition of peptidoglycan synthesis at the point where septation is initiated, thus leading to localised weakening of the cell wall.

**Table 28. Morphology of *P. aeruginosa* PAO1 during carbapenem PAE evaluation.**

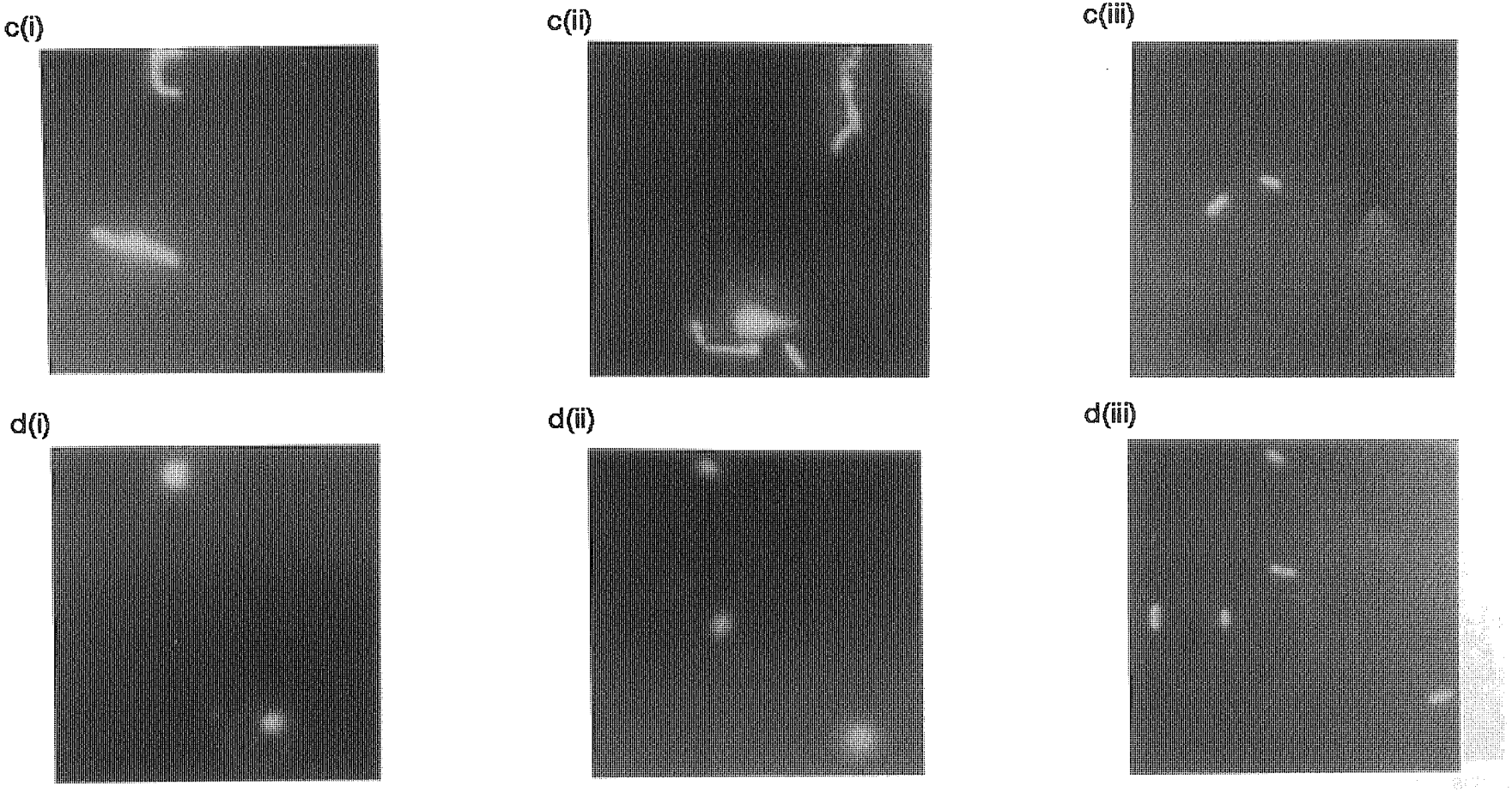
| Carbapenem (PAE h) | Time (h) | Predominant morphology (≥ 90%) |
|---|---|---|
| Imipenem (2.6) | -0.5 | Spherical cells |
| | 0 | Spherical cells |
| | 1 | Spherical cells |
| | 2 | Spherical cells |
| | 3 | Rods (few spherical cells) |
| | 4 | Rods |
| | 5 | Rods |
| | 6 | Rods |
| Meropenem (1.2) | -0.5 | Individual bulge cells |
| | 0 | Individual bulge cells and filaments |
| | 1 | Filaments |
| | 2 | Rods (few bulge cells) |
| | 3 | Rods |
| | 4 | Rods |
| | 5 | Rods |
| | 6 | Rods |
| Biapenem (2.6) | -0.5 | Spherical cells |
| | 0 | Spherical cells |
| | 1 | Spherical cells |
| | 2 | Spherical cells |
| | 3 | Rods |
| | 4 | Rods |
| | 5 | Rods |
| | 6 | Rods |

Figure 23. Epi-fluorescent morphology of *P. aeruginosa* PAO1 during carbapenem PAE phase.




Acridine orange stained *P. aeruginosa* PAO1 control (a) and following exposure to imipenem at concentration equivalent to 4 X MIC and subsequent removal by inactivation (b). At 0 h (i), 1 h (ii) and 3 h (iii) at 1250 X magnification.

Figure 23. Epi-fluorescent morphology of *P. aeruginosa* PAO1 during carbapenem PAE phase.

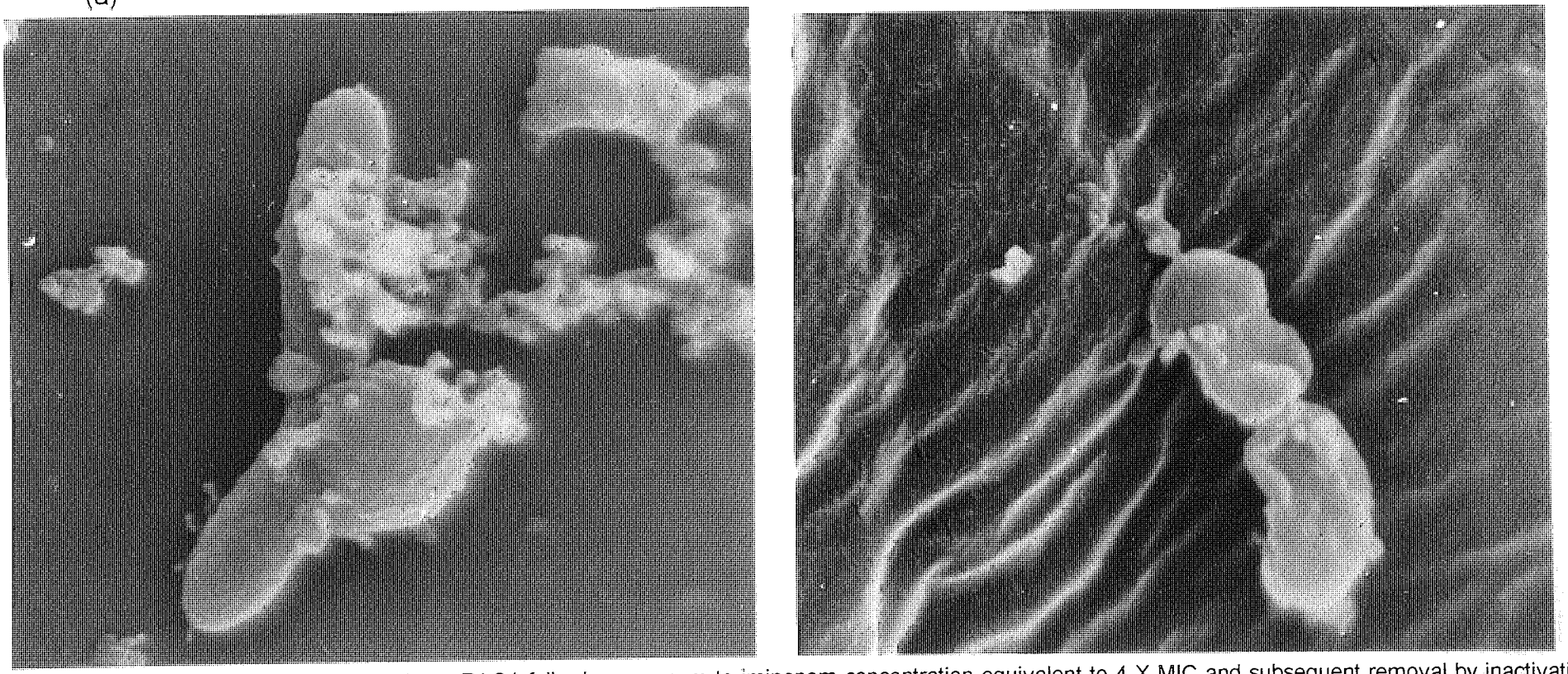



Acridine orange stained *P. aeruginosa* PAO1 following exposure to meropenem (c) and biapenem (d) at concentration equivalent to 4 X MIC and subsequent removal by inactivation. At 0 h (i), 1 h (ii) and 2 h (iii) for meropenem and 0 h (i), 1 h (ii) and 3 h (iii) for biapenem at 1250 X magnification.

**Figure 24. Morphology of *P. aeruginosa* PAO1 during imipenem PAE evaluation.**

(a)

(b)

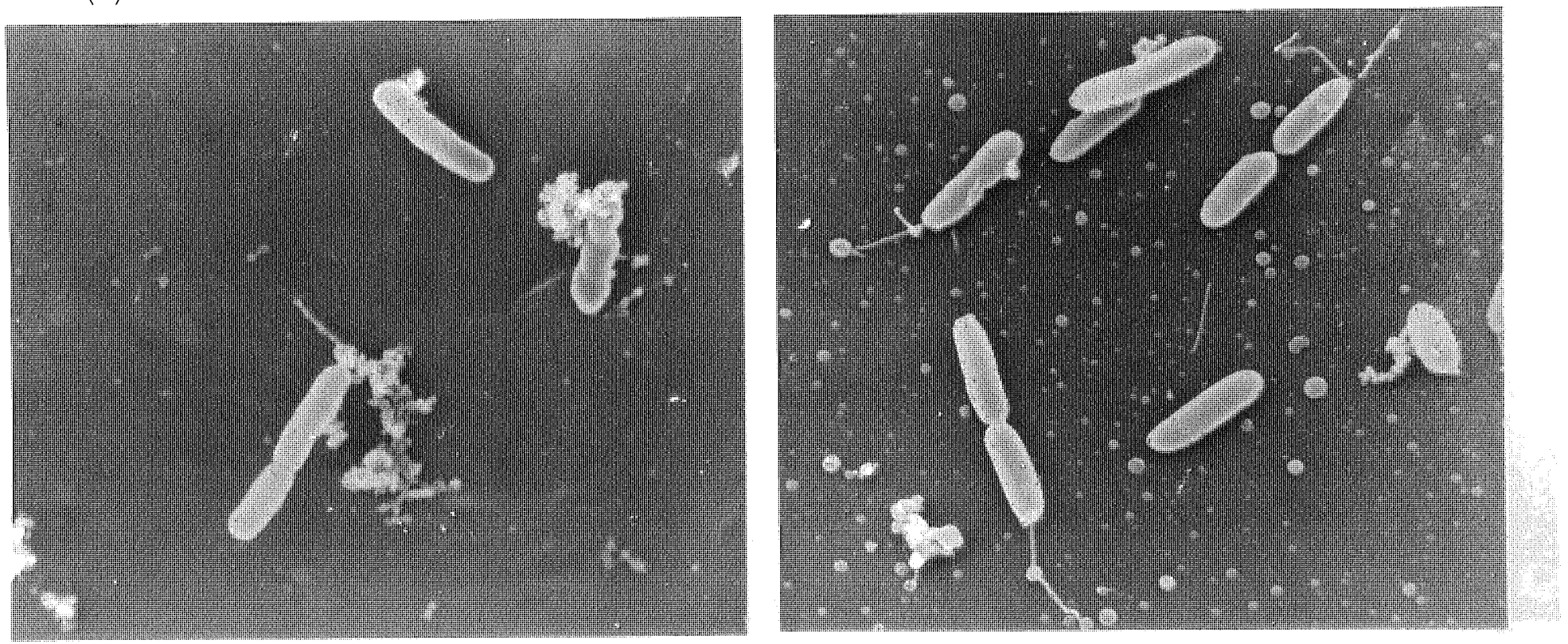


Electron micrographs of *P. aeruginosa* PAO1 following exposure to imipenem concentration equivalent to 4 X MIC and subsequent removal by inactivation with ß-lactamase. (a) 0 h, 44,800 X magnification, (b) 1 h, 44,800 X magnification,

**Figure 24. Morphology of *P. aeruginosa* PAO1 during imipenem PAE evaluation.**

( c)

(d)

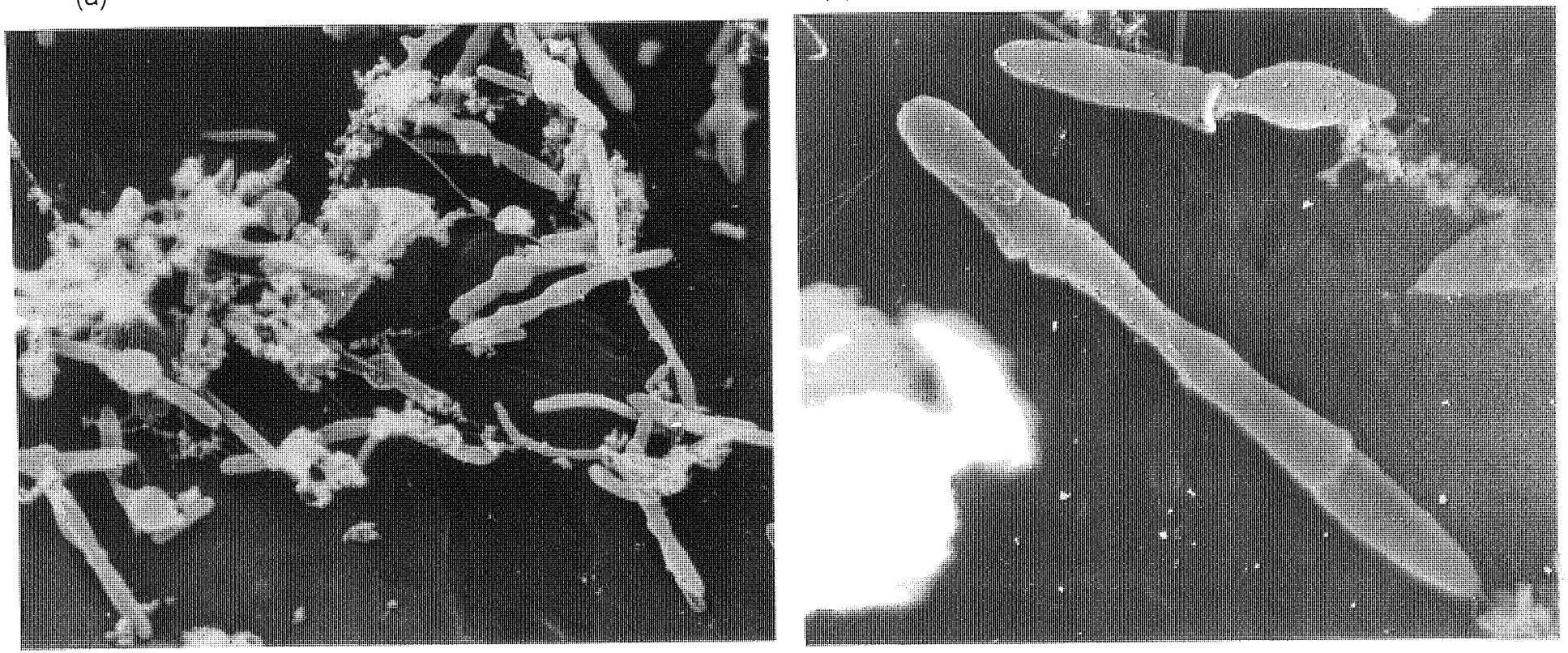


Electron micrographs of *P. aeruginosa* PAO1 following exposure to imipenem concentration equivalent to 4 X MIC and subsequent removal by inactivation with ß-lactamase. (c) 2 h, 17,900 X magnification and (d) unexposed control, 4 h, 13,400 X magnification.

**Figure 25. Morphology of *P. aeruginosa* PAO1 during meropenem PAE evaluation.**

(a)

(b)

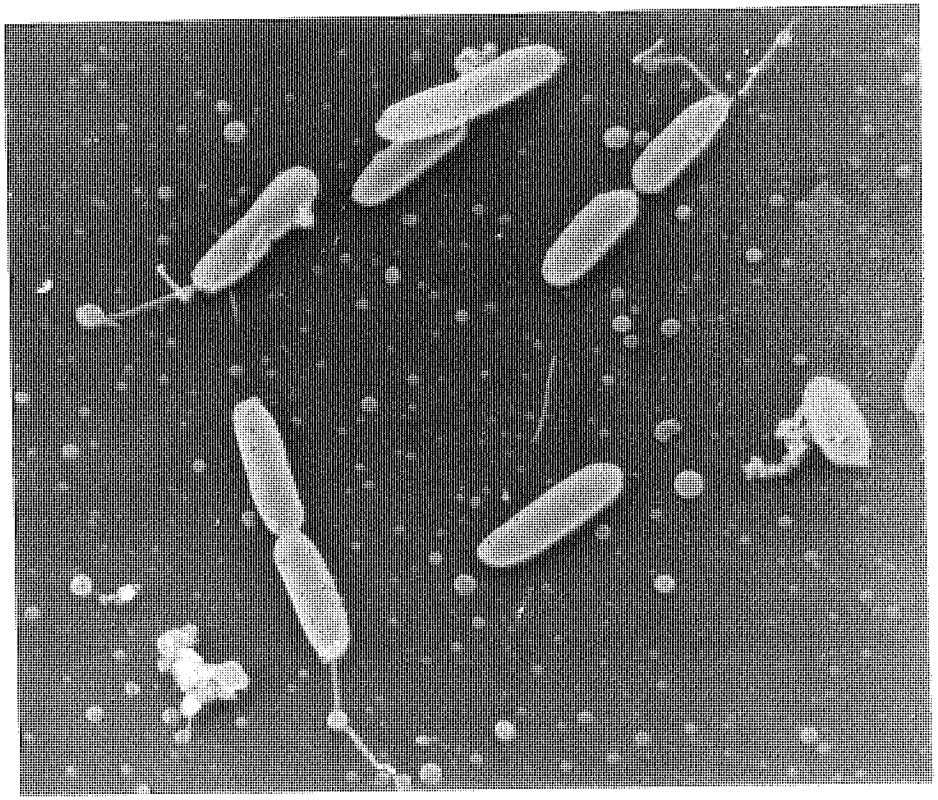


Electron micrographs of *P. aeruginosa* PAO1 following exposure to meropenem concentration equivalent to 4 X MIC and subsequent removal by inactivation with ß-lactamase. (a) 0 h, 6,700 X magnification, (b) 1 h, 17,900 X magnification,

**Figure 25. Morphology of *P. aeruginosa* PAO1 during meropenem PAE evaluation.**

( c)

(d)



Electron micrographs of *P. aeruginosa* PAO1 following exposure to meropenem concentration equivalent to 4 X MIC and subsequent removal by inactivation with ß-lactamase. (c) 2 h, 8,900 X magnification and (d) unexposed control, 4 h, 13,400 X magnification.

# 4. DISCUSSION

The conventional design of antimicrobial dosing regimens that maintain serum concentrations above the MIC/MBC of common microorganisms for most of the dosing interval, has been challenged despite having been used for over five decades (Ebert *et al.*, 1988; Vogelman *et al.*, 1988). Early clinical experience with penicillin G demonstrated that it was not necessary to maintain levels above the MIC *in vivo* to achieve efficacy (Tompsett *et al.*, 1949). However, most current regimens are empirical and have not been evaluated by classic dose response trials in humans due to ethical and economic difficulties (Kunin, 1981). Hence, in some instances agents whose serum half-lives differ by as much as 10-18 fold are given at the same dosage with the same dosage interval (Kunin, 1981).

It has been suggested that pharmacodynamic parameters, such as PAE and the extent and rate of bacterial killing, provide a more appropriate representation of the duration of antimicrobial activity than the MIC and should be used to design antimicrobial dosing regimens (Zhanel *et al.*, 1991a). Consequently, PAE quantification is currently recommended in the Infectious Diseases Society of America guidelines for the preclinical evaluation of new antimicrobial agents (Beam *et al.*, 1992). If optimal dosing intervals are to be predicted in individual patients the following information would be required: the pharmacokinetic behaviour of the antimicrobial; the duration of *in vitro* and *in vivo* PAE; the MIC and other factors that may affect efficacy, for example, protein binding and PALE (Turnidge, 1991).

Hence, optimal treatment of infections may require different dosage schedules depending on the microorganism, antimicrobial and the status of host defense system (Odenholt-Tornqvist *et al.*, 1991). However, if PAE is to be used for determining dosing regimen design several details must be addressed. Firstly, the measurement must be reliable, reproducible, consistent (unchanged throughout the course of therapy) and standardised. Secondly, the amount of inter-strain variation must be known, as it is unlikely that routine microbiology laboratories would undertake PAE measurement for individual patient isolates (Xue *et al.*, 1996).

Furthermore, PAE is a highly artificial phenomenon which may never exist alone *in vivo*, because a suprainhibitory concentration of antimicrobial will always be followed by a subinhibitory concentration. Thus, the logic of extrapolating data produced by non-kinetic *in vitro* models which apply rapid removal of antimicrobials to the *in vivo* situation is questionable. In addition, although PAE confers an advantage to the host, it is unlikely that it is an adequate explanation for successful discontinuous antimicrobial therapy as generally the sum of the time that the antimicrobial concentration is above the MIC and the PAE does not cover the whole dosing interval, and both host defences and sub-MIC concentrations will also contribute. In practice, it may be a matter of semantics whether a delayed recovery of surviving microorganisms is referred to as either PAE, sub-MIC effects, irreversible binding of the antimicrobial or the co-operative action of the host defences.

Therefore, it would be unwise to implement a dosing regimen based solely on PAE as the actual link between the demonstration of an *in vitro* PAE and the efficacies of more prolonged dosage intervals has not been proven (McGrath *et al.*, 1993).

Intermittent antimicrobial dosage schedules are potentially advantageous compared with continuous administration due to their improved penetration into tissues, convenience of administration, lower frequency of adverse reactions, reduced cost and better patient compliance.

With Gram-negative bacilli, the efficacy of aminoglycosides and fluoroquinolones in animal infection models has demonstrated little dependence on the dosing interval (Craig and Gudmundsson, 1996). The total dose or AUC is the major pharmacokinetic parameter correlating with efficacy of these antimicrobials. In fact, once-daily dosing of aminoglycosides has demonstrated similar or greater efficacy and lower frequency of toxicity than more frequent dosing regimens in some *in vivo* models. Thus, partly on the basis of PAE, once-daily dosing of aminoglycosides was introduced into clinical practice (den Hollander *et al.*, 1996a). However, den Hollander and coworkers (1996a) examined the tobramycin PAE on *P. aeruginosa* using an *in vitro* pharmacokinetic model and found the PAE to be zero at 12 h. Therefore, the emphasis placed on PAE in discussions of once-daily aminoglycoside therapy was questioned.

Imipenem and meropenem are usually administered three or four times daily, but due to their PAEs and concentration dependent bactericidal activity, it may be feasible to use less frequent dosing. In animal models of imipenem, meropenem and biapenem, Walker and coworkers (1994) demonstrated that the time for which serum levels exceed the MIC was the major pharmacodynamic/pharmacokinetic parameter correlating with *in vivo* efficacy. Furthermore, maximum *in vivo* carbapenem efficacy was observed with time above MIC values of 30-40% of the dosing interval (Walker *et al.*, 1994). The free imipenem and meropenem serum concentration exceeded the $MIC_{90}$ of *E. coli* and *P. aeruginosa* after a 1 g dose for 10.1 h and 11.5 and 4.5 and 4.6 h, respectively (Drusano, 1988, Nyhlen *et al.*, 1995). Thus the strain dependent carbapenem PAE will give a margin of safety in dosing schedules but treatment of serious infection should ensure bactericidal levels are maintained at the site of infection for the whole dosing interval (Drusano, 1991). Once-daily dosing of meropenem was employed by MacGowan and coworkers (1996) to successfully treat a chronic *P. aeruginosa* pulmonary infection. However, whether the presence of a PAE will ultimately allow other antimicrobials to be dosed less frequently in the future can only be demonstrated by further human clinical trials.

The MIC data presented here, demonstrated that meropenem had the greatest *in vitro* activity followed by biapenem and then imipenem against *E. coli*. For *P. aeruginosa* meropenem and biapenem had equal $MIC_{90}$s which were four times less than that of imipenem. The MICs were all below or equal to the Working Party of the British Society of Antimicrobial Chemotherapy (1991) susceptibility

breakpoint of 4 mg/L for both imipenem and meropenem. All three carbapenems had greater activity against *E. coli* than *P. aeruginosa*. Similar results have been reported, for example, Livermore and Williams (1996) reported meropenem to be four to eight fold more active than imipenem, and Bush (1997) reported the *in vitro* activity of biapenem to be equivalent or slightly more active than imipenem. Catchpole and coworkers (1992) observed that meropenem and biapenem exhibit similar activity on *P. aeruginosa*. Imipenem and meropenem exhibited bactericidal activity at concentrations equivalent or within one dilutional step of their MICs. The *in vitro* activity of both imipenem and meropenem is usually unaffected by an increase in inoculum size up to $10^7$ CFU/mL (Bush, 1997) therefore, the lack of inoculum effect observed was expected. In complex media, such as, Iso-Sensitest, basic amino acids competitively inhibit carbapenem permeation through the D2 porin channel (Trias and Nikaido, 1990a). Therefore, in media with a low amino acid content such as, minimal media broth carbapenem susceptibility increases (Fukuoka *et al.*, 1991). *P. aeruginosa* PAO1 demonstrated an increase in imipenem susceptibility in minimal media broth.

In general, antimicrobial agents are evaluated against microorganisms in logarithmic phase of growth in order to obtain the maximum antimicrobial effect. Logarithmic phase cultures in either Iso-Sensitest broth or minimal media broth were used throughout (except Section 2.9.6). Due to its reduced nutritional value the growth rate of *P. aeruginosa* in minimal media broth was reduced compared with the growth rate in Iso-Sensitest broth.

The evaluation of the effect of exposure to pooled human serum on the growth of twenty two clinical strains of *P. aeruginosa* demonstrated considerable inter-strain variation. Those strains least affected by the inhibitory nature of serum were studied in PAE investigations where serum was used.

The time-kill kinetics of imipenem, meropenem and biapenem on *P. aeruginosa* PAO1 demonstrated a 3 $\log_{10}$ decrease in viable count at 2.9, 4.2 and 4 h, respectively. These results are similar to the carbapenem time-kill kinetics observed by Ubukata (1990), Neu (1989) and White (1996) and their coworkers.

After preliminary experiments into the carbapenem *in vitro* activity, microorganism growth rates and the effect of serum on growth further investigations were performed towards standardising a method for carbapenem PAE determination.

## 4.1 *In vitro* PAE method development.

Both the presence and duration of PAE can be affected by many factors associated with the microorganism, antimicrobial agent, method of antimicrobial removal and other experimental conditions. Many of these parameters were evaluated to assist in the development of a standardised method to study the carbapenem PAE on the Gram-negative microorganisms, *E. coli* and *P. aeruginosa*.

Three different methods of carbapenem removal were employed, inactivation by ß-lactamase, repeated washing and dilution (which was predominantly used). The direct comparison of these three methods of removal demonstrated the significant imipenem PAEs on *P. aeruginosa* PAO1 differed only by 5%. This was much less than the 30% variability found by Bundtzen and coworkers (1981) for penicillin and ampicillin PAE. Adequate removal was achieved by inactivation with ß-lactamase (of at least 1000 mg/L of imipenem) (Odenholt *et al.*, 1989a), washing twice with complete removal of the supernatant, which reduced the antimicrobial concentration by ten-thousand fold (Craig and Gudmundsson, 1996), or by a one-thousand fold dilution, which was sufficient for concentrations greater than the MIC (Craig and Gudmundsson, 1996). Additionally, any subinhibitory carryover effect was reduced further by the serial dilutions made prior to viable counting. Furthermore, employing the spiral plater aided carryover detection, in that, if a subinhibitory concentration was present, as it decreased towards the edge of the spiral, an increase in growth would be observed.

Both dilution and washing are universal methods of antimicrobial removal, as they can be used for any class of antimicrobial, whereas, inactivation is class limited. Residual antimicrobial controls can be more easily calculated and performed for dilution than for the other two methods. Of the methods evaluated here, inactivation appears to have the least traumatic effect on growth, whereas, washing and dilution may lyse cells as well as cause a lag in growth. However, dilution is not only universal, but is the most rapid method and is the most analogous to *in vivo* conditions.

MacKenzie and Gould (1993) stated that viable counts are erroneous due to the use of inappropriate diluents, such as phosphate buffered saline or water, in which microorganisms with weakened cell walls may lyse by osmotic shock and hence it is essential to employ optimum techniques for the recovery of antimicrobially exposed microorganisms. The use of different viable count diluents; distilled water, 0.9% sodium chloride and PBS had no effect on the carbapenem PAE, therefore, PBS was subsequently used throughout.

It is important to examine the concentration dependence of pharmacodynamic parameters, such as PAE, since the correlation between the PAE and MIC between species has been shown to differ (Isaksson *et al.*, 1991b). The amikacin PAEs on *P. aeruginosa* and *S. marcescens* strains were much longer at low multiples of MIC than PAE on *E. coli* (Isaksson *et al.*, 1991b). In addition, the exposure duration and concentration required to induce a quinolone PAE in *E. faecalis* was twice that required in *E. coli*, *P. aeruginosa* and *S. aureus* (Sandford

and Jones, 1993). MacKenzie and Gould (1993) suggested that the exposure concentration should be standardised to 0.1, 1, 10 and 100 X MIC. Although, to mimic *in vivo* conditions the exposure concentration should be pharmacologically achievable. It has been recommended that antimicrobials are administered in doses that achieve a peak serum concentration that exceeds the MIC for the pathogen by a factor of four to ten (inhibitory quotient) (Ellner and Neu, 1981), therefore, perhaps these concentrations should be used to evaluate PAE.

The increase in PAE with increasing concentration was first observed by Eagle (1949) and has since been observed by most investigators. The results from both direct investigations into the effect of exposure concentration demonstrated that the imipenem and meropenem PAEs on *P. aeruginosa* PAO1 in Iso-Sensitest broth were concentration dependent. This was also observed with three carbapenems during the evaluations of pooled human serum effect and carbapenem PAE comparison, and during strain variation investigations using biapenem.

Generally, PAEs have been observed at concentrations equivalent to and above the MIC for most antimicrobials and at sub-MIC concentrations for aminoglycosides (Isaksson *et al.*, 1988). Imipenem and meropenem were found to produce significant PAEs on *P. aeruginosa* PAO1 at concentrations of $\geq$0.5 X MIC and $\geq$1 X MIC, respectively. Although these concentrations were not equipotent (bioequivalent) they were equimolar. The variation in PAE of different carbapenem concentrations observed here may demonstrate differences between

the affinities of the three carbapenems for non-essential PBPs. In the case of meropenem, it is possible that it binds to non-essential PBPs prior to essential PBPs, thus, a higher concentration of meropenem is required to cause PAE. *In vivo*, the effects of sub-MIC concentrations achieved between doses are important factors for preventing the regrowth of the infecting microorganism. Consequently, if a PAE is produced at low concentrations it may be a significant factor in preventing regrowth.

Imipenem and meropenem both achieved maximal PAEs on *P. aeruginosa* PAO1 at concentrations of 4 and 2 X MIC, respectively. This could relate to the fact that the rate and extent of killing of ß-lactams is constant when the concentration is about 4 X MIC (Nicolau, 1997). Maximal PAEs have been observed with other antimicrobials at concentrations of 10 X MIC (Vogelman and Craig, 1985). However, Trias and Nikaido (1990) observed maximal imipenem and meropenem PAEs on *E. coli* and *P. aeruginosa* at concentrations of 4 X MIC except imipenem on *E. coli* where a maximal PAE was observed at concentration of 64 X MIC. During the evaluation of the effect of pooled human serum, clinical strain *P. aeruginosa* G341 demonstrated maximal carbapenem PAEs at higher concentrations than did PAO1. The concentrations of imipenem (16 mg/L) and meropenem (4 mg/L) that gave maximal PAEs on *P. aeruginosa* PAO1 are considerably less than the $C_{max}$ values of 69.9 mg/L and 61.6 mg/L, respectively, observed after IV infusion (Meropenem Technical Monograph, 1997).

Many investigators have demonstrated that increasing duration of exposure coincided with an increase in the duration of PAE, with maximal PAEs usually being achieved with an exposure of 2 h (Vogelman and Craig, 1985). Results from increasing the duration of carbapenem exposure from 1 to 2 h demonstrated the anticipated increase in PAE. Correlation between PAE and AUC (mg/L.h) has been observed, which demonstrates the effect of concentration at exposure and duration of exposure combined (Bundtzen *et al.*, 1981).

The three carbapenem PAEs were found to be inoculum dependent, that is, PAE decreased as *P. aeruginosa* inoculum increased which corresponded to a decrease in bactericidal effect. Odenholt and coworkers (1989a) also found imipenem PAE to be inoculum dependent and did not observe a PAE at $10^8$ CFU/mL with *P. aeruginosa*. Various explanations have been proposed for the inoculum effect for instance, a higher quantity of ß-lactamase in high inoculum cultures or the amount of crucial PBPs, for example, Gram-negative bacilli in slow growth phase have much lower levels of PBP 3 (Gould and MacKenzie, 1997). A microorganism inocula of $10^{6-9}$ CFU/mL has been observed in endocarditis caused by *P. aeruginosa* (Korvick and Yu, 1991) and in abscesses $10^{7-8}$ CFU (Faro, 1989) have been recorded, however, a therapeutically 'correct' inocula has yet to be established.

The rate of growth of the microorganism is one of the many parameters of PAE methodology that requires standardising. Generally, antimicrobial agents are evaluated against microorganisms in logarithmic phase of growth in order to

obtain the maximum antimicrobial effect, although, antimicrobial growth *in vivo* may be less than optimal (Ashby, 1993). A slow growth rate and restricted availability of iron and possibly other nutrients appear to be a characteristic of many infections *in vivo* (Cozens *et al.*, 1986). As a general rule antimicrobial agents kill bacteria at a rate which is strictly proportional to the rate of bacterial growth such that a constant proportion is killed per generation (Cozens *et al.*, 1989). However, an ideal antimicrobial agent would kill bacteria independently of the rate of growth and thus retain a high bactericidal activity against both slow growing and non growing bacteria. The carbapenems have been shown to have activity against non growing microorganisms (Cars and Odenholt-Tornqvist, 1993). However, the imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1 in stationary (longer generation time) growth phase demonstrated a negative PAEs on *P. aeruginosa* PAO1. Although, initial kill was similar therefore, whilst activity was retained PAEs were not induced.

Fuursted and coworkers (1995) analysed the PAE of penicillin, erythromycin and gentamicin on *S. aureus* and ampicillin, ciprofloxacin and gentamicin on *E. coli* recovering at different temperatures representing different generation times (0.3 to 4 h). In general, they observed that the duration of PAE directly correlated with generation time, that is, PAE increased as generation time increased, apart from *E. coli* and ampicillin where no PAE was observed. However, Svensson and coworkers (1993) noted a reduced imipenem PAE with non growing *P. aeruginosa* and Wu and Livermore (1990) reported that in a chemostat continuous culture system, imipenem exerted a PAE on *P. aeruginosa* but

meropenem did not. Expression of PBPs is highly growth rate dependent, it has been noted that as generation time increases *E. coli* PBP 1a/b and 4 decreased (Tuomanen *et al.*, 1986). However, carbapenems are thought to be most active of all ß-lactams against slow growing or stationary phase Gram-negative bacilli because they have higher affinities for PBP 7 which at high inocula is expressed in greater amounts and takes on a more crucial role in maintaining cell integrity (Gould and MacKenzie, 1997).

Although continuous shaking of cultures facilitates sufficient antimicrobial contact (National Committee for Clinical Laboratory Standards, 1992), it also could cause lysis of fragile microorganisms. Only a few investigators have stated whether cultures were shaken or stationary during antimicrobial exposure. Culture agitation during exposure had no effect on carbapenem PAE. In concordance, Wilson and Rolinson (1979) found no significant difference in the penicillin PAE on *S. aureus* with shaking, however, Lee and coworkers (1982) found that the ampicillin PAE on *E. coli* decreased with shaking.

It is important to note that the results of any *in vitro* study will not accurately reflect those which would be observed under *in vivo* conditions. Due to the effect of several factors at infected tissue sites on the interaction between microorganisms and antimicrobial agents. For example, antimicrobials which exhibit high protein binding may have significantly less antimicrobial action (than predicted from *in vitro* susceptibility results) in human serum, where the amount of unbound active antimicrobial is only a small fraction of the total antimicrobial concentration (Bush

*et al.*, 1989). Thus, if the presence of a PAE is to be used in the design of dosing regimens, the conditions for *in vitro* PAE determination must attempt to resemble those *in vivo*. The use of bodily fluids in *in vitro* studies allows their biological significance to be assessed. Therefore, the effect of pooled human serum on the carbapenem PAE of both *E. coli* and *P. aeruginosa* was evaluated. These studies demonstrated that heat-inactivated pooled human serum enhanced the imipenem PAE but not the meropenem and biapenem PAEs on *P. aeruginosa*. This increase in imipenem PAE may be due to some heat stable protein component present in the serum interacting with the imipenem to increase PAE.

Both Hessen (1988) and Manek (1986) and their coworkers observed that the imipenem PAEs on *P. aeruginosa* were less in broth than serum thus confirming the results presented here. The effect of serum on PAE appears to be dependent on the antimicrobial evaluated, for example, Bundtzen and coworkers (1981) found the rifampin and tetracycline PAEs to be equivalent in both broth and serum. Whereas, Davidson (1991a) and Boswell (1994) and their coworkers noted that serum increased the fluoroquinolone and streptogramin PAEs, respectively.

If the growth of the RAC is identical to that of the non-exposed control culture, then any PAE observed is not attributed to any persisting extracellular subinhibitory antimicrobial concentration. However, RACs are not ideal controls as the effect of the residual antimicrobial concentration on previously antimicrobial exposed microorganisms is not determined. Odenholt and

coworkers (1989) observed an increase in susceptibility of microorganisms in PAE phase to a rechallenge with sub-MIC concentrations of antimicrobial. Subsequently, Winstanley and coworkers (1991) exposed microorganisms in teicoplanin PAE phase to a previously used residual antimicrobial concentration (Cooper *et al.*, 1990), and demonstrated that the non-PAE phase control grew identically to the non-antimicrobial exposed control. However, the PAE determined with antimicrobial exposed microorganisms recovered in the residual antimicrobial concentration was about five-fold greater than the PAE determined with recovery in antimicrobial-free media.

If appropriate antimicrobial dosing regimens are to be designed on the basis of *in vitro* PAE data, the reproducibility of the PAE for various antimicrobial-microorganism combinations must first be evaluated. The accuracy with which PAE can be determined is influenced by a number of factors, these include, errors connected with the laboratory procedure itself and those which can occur during the calculation of the actual values from original data (MacKenzie and Gould, 1993). Errors are inherent in all methods, for example, not all bacterial activities used to follow bacterial growth kinetics will be suppressed for equal durations (Craig *et al.*, 1991).

The majority of investigators use viable counts, which are laborious and usually only one or two experiments are performed for each antimicrobial-microorganism combination. Errors which are a consequence of counting small numbers, in accordance with a Poisson distribution, may also be introduced. For example, if *n*

colonies are being counted the SD is $n^{1/2}$ and the relative error is therefore $n^{1/2}/n$. Generally the SD of viable counts is such that there may be a discrepancy in a single count by as much as $\pm$ 0.5 $\log_{10}$ (MacKenzie and Gould, 1993).

To date, only Zhanel and coworkers (1990) have extensively studied the reproducibility of fluoroquinolone PAEs. The evaluation of the reproducibility of carbapenem PAE on *P. aeruginosa* PAO1 demonstrated intra run and inter run standard deviation ranges of 0.15-0.28 and 0.22-0.36 h, respectively. Both Kuenzi in a smaller study (1987) and Zhanel (1990) and their coworkers found reproducibility varied with the concentration studied, such that, as concentration increased the standard deviation decreased. Zhanel (1990) and Kuenzi (1987) and their coworkers found variations of no more than 15% and at concentrations of 4-10 X MIC and up to 50% at concentrations of 1 X MIC. At 16 X MIC the carbapenem intra and inter run coefficients of variance were 21 and 28%, respectively. Fuursted (1997a) also observed a coefficient of variance of 20% for most assay conditions.

However, diversity in the reproducibility has been observed, for example, using high meropenem concentrations Bowker and coworkers (1996) found the PAE of *P. aeruginosa* to be 0.72 h and the standard deviation to be 0.77. In addition, imipenem *in vivo* PAE ranged from 1.4-4.6 h between experiments (Gudmundsson *et al.*, 1986). Van der Auwera and coworkers (1991) stated that reproducibility was strain dependent and good if $\leq$0.5 h between duplicates, moderate if 0.5 to 1 h between duplicates and poor if $\geq$1 h between duplicates.

These results are in concordance with Van der Auwera and coworkers (1991) in that the reproducibility for *P. aeruginosa* carbapenem PAE is moderate (0.4-0.8 h).

There has been concern particularly with bactericidal antimicrobials that PAE is due to the selection of a slow-growing resistant subpopulation of microorganisms in the exposed cultures (Craig and Gudmundsson, 1996). There are several reasons why this population shift is an unlikely explanation for PAE. Firstly, the growth curves of the exposed and control cultures are in parallel in post PAE phase, for example, strain G318 (Figure 20). Secondly, no correlation is observed between the extent of bactericidal activity and the duration of PAE (Figure 16c), correlation coefficients ($r^2$) were 0.6 and 0.14 for imipenem and meropenem, respectively. In accordance, Erlendsdottir and Gudmundsson (1988) observed poor correlation ($r^2$ of 0.2) with bactericidal rate and imipenem PAE on *P. aeruginosa*. Furthermore, Gudmundsson and coworkers (1991a) found poor or no correlation ($r^2$) between duration of penicillin, dicloxacillin, gentamicin, rifampin and ciprofloxacin PAEs and bactericidal rates for *E. coli* (0.439), *S. aureus* (0.004), *K. pneumoniae* (0.069) and *P. aeruginosa* (0.015). However, a relationship has been observed between fluoroquinolone bactericidal activity and PAE on *S. aureus* and *E. coli* (Minguez *et al.*, 1991). Furthermore, Munckhof and coworkers (1997) concluded that the AUC was the best predictor of imipenem PAE on *E. coli*. This is unexpected as the time above the MIC is the major pharmacodynamic/pharmacokinetic predictor of efficacy for ß-lactams (Leggett *et al.*, 1989). Thirdly, the ultrastructural alterations that occur in bacteria during the

PAE phase return to normal prior to regrowth (Gottfredsson *et al.*, 1989a). In addition, DNA-synthesis measured by $^{3}$H-thymidine uptake is decreased during the vancomycin PAE and recovers about 20-40 minutes prior to regrowth (Craig and Gudmundsson, 1991).

Susceptibility tests performed prior to and after PAE determination verified that the carbapenem PAEs observed here were not due to the selection of a resistant slow-growing subpopulation of *P. aeruginosa*.

Only a few studies have investigated the important effect on PAE of antimicrobial reexposure. The imipenem, meropenem and biapenem PAEs on *P. aeruginosa* remained constant after three consecutive exposures. These results are in concordance with those previously reported, such that, the imipenem PAE on *P. aeruginosa* and *E. coli* remained constant after three consecutive exposures (McGrath *et al.*, 1993) and even after six successive exposures (Majcherczyk *et al.*, 1994).

Both the presence and duration of PAE are dependent on the specific microorganism-antimicrobial combinations evaluated. The carbapenems and penems are the only members of the ß-lactam group which produce significant PAEs with Gram-negative microorganisms, but their PAEs are not consistent. Comparison of the three carbapenems demonstrated that they did not produce similar PAEs to each other and that they also exhibited genera variations. The imipenem PAE observed was longer than that observed with biapenem and

considerably longer than that of meropenem for *P. aeruginosa*. Nadler and coworkers (1989) observed that meropenem exhibited similar PAEs on both *E. coli* and *P. aeruginosa*, and imipenem only exhibited a PAE similar to meropenem but only with *P. aeruginosa*. Totsuka and coworkers (1996) also found the imipenem PAE to be less with *E. coli* than with *P. aeruginosa*. Furthermore, Odenholt-Tornqvist (1993) observed similarities with the data in this thesis, such that, negative or very low meropenem PAEs for both *E. coli* and *P. aeruginosa* were demonstrated and Odenholt and coworkers (1996) observed a lack of PAE with *E. coli* and the novel carbapenem L 749-345. MacKenzie and coworkers (1994a) also found carbapenem PAE to be variable and method dependent. They observed negative and very low meropenem PAEs with *E. coli* by viable count methodology, but, they observed significant positive PAEs using either morphology, bioluminescence or impedance and bioluminescence combination methods. The three carbapenems gave mean negative PAEs for *E. coli*, which may have been due to an apparent increase in CFU/mL due to the separation of antimicrobial induced filaments into individual cells. Filamentation is due to the inhibition of the synthesis of division proteins (PBP 3) which ultimately prevents separation into normal sized bacilli (Lorian *et al.*, 1979). Alternatively, the negative PAE values observed may be due to an overestimation of the initial decrease in CFU/mL by viable count methods, due to microorganisms having difficulty forming colonies on agar plates which directly causes an underestimation of the PAE. Hence, interpretation of negative PAEs is very difficult.

Pharmacodynamic studies have demonstrated that optimal dosing regimens will not only vary between different classes of antimicrobials but also between different bacterial species however, few studies have compared the pharmacodynamics of native strains with those of resistant. Odenholt and coworkers (1997) observed that penicillin resistant strains of *S. pneumoniae* exhibited a reduce penicillin PAE compared to sensitive strains at equipotent concentrations. Whereas, D2 mutants of *P. aeruginosa* exhibited imipenem and biapenem PAEs similar to their parent strains (Watanabe *et al.*, 1992). In addition, Slavin and Tureen (1997) reported that mucoid *P. aeruginosa* strains had shorter tobramycin PAEs than non-mucoid strains.

The degree of inter-strain variation significantly increases the difficulty of using PAE in the design of optimal dosing regimens. It is essential to characterise the microbial genera that consistently exhibit a PAE to confirm the clinical relevance of PAE. It may be practical to consider inter-strain variation by using the population statistics of $PAE_{50}$ and $PAE_{90}$, that is, the PAE for 50% and 90% of the strains tested, respectively (Xue *et al.*, 1996). Considerable inter-strain variation was observed with the biapenem PAE on eleven strains of *P. aeruginosa*, but they all demonstrated significant PAEs, with a $PAE_{90}$ value of 1.8 h after exposure to 5 mg/L for 1 h. Correspondingly, Nadler and coworkers (1989) noted differences in imipenem PAE on *P. aeruginosa* due to inter-strain variation. Furthermore, Gudmundsson and coworkers (1986) observed imipenem PAEs ranging from 0.1 to 1.9 h for 8 strains of *P. aeruginosa* and Erlensdottir and Gudmundsson (1988) observed an imipenem PAE with most but not all 20 *P.*

*aeruginosa* strains studied. However, meropenem PAEs of ≥0.5 h were obtained with 90% of strains tested ($n$ = 31), compared with 63% of strains with imipenem, illustrating that meropenem has a broader PAE spectrum than that of imipenem (Meropenem Technical Monograph, 1997). The factors that control the magnitude of PAE are recovery and repair of the microbial cell and these will be inherently different in each strain-antimicrobial combination. Hence, Fantin and Craig (1988), concluded that PAE duration can vary 2-4 fold amongst strains. Furthermore, it has been stated that the duration of carbapenem PAE was not related to the MIC of the strains concerned, that is, strains with low MICs did not have longer PAEs (Meropenem Technical Monograph, 1997), this statement also appears to be true for biapenem PAE on *P. aeruginosa*.

In this study viable counts were employed to follow bacterial growth kinetics. They have been used by the majority of investigators and were described by Mattie (1981) as the most reliable method of determining microbial growth in the presence of antimicrobial agents, especially ß-lactams. However, MacKenzie and Gould (1993) stated that the standard viable count method is gradually being replaced by more novel techniques. They advocate that the optimum method for PAE determination would be a combination of both bioluminescence and impedance techniques, due to its reliability, the fact that the presence of filaments and spheroplasts have little effect and that it is not labour intensive, although, it would be both unfeasible and uneconomical for most laboratories. However, viable counts are universally available and have a favourable lower limit of detection of $<10^3$ CFU/mL, compared to approximately $10^6$ CFU/mL for both

impedance and spectrophotometric methods. Additionally, due to the importance of the number of viable microorganisms to the progression of an infection, viable counts have an advantage over spectrophotometric and other non-metabolic methods of growth measurement (Bergan *et al.*, 1980). However, viable counts are retrospective compared to other methods of growth kinetics, that is, the results are only available eighteen hours or more after sampling. Ideally, PAE should be determined by directly measuring the true number of surviving and regrowing microorganisms in the culture media after antimicrobial exposure (Odenholt *et al.*, 1989a).

PAE = *T* - *C*, was the principal method of PAE quantification used throughout. This is still the most commonly used quantification method despite its obvious flaws. CERT has also been used to quantify PAE (Nilsson *et al.*, 1995) it allows the antimicrobial activity to be assessed independently of method and temporary differences in growth phase and accounts for microbial killing as well as PAE (Hanberger *et al.*, 1995). PAE, CERT and initial kill increase with increasing carbapenem concentration.

Kroeker and coworkers (1995) recommended that for the calculation of PAE of ß-lactams and Gram-negative bacteria, after CFU data is plotted, the regrowth curve of the exposed culture should be extrapolated from the portion of curve demonstrating conventional regrowth kinetics. Using this method they found the 'true' ceftazidime PAE of *P. aeruginosa* to be 0 h compared to -0.8 h by conventional PAE quantification (Kroeker *et al.*, 1995). Using the method of

Kroeker and coworkers (1995) the meropenem PAE on *E. coli* I425 (Figure 19) was found to be 0.14 h and -1.2 h by conventional PAE quantification. This discrepancy is probably due to the separation of filaments into cells causing a rapid increase in CFUs in the meropenem exposed culture compared to the non-exposed culture.

### 4.2 Standardised method.

Due to the variation and complexity of the techniques currently in use, many authors have expressed a need for the method of determining PAE to be regulated and standardised. However, controversy exists over which method would be the most appropriate (MacKenzie & Gould, 1993), and an ideal faultless method has yet to be established. If the data is to be used for clinical interpretation then it is essential for *in vitro* study design to simulate *in vivo* conditions as closely as possible. However, the action of antimicrobial agents on microorganisms *in vivo* is complex. It depends on factors such as activity, pharmacokinetics and host defences acting together therefore it is impossible to simulate all of these parameters in an *in vitro* model. Although, MIC values alone completely neglect the pharmacological properties of antimicrobials.

The initial aim of this thesis was to develop a method for the determination of carbapenem PAE under reproducible laboratory conditions which simulate those *in vivo*. The following recommendations can be made from the data in this thesis. Dilution is advocated as the method of antimicrobial removal, due to this technique being universal, rapid and comparable to the *in vivo* situation. Viable

counting with PAE quantification by PAE = $T$ - $C$ is the recommended method for following bacterial growth kinetics because of its favourable lower limit of detection and universal availability. The exposure concentrations should be 4 and 10 X MIC, as these concentrations should be equal to the peak serum concentration (Ellner and Neu, 1981) and also, maximal PAEs are usually observed at concentrations of 10 X MIC. The length of duration of exposure should be 1 h, because in the case of bactericidal antimicrobial agents this will limit their bactericidal effect, so that the CFU/mL is above the lower limit of detection. Furthermore, PAE determinations are inherently time consuming and this will restrict the experimental duration so that an extended working day is not required.

The recommended inoculum size is approximately $10^6$ CFU/mL as this is more comparable with the *in vivo* situation than the inocula ($10^4$ CFU) used for agar MIC determinations, but not so high as to eliminate PAE. The use of logarithmic phase of growth is suggested for the standardised method because, although it is less akin to the *in vivo* situation, most PAE and susceptibility determinations have been performed with this phase of growth and maximal antimicrobial effects will be observed.

The media ordinarily used for antimicrobial susceptibility testing has little in common with the serum and interstitial fluid of the patient (Stratton and Reller, 1977). Therefore, either bodily fluids or a commercial defined medium that resembles the *in vivo* conditions should be used to determine PAE. Iso-Sensitest,

is a commercially available, defined, synthetic, complex medium with a physiological pH (7.4) and was used throughout this thesis. Iso-Sensitest broth has a lysine level approximately 2.5 times that of serum (which is 0.172 mM (Gitlitz *et al.*, 1974)) but considerably less than that of MHB (Fukuoka *et al.*, 1991). In addition, the calcium and magnesium ion concentrations of Iso-Sensitest broth are approximately one fifth and equal to that of normal human serum, respectively. Furthermore, the osmolarity of Iso-Sensitest (328 mOsm/kg) is close to value of normal serum (Bergan and Carlsen, 1980). Pooled human serum has several disadvantages namely, its instability of pH, risk of infection, inherent antibacterial activity, cost, availability and poor support of growth of some microorganisms (Stratton and Reller, 1977). In addition, a decrease in protein binding is observed in heat inactivated serum compared to untreated serum (White *et al.*, 1991). Albumin supplemented broth can be used as a human serum equivalent however, it does not perform the same as human serum (Stratton and Reller, 1977).

Several infections involve microorganisms in biofilms for example, pneumonia in cystic fibrosis patients, cystitis, endocarditis, osteomyelitis, and infections related to medical implants and prosthetic devices (Gilbert *et al.*, 1987). Hence, in some instances, the use of membrane filters for exposure and as a solid support surface for the growth of microorganisms provides a greater approximation of the *in vivo* conditions than does the broth milieu (Stratton, 1996).

Furthermore, any study of PAE must be assessed for reproducibility and the genera studied must be characterised by studying a considerable number of recent clinical strains.

### 4.3 Mechanism of PAE.

Although a vast amount of knowledge has been obtained on PAE, the precise molecular mechanisms by which antimicrobials induce PAEs are still unknown. The unique PAEs observed with the carbapenems and penems were initially thought to be due to their high affinity to PBP 2. Furthermore, Gould and coworkers (1989) noted that mecillinam which also binds to PBP 2 demonstrated a brief PAE with *E. coli*. However, this was disproved by Majcherczyk and Livermore (1990) who demonstrated that an *E. coli* mutant with a thermosensitive PBP 2 (which does not bind ß-lactams at 44°C) exhibits carbapenem PAEs (of similar duration to the parent strain) at both 30 and 44°C. Additionally, Erlensdottir and Gudmundsson (1992) noted that mecillinam did not produce a PAE on *P. aeruginosa* and concluded that PBP 2 does not mediate PAE.

Alternatively, PAE may be either the period of time during which microorganisms synthesis new PBPs or the effect of carbapenem which has remained in the periplasmic space where it continues to inhibit the enzyme activities of newly formed PBPs (Bush *et al.*, 1989). Tuomanen (1986) demonstrated that the regrowth of *S. pneumoniae* and *E. coli* after removal of penicillin was dependent on resynthesis of PBPs. Additionally, it has been suggested that the PAE of penicillin on *Streptococcus pyogenes* was caused by irreversible binding of

penicillin to PBPs 1-3 and represents the time necessary for synthesis of new PBPs required for normal growth (Yan *et al.*, 1994).

Theoretically if the PAE reflects the duration of cellular recovery, the extent of cellular damage inflicted on microorganisms by an antimicrobial (as suggested by the extent of bactericidal activity) should reflect length of PAE. To investigate this hypothesis Li and coworkers (1997) correlated the PAEs and bactericidal rates of five antimicrobial agents with different mechanisms of action; tobramycin and ciprofloxacin on *P. aeruginosa*, dicloxacillin on *S. aureus* which binds irreversibly, trimethoprim on *Enterococcus faecalis* and tetracycline on *E. coli* which binds reversibly. PAE and bactericidal activity increased non-linearly with an increase in concentration. The strong correlations observed suggest that cellular recovery from non-lethal damage following antimicrobial exposure may be a major determinant of PAE. However, Jason and coworkers (1994) suggested that the meropenem PAE on *E. coli* may be ascribed entirely to a prolongation of lag time.

In an attempt to elucidate the mechanism of PAE, Majcherczyk and coworkers (1994) isolated and further studied a non-PAE producing mutant. This imipenem PAE lacking mutant of *P. aeruginosa* PAO1 exhibited reduced expression of a 52 kDa outer membrane protein and had a smaller cell volume than the parent strain, however these observations did not further define the mechanism of PAE.

Hence, investigations were performed in an attempt to determine the mechanism behind carbapenem PAE in *P. aeruginosa*. Carbapenems and other hydrophilic antimicrobials of small molecular size with a dipolar ion permeate the outer membrane of *P. aeruginosa* through the D2 protein channel (Trias and Nikaido, 1990). It has been demonstrated that basic amino acids such as L-lysine competitively inhibit carbapenem permeation through the D2 porin to the periplasm in *P. aeruginosa* by binding to a site in the D2 channel (Trias and Nikaido., 1990a). Hence, the carbapenem susceptibility of *P. aeruginosa* has been shown to increase in low basic amino acid media, such as, minimal media (Fukuoka *et al.*, 1991). Increasing the L-lysine monohydrochloride concentration present during carbapenem exposure decreased the imipenem, meropenem and biapenem PAEs on *P. aeruginosa* PAO1. This decrease in PAE was presumed to be due to competition occurring between L-lysine and carbapenem for permeation through the D2 porin and thus reducing carbapenem permeation into the microorganism. The use of complex media as opposed to human serum may therefore reduce the *in vitro* activity of the carbapenems due to the higher basic amino acid concentration in complex media (Fukuoka *et al.*, 1991). For example, the L-lysine concentration in MHB is at least forty times higher than that of human serum (Fukuoka *et al.*, 1991). Thus, the effect of carbapenems could be greater *in vivo* than expected from the *in vitro* investigations performed in complex media.

If the duration of PAE is due to the time required for the imipenem to leave the cell, (assuming the mechanism of influx and efflux are the same) recovery in the presence of L-lysine should be longer, due to competition in the porins. Increasing the concentration of L-lysine present during the recovery of cells had no significant effect on the duration of imipenem PAE (biapenem and meropenem not studied). Therefore, either the duration of PAE is not due to the time required for the imipenem to leave the cell or imipenem efflux occurs by another method. The latter appears to be correct, because the outer membrane slows the imipenem influx to such a level that periplasmic clearance mechanisms including hydrolysis, covalent binding by ß-lactamases and non-essential PBPs and efflux can occur. Furthermore, recent data produced by Yoneyama and coworkers (1998) demonstrates that *P. aeruginosa* encodes for three antimicrobial extrusion proteins designated MexA,B-OprM, MexC,D-OprJ and MexE,F-OprN which are regulated by the *nalB*, *nfxB* and *nfxC* genes, respectively. The tripartite extrusion protein is necessary for efflux, with the inner membrane component (MexB, D, F) functioning as a rnd family antiport exporter and outer membrane component (OprM, J, N) forming the exit channel with a membrane fusion protein linking the membrane associated efflux components MexA, C or E (Srikumar *et al.*, 1998).

Information on the nature of the recovery process may help to elucidate the mechanism behind PAE hence, Fuursted (1997a) examined the effect of postexposure conditions on penicillin G, ampicillin, erythromycin, ciprofloxacin and gentamicin PAE on *S. aureus* and *E. coli*. The various postexposure conditions evaluated included incubation temperatures of 20, 25, 30 or 35°C,

shaken or unshaken cultures, alterations in oxygen tension, pH 6.0, 7.4 or 9.0 and sodium chloride concentrations of 0, 1, 3 or 6% (Fuursted, 1997a). PAE increased as temperature decreased and PAE correlated with generation time (correlation coefficient $r^2$, 0.82-0.97), therefore it was concluded that PAE was not a consequence of antimicrobial dissociation. Minor differences in PAEs related to oxygen tension were observed, however anaerobic incubation extended the PAE of gentamicin on *S. aureus* and ciprofloxacin on *E. coli* suggesting that the recovery process of some antimicrobial-microorganism combinations partly requires metabolic energy from the electron transport system. Penicillin PAE on *S. aureus* increased at pH 6.0 and 9.0, however, the significance of pH on the recovery process is difficult to explain but could be related to pH sensitive enzymes associated with cell wall or cell membrane such as PBPs (Fuursted, 1997a). PAE increased significantly with increasing salinity therefore this loss of salt tolerance suggested some sub-lethal damage of the component(s) of the cell wall or cell membrane which is associated with various permeability functions including the permeation of sodium ions (Fuursted, 1997a).

Increasing sodium chloride concentration during recovery had no effect on the meropenem and biapenem PAEs but significantly increased the imipenem PAE. Implying that salt tolerance was apparently not lost and sub-lethal damage of the components of the cell wall did not occur during either meropenem or biapenem PAE. However, the mechanism behind imipenem PAE could reflect the time required to repair or reorganise the antimicrobial-induced sub-lethal damage of the cell wall.

It is thought that the classical PAE duration is proportionally shorter than the actual recovery time, that is, the previously exposed microorganisms may still be physiologically abnormal. Hence, Bergeron (1992) broadly defined PAE as the persistence of an antimicrobial effect for a variable period after cessation of exposure of a microorganism to an effective antimicrobial agent. Bergeron (1992) fundamentally criticised the currently used definition of PAE as it assumes the cell population is homogenous and that recovery of each individual cell follows an identical time course.

Grimwood and coworkers (1989) found that *P. aeruginosa* exoenzyme expression can be down regulated both *in vitro* and *in vivo* by an antimicrobial concentration which does not inhibit bacterial growth. Furthermore, even in the presence of multiplying microorganisms, a subinhibitory concentration of antimicrobial protected the lungs from significant histological damage (Grimwood *et al.*, 1989). Hence, Gerber (1993) suggested that a major contribution to the information on PAE would be the assessment of virulence factors and microbial toxicity of microorganisms in the PAE phase. In addition, Miller (1997) stated that the rate of bacterial eradication may not be the factor which best correlates with outcome in patients with selected Gram-negative infections, for ß-lactams a possible relationship has been proposed between PBP binding affinity, relative endotoxin release and outcome.

Garcia and coworkers (1995) observed during the penicillin PAE on Group A streptococci that exposed and non-exposed control cultures had the same cell surface hydrophobicities, but that extracellular deoxyribonuclease and hyaluronidase were produced in larger amounts in the exposed culture. In addition, Shibl and coworkers (1995) observed that macrolide treated streptococci cells failed to exhibit normal extracellular haemolytic activity for 2 h longer than the roxithromycin PAE and that cell surface charge was altered in PAE. The examination of the virulence factors of *P. aeruginosa* during carbapenem PAE phase may determine if PAE is due to recovery from non-lethal damage. However, no apparent change in *P. aeruginosa* virulence factor production during carbapenem PAE phase was observed. Although, this method was only semi-quantitative hence small differences may not have been detected. In addition, Stenhem and coworkers (1997) studied endotoxin release from *E. coli* and *P. aeruginosa* during imipenem and meropenem PAE phase and found that endotoxin release occurred during exposure but ceased on antimicrobial elimination.

Alternatively, carbapenem PAE could represent either the time required for spheroplasts to resynthesise the cell wall (because the preferential binding to PBP 2 inhibits the formation of bacillus shaped cells and leads to spherical cells) or the time required for filament reseptation (as binding to PBP 3 leads to filamentation) depending on the antimicrobial (Hanberger *et al.*, 1991b). Hanberger and coworkers (1993a) succeeded in eliminating spheroplasts by culturing *E. coli* in iso-osmotic MHB, treating samples from these cultures for 10

minutes in water-diluted hypo-osmotic MHB and also by performing both procedures in hypo-osmotic nutrient broth. The presence of long imipenem PAEs determined by bioluminescence for *E. coli* despite the lack of spheroplasts indicates that the PAE is probably due to some mechanisms that occur without morphological alterations detectable by light microscopy. The negative imipenem PAEs observed here are similar to those observed by Hanberger and coworkers (1993a) after spheroplast lysis when determined by viable counts. Furthermore, osmolarity *in vivo* would predispose to the lysis of spheroplasts (MacKenzie and Gould, 1993).

In an attempt to determine if the resynthesis of the cell wall of spheroplasts was responsible for carbapenem PAE recovery using media containing *N*-acetyl-D-glucosamine was evaluated. This selective medium has previously been used to culture *H. influenzae* spheroplasts (Roberts *et al.*, 1984). However the presence of *N*-acetyl-D-glucosamine in the media had no effect on the duration of carbapenem PAE. Therefore, assuming *N*-acetyl-D-glucosamine present in the media facilitated the culture of *P. aeruginosa* spheroplasts then carbapenem PAE is not due to spheroplast recovery. In concordance, Hanberger and coworkers (1991b) found that neither a certain multiple of MIC, the presence of spheroplasts nor strong initial killing could predict the length of ß-lactam PAEs on Gram-negative microorganisms.

The microorganism cellular morphology during PAE phase reflects the enzyme activity of the PBPs and may contribute to elucidating the mechanism of PAE. PBP 2 is the primary target for both imipenem and meropenem in *E. coli*, although in *P. aeruginosa* either PBP 2 and 3, or PBP 3 alone are the primary targets for meropenem (Edwards, 1995). Due to these differences in binding affinities meropenem tends to cause filamentation and imipenem tends to cause spherical cell formation in *P. aeruginosa*. Therefore, the primary morphological response of *P. aeruginosa* is due to PBP 3, even though the $IC_{50}$s are almost the same and the relative amount of PBP 3 is greater than PBP 2, this may be due to the location of the two PBPs at the membrane with PBP 3 being more accessible (Hancock, 1986). The binding profile of biapenem is more like imipenem than meropenem (Sumita and Fukasawa, 1995). It appears that the overall effect of a ß-lactam does not only depend on the type of PBPs that are affected, but the sequence in which these target proteins are acetylated.

*P. aeruginosa* PAO1 exhibited altered morphology under epi-fluorescence microscopic examination during the conventional imipenem, meropenem and biapenem PAE duration. In addition, the morphology of *P. aeruginosa* PAO1 during imipenem and meropenem PAE phase as determined by scanning electron microscopy demonstrated that imipenem and meropenem exhibited morphology alterations equivalent to the conventional PAE durations of 2.4 and 1.2 h, respectively. Gottfredsson and coworkers (1993) followed the ultrastructural changes using transmission electron microscopy of *P. aeruginosa* in imipenem PAE and observed an increase in intracellular electron-dense

aggregates. In concordance, although, these alterations were not uniform in every microorganism the persistence of these ultrastructural alterations correlated with the duration of PAE determined by viable counts. However, Guan and coworkers (1992) found quinolone PAE on *E. coli* defined by nucleoid morphology longer than that determined by viable count.

In conclusion, the mechanism behind carbapenem PAE in *P. aeruginosa* appears to be the true recovery from non-lethal morphological damage which is subsequently governed by the synthesis of PBPs and repair of the peptidoglycan in the cell wall. The experiments of Tuomanen (1986) and Yan and coworkers (1994) concur in that they demonstrated that penicillin PAE was dependent on the resynthesis of PBPs. In accordance, Li and coworkers (1997) also suggested that cellular recovery from non-lethal damage following antimicrobial exposure may be a major determinant of PAE. Hence, the difference in duration of imipenem and meropenem PAEs might be due to the fact that cell wall synthesis takes longer than reseptation.

**4.4 Further advances.**

This thesis has demonstrated that carbapenems do not exhibit similar PAEs on *P. aeruginosa* and *E. coli*, different carbapenems do not exhibit the similar PAEs to each other and apparently a higher concentration of meropenem is required to initiate a PAE on *P. aeruginosa* compared with imipenem and biapenem. These differences in carbapenem PAE may be due to a difference between the affinities of the three carbapenems for non-essential and essential PBPs. Furthermore, *E.*

*coli* PBPs spontaneously release inactive penicillin with half-lives ranging from 5 to >60 minutes (Spratt, 1977), thus the differences in the PAEs of the ß-lactams on Gram-positive and Gram-negative microorganisms could represent differences in the rates of antimicrobial turnover.

Therefore, by following the PBP profiles of *P. aeruginosa* in imipenem, meropenem and biapenem induced PAE phases at different antimicrobial concentrations it would be possible to ascertain which PBPs are involved and to produce data on PBP affinity versus PAE. Consequently, this may help to clarify the mechanism of PAE, however, it would be difficult to determine whether any correlation between PBP affinity and PAE was causal. Following antimicrobial treatment discontinuation the microorganisms will begin to synthesise new PBPs and existing PBP-carbapenem complexes will gradually breakdown thus, increasing the apparent amount of unbound PBP and obscuring any conclusions. However, this could be overcome by recovering the PAE phase microorganisms in the presence of a protein synthesis inhibitor, therefore elucidating whether any increase in PBPs observed was due to PBP synthesis or PBP-carbapenem bond breaking. By examining PBP profiles and using a protein synthesis inhibitor Iida and coworkers (1982) concluded that resumption of synthesis of PBP 3 is essential for resumption of cell division in piperacillin induced filaments.

Studies on microbial metabolism during PAE phase are limited, even though they provide an insight into the mechanism of PAE. For example, DNA, RNA, protein and peptidoglycan synthesis can be measured in recovering cells by monitoring

uptake of the radiolabelled precursors of these macromolecules (Majcherczyk, 1996). Furthermore, these results would not be artefactually affected by cellular morphology and would provide a measure of the recovery of cellular metabolism. The study of macromolecular synthesis during tobramycin induced PAE on *E. coli* demonstrated that the duration of PAE correlated with inhibition of total functional protein synthesis but not RNA or DNA synthesis (Barmada *et al*, 1993). Conversely, Guan and coworkers (1992) suggested that DNA synthesis provided an accurate measure of the time required for quinolone treated *E. coli* cells to recover fully. Additionally, DNA synthesis (rate of $^{3}H$ adenine incorporation) was enhanced during imipenem PAE on *P. aeruginosa* probably due to continued DNA replication, inhibition of bacterial septation and multiplication (Gottfredsson *et al.*, 1995).

Imipenem is analogous to mecillinam which binds to PBP 2 resulting in the formation of spherical cells which rapidly lyse by a process that requires cyclic AMP (Aono *et al.*, 1979). Bustamante and coworkers (1984) suggested that the determination of damage to this particular cyclic AMP pathway by imipenem and its correlation with the presence of a PAE on *P. aeruginosa* required further investigation.

During the course of normal growth the synthesis of peptidoglycan in *E. coli* is thought to be regulated by a stringent control mechanism (regulated by *relA* gene), thus when the cell undergoes stress (amino acid deprivation) this control mechanism is invoked leading to a rapid accumulation of guanosine-5-

diphosphate-3-diphosphate resulting in the shutdown of RNA and peptidoglycan synthesis and presumably hydrolase activity (Bryan and Godfrey, 1991). Stratton (1996) stated that aberrant forms for example, spheroplasts or filaments might represent the entry of the microorganism into the stringent response phase. Therefore the study of mutants lacking a stringent control mechanism might provide an insight into the mechanism of PAE.

In addition, if the growth medium is exhausted of an essential nutrient the cells enter stationary phase where the *rpoS* gene is apparently essential for continued cell survival (Dougherty and Pucci, 1994). Many genes are under *rpoS* control including, *katE* coding for catalase, *xthA* coding for exonuclease III involved in DNA repair, *dps* coding for DNA-binding protein for resistance to hydrogen peroxixde and *otsBA* involved in trehalose synthesis and osmotic stability (Smith, 1995). Futhermore, *rpoS* down regulates PBP 3 and therefore ceases cell division (Dougherty and Pucci, 1994). Thus the expression of the *rpoS* gene and survival during PAE phase could be related, however this has not been investigated.

Furthermore, real time metabolic monitoring can be performed using a microphysiometer which measures changes in pH or redox potential in the cellular environment and thus reflects the metabolic activity of cells. Using this method the piperacillin and cefotaxime PAEs on *E. coli* were found to be similar to those determined by bioluminescence but longer than those determined by viable count (Libby, 1998).

The extent of damage to the microbial structure could be determined by the extracellular presence (cell leakage) of nucleotides, ATP, cytochromes, potassium ions, oxidase or amino acids (Davies, 1987). For example, ATP released during penicillin, aminoglycoside, quinolone and carbapenem PAE phase was measured by Winstanley and Hastings (1989), Ishihara and coworkers (1993) and Stenhem and coworkers (1997), respectively. Whereas, Curtis and coworkers (1985) observed that on filament and spherical cell formation of *E. coli* after antimicrobial exposure a rapid release of periplasmic ß-lactamase occurred. In addition, Hancock and Wong (1984) used rate of permeabilisation by lysozyme to determine the state of outer membrane.

In summary, the carbapenems evaluated exhibited PAEs that were reproducible, constant over successive exposures, dependent on genera, concentration, duration of exposure, inoculum size and growth phase. Furthermore, the recovery of the penicillin binding protein target enzymes appears to be the mechanism underlying carbapenem PAE in *P. aeruginosa* PAO1. However, subsequent investigations may further explicate the mechanisms behind PAE and the differences in PAE exhibited by different genera. A considerable amount of information on PAE has been published over the last 50 years, however the disparate nature of the data is due to the diverse methodological procedures used. Therefore, if PAE is to merit further evaluation and have any clinical implications its methodology needs to be standardised due to the plethora of variables that affect the duration of PAE and the introduction of standardisation into related laboratory methods (NCCLS, 1992; Working Party of BSAC, 1991).

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# 6. APPENDICES

## Appendix 1. MOPS buffer preparation.

To prepare 0.02 M 3-(*N*-Morpholino) propane-sulphonic acid (MOPS) pH 7 buffer, dissolve 0.419 g of MOPS (molecular weight 209.3) in 100 mL of distilled water and adjust to pH 7 dropwise with 0.1 M sodium hydroxide. Sterilise by autoclaving at 121°C for 15 minutes.

## Appendix 2. Phosphate buffered saline composition.

| Formula | grams per litre |
|---|---|
| Sodium chloride | 8.0 |
| Potassium chloride | 0.2 |
| Disodium hydrogen phosphate | 1.15 |
| Potassium dihydrogen phosphate | 0.2 |
| pH | 7.3 |

**Appendix 3. Iso-Sensitest agar and broth composition.** (Oxoid manual)

Oxoid **Iso-Sensitest agar** was developed specifically for antimicrobial susceptibility tests. Its formulation was carefully constructed to give a reproducible, closely semi-defined medium with stabilised mineral content and in which the undefined components were kept to a minimal level. However, it allows the growth of the great majority of microorganisms without further supplementation. The formula is defined by the BSAC.

Suspend 31.4 g in 1 litre of distilled water and bring to the boil to dissolve the agar. Sterilise by autoclaving at 121°C for 15 minutes.

Oxoid **Iso-Sensitest broth** is produced in parallel with Iso-Sensitest agar. The broth has an identical nutrient formulation to the agar, without the specially purified agar. Add 23.4 g to 1 litre of distilled water. Boil to dissolve starch and aliquot as necessary. Sterilise by autoclaving at 121°C for 15 minutes.

| Formula | grams per litre |
|---|---|
| Hydrolysed casein | 11.0 |
| Peptones | 3.0 |
| Glucose | 2.0 |
| Sodium chloride | 3.0 |
| Soluble starch | 1.0 |
| Disodium hydrogen phosphate | 2.0 |
| Sodium acetate | 1.0 |
| Magnesium glycerophosphate | 0.2 |
| Calcium gluconate | 0.1 |
| Cobaltous sulphate | 0.001 |
| Cupric sulphate | 0.001 |
| Zinc sulphate | 0.001 |
| Ferrous sulphate | 0.001 |
| Manganous chloride | 0.002 |
| Menadione | 0.001 |
| Cyanocobalamin | 0.001 |
| L-Cysteine hydrochloride | 0.02 |
| L-Tryptophan | 0.02 |
| Pyridoxine | 0.003 |
| Pantothenate | 0.003 |
| Nicotinamide | 0.003 |
| Biotin | 0.0003 |
| Thiamine | 0.00004 |
| Adenine | 0.01 |
| Guanine | 0.01 |
| Xanthine | 0.01 |
| Uracil | 0.01 |
| Agar No. 1 | 8.0 |

pH 7.4 $\pm$ 0.2

**Appendix 4. Minimal media broth** (Fukuoka *et al.*, 1991)

| Formula | grams per litre |
|---|---|
| Dipotassium hydrogen phosphate | 10.5 |
| Potassium dihydrogen phosphate | 4.5 |
| Ammonium sulphate | 1.0 |
| Magnesium sulphate | 0.1 |
| Sodium gluconate | 4.4 |

(all from BDH Chemicals Ltd., Poole, UK.)

pH 7.0

L-lysine monohydrochloride (molecular weight 182.6 g, Sigma-Aldrich Company Ltd.)

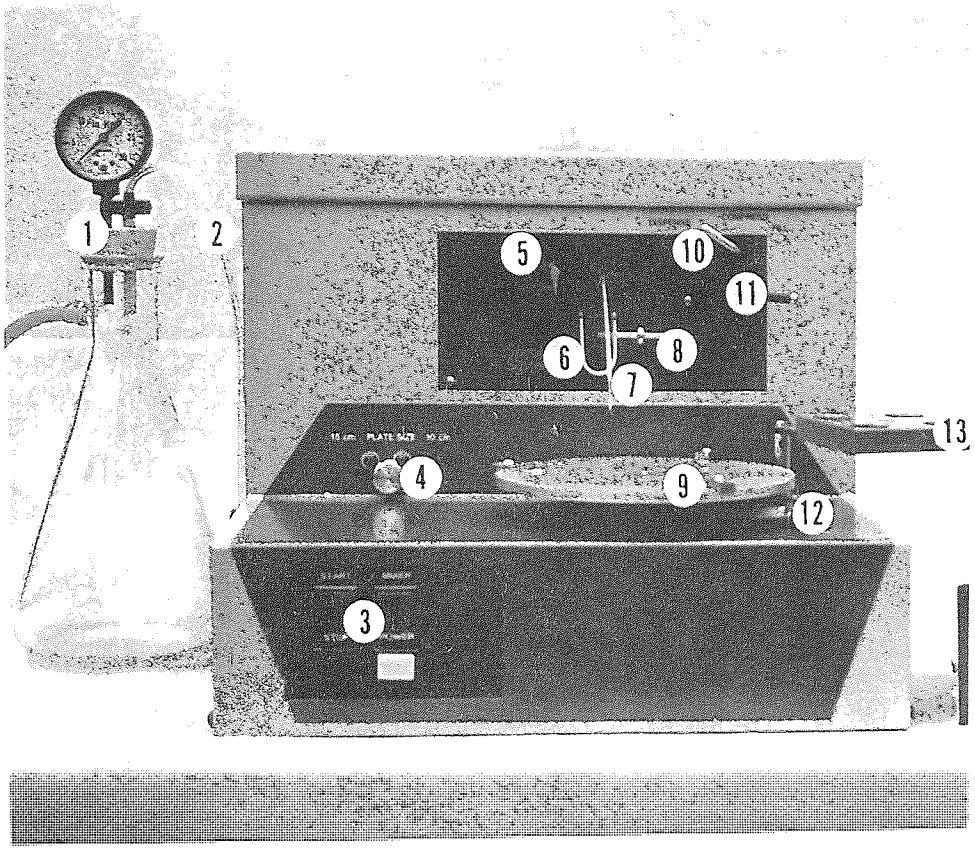
1.826 g in 10 mL sterile distilled water = 1000 mM
Adjust pH of minimal media broth with L-lysine to pH 7.0 dropwise with 0.1 M sterile sodium hydroxide.

## Appendix 5. Microtitre tray grid.

Carbapenem concentration mg/L

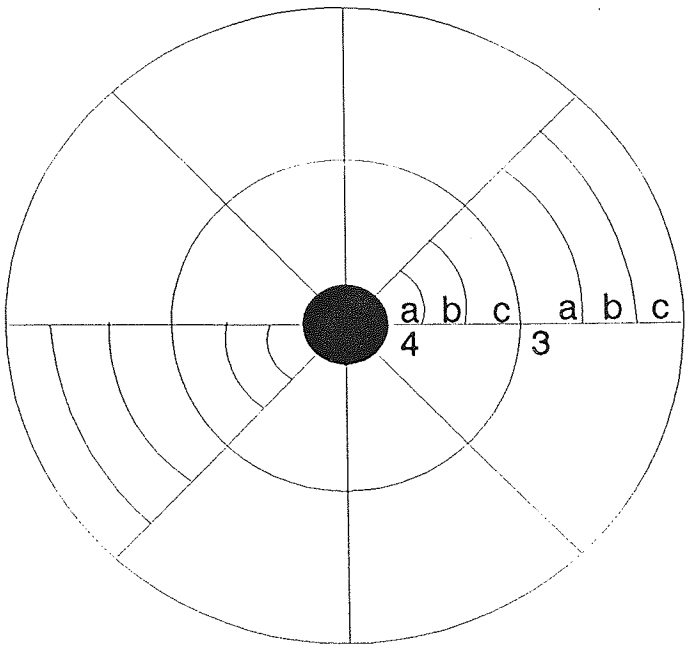
| Strain | .008 | .015 | .03 | .06 | .12 | .25 | .5 | 1 | 2 | 4 | 8 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

## Appendix 6. Spiral plater - Model B




1. Vacuum controls
2. Vacuum tube
3. Switches
4. Plate size adjuster
5. Vacuum filling valve
6. Sight glass
7. Stylus
8. Stylus lift arm
9. Turntable
10. Micro-diluter control
11. Carriage return levers
12. Turntable height adjuster
13. Sample cup holder

## Appendix 7. Spiral plater counting grid




| Area code | Volume (mL) |
|---|---|
| Total | 0.0360 |
| 4a | 0.0090 |
| 4b | 0.0060 |
| 4c | 0.0038 |
| 3a | 0.0025 |
| 3b | 0.0015 |
| 3c | 0.0005 |

Example of calculation.

If 27 and 34 colonies are counted in the two opposite segments marked 4a of a 10-fold diluted culture.

$$\frac{27 + 34 \times 10}{0.009} = 6.8 \times 10^4 \text{ CFU/mL}$$

## Appendix 8. ß-lactamase - broad spectrum mixture.
(Genzyme Biochemicals Limited).

ß-lactamase (penicillin amido-ß-lactam hydrolase E.C.3.5.2.6) prepared from *Bacillus cereus* 569/H9 and supplied in the form of a freeze dried powder containing buffer and zinc salts.

One vial contains a minimum of 500 units of ß-lactamase I and 50 units of ß-lactamase II and is reconstituted in 5 mL of sterile distilled water.

One unit of activity is defined as that amount of enzyme which will catalyse the hydrolysis of 1 µmole of substrate per minute.

## Appendix 9. Motility media composition.

Suspend 13 g of nutrient broth (Oxoid) and 2 g agar bacteriological (agar No. 1, Oxoid) in 1 litre of distilled water. Sterilise by autoclaving at 121°C for 15 minutes. When cooled to 50°C supplement with 0.05 g of tetrazolium salt (BDH Chemicals Ltd.). Dispense in 3 ml volumes into glass bijoux and reautoclave.

## Appendix 10. Acridine orange staining method.

Acridine orange is a fluorochrome that causes DNA to fluoresce green and RNA to fluoresce orange-red.

Air dried slides are covered with acridine orange stain (Sigma) (140 mg/mL PBS) for 45 seconds. Excess stain is drained off and slides were washed in PBS.

Bacterial nuclei, appear a bright greenish yellow by fluorescence microsopy after staining with acridine orange, whereas the cytoplasm appears flame-red.

## Appendix 11. Table of viable counts of *P. aeruginosa* G318 from Section 2.11.

| | Time (h) | Colony count | Area | Dilution | CFU/mL | Log CFU/ml | Mean Log CFU/mL |
|---|---|---|---|---|---|---|---|
| Control | 0 | 45 | 4a | 1E-01 | 5.00E+04 | 4.70 | 4.81 |
| | | 30 | T | 1E-02 | 8.33E+04 | 4.92 | |
| | 1 | 90 | 3b | 1E-01 | 6.00E+05 | 5.78 | 5.64 |
| | | 19 | 4b | 1E-02 | 3.17E+05 | 5.50 | |
| | 2 | 118 | 4a | 1E-02 | 1.31E+06 | 6.12 | 5.91 |
| | | 18 | T | 1E-03 | 5.00E+05 | 5.70 | |
| | 3 | 23 | 3a | 1E-03 | 9.20E+06 | 6.96 | 6.83 |
| | | 18 | T | 1E-04 | 5.00E+06 | 6.70 | |
| | 4 | 48 | 4c | 1E-03 | 1.26E+07 | 7.10 | 7.29 |
| | | 18 | 4b | 1E-04 | 3.00E+07 | 7.48 | |
| | 5 | 20 | 4b | 1E-04 | 3.33E+07 | 7.52 | 7.76 |
| | | 36 | T | 1E-05 | 1.00E+08 | 8.00 | |
| | 8 | 42 | 4c | 1E-04 | 1.11E+08 | 8.04 | 7.97 |
| | | 29 | T | 1E-05 | 8.06E+07 | 7.90 | |
| | 24 | 53 | 4b | 1E-05 | 8.83E+08 | 8.95 | 9.10 |
| | | 64 | T | 1E-06 | 1.78E+09 | 9.25 | |
| Biapenem | 0 | 24 | 4b | 1E+00 | 4.00E+03 | 3.60 | 3.67 |
| 1mg/L | | 20 | T | 1E-01 | 5.56E+03 | 3.74 | |
| | 1 | 19 | 4c | 1E+00 | 5.00E+03 | 3.70 | 3.77 |
| | | 25 | T | 1E-01 | 6.94E+03 | 3.84 | |
| | 2 | 16 | 3b | 1E+00 | 1.07E+04 | 4.02 | 3.91 |
| | | 23 | T | 1E-01 | 6.39E+03 | 3.80 | |
| | 3 | 51 | 4a | 1E-01 | 5.67E+04 | 4.75 | 4.82 |
| | | 28 | T | 1E-02 | 7.78E+04 | 4.89 | |
| | 4 | 78 | 4c | 1E-01 | 2.00E+05 | 5.30 | 5.40 |
| | | 114 | T | 1E-02 | 3.17E+05 | 5.50 | |
| | 5 | 38 | 4c | 1E-02 | 1.00E+06 | 6.00 | 6.14 |
| | | 17 | 4a | 1E-03 | 1.69E+05 | 6.28 | |
| | 6 | 24 | 3b | 1E-02 | 1.60E+06 | 6.20 | 6.41 |
| | | 38 | 4a | 1E-03 | 4.22E+06 | 6.62 | |
| | 24 | 35 | 3a | 1E-05 | 1.40E+09 | 9.15 | 9.05 |
| | | 32 | T | 1E-06 | 8.89E+08 | 8.95 | |
| Biapenem | 0 | 32 | 4b | 1E+00 | 5.33E+03 | 3.73 | 3.59 |
| 5mg/L | | 10 | T | 1E-01 | 2.78E+03 | 3.45 | |
| | 1 | 24 | 4b | 1E+00 | 4.00E+03 | 3.60 | 3.59 |
| | | 14 | T | 1E-01 | 3.89E+03 | 3.58 | |
| | 2 | 15 | 4c | 1E+00 | 3.95E+03 | 3.60 | 3.74 |
| | | 27 | T | 1E-01 | 7.50E+03 | 3.88 | |
| | 3 | 65 | 3a | 1E+00 | 2.64E+04 | 4.42 | 4.52 |
| | | 38 | 4a | 1E-01 | 4.22E+04 | 4.62 | |
| | 4 | 15 | 3b | 1E-01 | 1.00E+05 | 5.00 | 5.17 |
| | | 79 | T | 1E-02 | 2.19E+05 | 5.34 | |
| | 5 | 36 | 4a | 1E-02 | 4.00E+05 | 5.60 | 5.81 |
| | | 38 | T | 1E-03 | 1.06E+06 | 6.02 | |
| | 6 | 19 | 3b | 1E-02 | 1.27E+06 | 6.10 | 6.30 |
| | | 28 | 4a | 1E-03 | 3.11E+06 | 6.50 | |
| | 24 | 18 | 3a | 1E-05 | 7.20E+08 | 8.85 | 9.00 |
| | | 51 | T | 1E-06 | 1.42E+09 | 9.15 | |

## Appendix 12. Publications resulting from this thesis.

Boswell, F.J., Andrews, J.M. and Wise, R. (1994). Postantibiotic effect of Biapenem (L-627) on *Pseudomonas aeruginosa*. In *Recent Advances In Chemotherapy. Proceedings of the Eighteenth International Congress of Chemotherapy, Stockholm, Sweden,* p 372-3. American Society of Microbiology, Washington, DC.

Boswell, F.J., Andrews, J.M., Gill, M.J. and Wise, R. (1995). Postantibiotic effects of three carbapenems on *Pseudomonas aeruginosa* in the presence of lysine. *Journal of Antimicrobial Chemotherapy* **35**, 232-3.