Plasma concentrations might lead to overestimation of target site activity of piperacillin in patients with sepsis

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Objectives: Pharmacokinetic (PK)/pharmacodynamic (PD) models have become increasingly important in optimizing antimicrobial therapy. This approach is highly recommended by regulatory authorities intending to force the evaluation of antimicrobial action at the site of infection.

Methods: Clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus with MICs of 4, 8 and 16 mg/L for piperacillin were used in an in vivo PK/in vitro PD model. Bacteria were exposed in vitro to the concentration-versus-time profiles of piperacillin in plasma and subcutaneous adipose tissue measured in vivo in septic patients. Samples were withdrawn at defined intervals and the numbers of bacteria per mL were counted and plotted against time.

Results: Piperacillin levels determined in plasma were able to effectively inhibit bacterial growth of all bacterial strains used in the present study (MIC ranged from 4–16 mg/L). In contrast, concentration-versus-time profiles of subcutaneous adipose tissue were effective in killing isolates with MICs of 4 and 8 mg/L only, while bacterial growth of S. aureus and P. aeruginosa with MICs of 16 mg/L was not inhibited.

Conclusions: Bacteria with MICs < 16 mg/L were effectively inhibited in subcutaneous adipose tissue in patients with sepsis. The prediction of microbiological outcome based on concentrations of piperacillin in plasma resulted in a marked overestimation of antimicrobial activity at the site of infection.

Keywords: pharmacokinetics, pharmacodynamics, PK/PD, β-lactams, in vitro

Introduction

Pharmacokinetic (PK)/pharmacodynamic (PD) models have attracted increasing attention during the last decade. These PK/PD models are commonly based on easily available serum concentrations of a healthy population and are used for the evaluation of dosing regimens and for the determination of susceptibility breakpoints. However, this approach suffers from certain limitations. Most importantly, infections are primarily localized in the interstitial space fluid of tissues rather than in plasma. In addition, it has been shown that conditions such as sepsis might severely hamper concentrations of antimicrobials in target tissues when compared with healthy controls. Therefore, regulatory authorities such as EMEA (European Agency for the Evaluation of Medicinal Products) and the US-FDA (Food and Drug Administration) recommend the evaluation of target site concentrations of antimicrobials instead of using plasma concentrations in PK/PD models. Concentrations of the β-lactam antibiotic piperacillin were recently determined in plasma and the interstitial space fluid of subcutaneous adipose tissue of septic patients by use of microdialysis. In this previous study, significant impairment of plasma-to-tissue equilibration was found, i.e. a finding likewise seen in numerous other infections. Against this background, we performed the present study to evaluate the impact of impaired tissue penetration of piperacillin on antimicrobial killing in subcutaneous
adipose tissue, which represents a potential site of source of infections in patients with sepsis. For this purpose an established in vivo PK/in vitro PD model, which allows for the estimation of the effect of an antibiotic at the target site, was employed.\textsuperscript{9–12} Dynamic time–kill curves were generated by exposing clinical isolates of \textit{Pseudomonas aeruginosa} and methicillin-susceptible \textit{Staphylococcus aureus} to changing piperacillin levels according to the concentration-versus-time profiles measured in plasma and subcutaneous adipose tissue in vivo.

**Materials and methods**

\textit{In vivo PK of piperacillin}

In a recent study, free concentrations of piperacillin in plasma and the interstitial space fluid of subcutaneous adipose tissue of septic patients were determined by ultrafiltration and microdialysis, respectively.\textsuperscript{6} Six patients were enrolled in the study. Patients had an age of 64.2 ± 16.2 years (mean ± SD), a weight of 71.8 ± 12.0 kg and a height of 170.2 ± 6.9 cm. The mean arterial pressure was 80.7 ± 9.7 mmHg, the mean C-reactive protein was 24.8 ± 9.0 mg/dL and leucocytes were 18.1 ± 9.7 G/L. Sepsis was diagnosed according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee.\textsuperscript{13} systolic blood pressure of <90 mmHg; tachycardia of >90 beats/min; respiratory rate of >20 breaths/min or a PaCO\textsubscript{2} of <32 mmHg; a temperature of >38.0°/C or <36.0°/C; a leucocytosis count of >12000/mm\textsuperscript{3} or a leucopenia count of <4000/mm\textsuperscript{3} or >10% immature (band) forms. Each patient was enrolled into the study after having met at least two of these criteria. Septic shock was defined as sepsis-induced hypotension along with the presence of organ dysfunction and hypoperfusion abnormalities. The mean Acute Physiology and Chronic Health Evaluation (APACHE) III score of the population was 52.8 ± 10.4. Patients received a single combined intravenous (iv) dose of 4.0 g of piperacillin and 0.5 g of tazobactam (Tazocin\textsuperscript{®}; Cynamid GmbH, Wolfratshausen, Germany) over a period of 10 min. Sampling of venous blood and dialysates was performed at 20 min intervals up to 240 min.

**Bacteria and antibiotic**

For the \textit{in vitro} experiments, clinical isolates of \textit{S. aureus} and \textit{P. aeruginosa} obtained at the general hospital of Vienna were chosen. The susceptibilities of the bacteria to piperacillin were determined by the broth microdilution method, according to the criteria of the NCCLS.\textsuperscript{14} Therefore, test strains were pre-cultured overnight on Columbia agar plates and were then introduced into Mueller–Hinton broth containing piperacillin in order to achieve an initial concentration of \(5 \times 10^5\) cfu/mL. The lowest concentration of piperacillin that inhibited visible bacterial growth after 18 h of incubation at 37°C was defined as the respective MIC. Conditions were controlled by using \textit{S. aureus} ATCC 29213 and \textit{P. aeruginosa} ATCC 27853 for comparison. \textit{S. aureus} and \textit{P. aeruginosa} strains with MICs of 4, 8 and 16 mg/L each were selected for PK/PD modelling, since they are representative of \textit{S. aureus} and \textit{P. aeruginosa} strains isolated in intensive care units and in patients presenting soft tissue infections at the general hospital of Vienna. All bacteria were stored frozen in nitrogen at –196°C until use. Piperacillin used for the \textit{in vitro} experiments was purchased from Sigma–Aldrich (St Louis, USA).

**In vitro PD**

Based on the PK data obtained \textit{in vivo}, we employed a combined \textit{in vivo} PK/\textit{in vitro} PD model, which allows for the estimation of the effect of an antibiotic at the target site.\textsuperscript{6,9–12} In order to generate time–kill curves, selected bacteria were exposed \textit{in vitro} to dynamically changing concentration-versus-time profiles of piperacillin measured in plasma and interstitial space fluid of subcutaneous adipose tissue. The concentration-versus-time profiles were measured \textit{in vivo} for a period of 240 min.\textsuperscript{6} However, bacterial time–kill curves were simulated \textit{in vitro} over a period of 480 min by use of the elimination half-life of piperacillin according to the one-compartment model observed \textit{in vivo}.\textsuperscript{5}

In brief, 3 mL of Mueller–Hinton broth (Merck, Darmstadt, Germany) kept at 37°C in a water bath was inoculated with different clinical isolates of \textit{S. aureus} and \textit{P. aeruginosa} in order to achieve a concentration of \(5 \times 10^7\) cfu/mL. Bacterial strains were exposed \textit{in vitro} to the dynamically changing concentrations of piperacillin determined \textit{in vivo} in plasma and the interstitial space fluid of subcutaneous adipose tissue. The concentration of piperacillin in broth was adjusted at time intervals as depicted in Figure 1. Increasing antibiotic concentrations were simulated by the addition of piperacillin. Decreasing concentrations were attained by adding Mueller–Hinton broth without antibiotic at appropriate volumes, according to the equation \(V_2 = (C_1/C_2) \times V_1\), where \(C_1\) and \(V_1\) are the current piperacillin concentration and the current volume in the test tube, respectively; \(C_2\) is the desired piperacillin concentration; and \(V_2\) is the volume in the test tube after the addition of adequate broth. Adjustment of piperacillin concentrations was performed after drawing samples for determination of bacterial counts. The test tubes were vortexed after the addition of piperacillin or pure broth. After the test tubes were vortexed, samples of 200 \(\mu\)L were drawn at 0, 40, 80, 140, 200, 240, 300, 360, 420 and 480 min to determine the bacterial counts. The samples were serially diluted with physiological saline solution and 20 \(\mu\)L of each dilution was plated onto Columbia agar plates (BioMerieux, Marcy l’Etoile, France). The agar plates were cultured overnight, and the bacterial counts were determined and back-extrapolated to the original volume to account for the respective dilution due to the addition of antibiotic and broth. Each simulation was performed six times. Bacterial growth control experiments were performed in culture tubes without antibiotic.

**Data analysis and statistical calculations**

All data are presented as means ± SD. Absolute log\textsubscript{10} differences in cfu/mL between initial and final inoculum after 8 h of simulation were calculated. For statistical significance testing of differences in bacterial killing or growth between simulations for plasma and subcutaneous
adipose tissue, Mann–Whitney U-tests were performed by using a commercially available computer program (Statistica®, StatSoft, Inc., Tulsa, USA). A two-sided $P$ value $<0.05$ was considered the level of significance.

Results

The present study simulated the bacterial killing of selected bacteria at the target site in septic patients by use of an established in vivo PK/in vitro PD model. Figure 1 depicts the simulated concentration-versus-time profiles of piperacillin for plasma and subcutis and presents respective MICs for bacteria used in the present experiments. In addition, the piperacillin NCCLS breakpoint for P. aeruginosa (64 mg/L) is shown. Mean time–kill profiles for S. aureus and P. aeruginosa are depicted in Figure 2 for plasma and subcutaneous adipose tissue of septic patients. The detection limits are depicted for plasma and subcutis and rise during the experiment due to the increasing dilution of the bacterial counts over time by addition of broth. Absolute log$_{10}$ differences in bacterial counts between the baseline and final inoculum in broth after 8 h are presented in Figure 3.

![Figure 2. In vitro time–kill curves of S. aureus (left-hand panels) and P. aeruginosa (right-hand panels) with MICs of 4 mg/L (squares, upper panels), 8 mg/L (circles, middle panels) and 16 mg/L (triangles, bottom panels) after exposure of bacteria to the concentration-versus-time profiles of piperacillin derived for plasma (filled symbols) and subcutaneous adipose tissue (open symbols). Bacterial growth controls for S. aureus and P. aeruginosa are shown as solid diamonds. All data are presented as means ± SD (n = 6). The detection limit is indicated for plasma by a dotted line and for subcutis by a dashed line.](image)
The exposure of *S. aureus* strains (MICs of 4 and 8 mg/L) to piperacillin concentration-versus-time profiles in plasma and subcutis effectively reduced bacterial counts during the observation period of 8 h. The mean log_{10} differences between initial and final bacterial concentrations after 8 h were −1.75 ± 0.29 and −1.00 ± 0.53 cfu/mL for the strain with an MIC of 4 mg/L and −1.39 ± 0.93 and −0.98 ± 0.51 cfu/mL for the strain with an MIC of 8 mg/L for plasma and subcutis, respectively. For the *S. aureus* strain with an MIC of 16 mg/L, the experiments resulted in a −4.85 ± 1.15 log_{10} reduction of cfu/mL when plasma concentrations were employed, whereas simulating concentration-versus-time profiles of subcutis led to growth of bacteria (1.18 ± 1.32 log_{10} cfu/mL, *P* < 0.05) at a magnitude similar to the control curve.

Exposure of clinical isolates of *P. aeruginosa* with MICs of 4, 8 and 16 mg/L to concentrations of piperacillin measured *in vivo* resulted in similar time–kill curves as observed for *S. aureus*. Mean log_{10} differences in cfu/mL were −1.53 ± 0.21 and −1.50 ± 0.48 for *P. aeruginosa* with an MIC of 4 mg/L and −3.33 ± 1.56 and −2.17 ± 0.60 for the strain with an MIC of 8 mg/L for plasma and subcutis, respectively. For *P. aeruginosa* with an MIC of 16 mg/L, a significant difference in bacterial killing was observed between the plasma compartment and subcutis (*P* < 0.05). The corresponding absolute values were log_{10} −4.33 ± 1.38 and log_{10} 3.28 ± 0.35 cfu/mL for plasma and subcutis, respectively.

### Discussion

From current literature there is circumstantial evidence that tissue penetration of antimicrobial agents is substantially impaired or delayed in septic patients compared with healthy controls.5,7,3,6,15,16 The authors of these reports suggested that this finding might result in inadequate antimicrobial killing at the target site in septic patients, despite proven susceptibility to the antibiotic. In the present study, we employed an *in vivo* PK/*in vitro* PD model to test this hypothesis. The PK/PD model used has been proven to be highly reproducible and suitable for the simulation of bacterial killing in plasma and target tissues.

The key finding of the present study was that the prediction of microbiological outcome based on plasma concentrations of piperacillin might overestimate its activity in subcutaneous adipose tissue, which is a potential source of infection in septic patients. Free piperacillin levels obtained in plasma effectively inhibited bacterial growth of all used *S. aureus* and *P. aeruginosa* strains, independently of their MICs (range 4–16 mg/L). In contrast, the concentration-versus-time profiles of piperacillin in subcutaneous adipose tissue were sufficient to kill isolates with MICs of 4 and 8 mg/L only, while bacterial growth of test strains with MICs of 16 mg/L was not inhibited (Figures 2 and 3).

While these findings are in agreement with the susceptibility breakpoints of 4–8 mg/L reported for *S. aureus* by the NCCLS, our results do not confirm previous data on susceptibility reported for *P. aeruginosa*, suggesting susceptibility of *P. aeruginosa* up to a breakpoint of 64 mg/L.14 A prime limitation in the interpretation of NCCLS breakpoints needs to be taken into account. For the determination of breakpoint concentrations, clinical data reporting on success of antimicrobial treatment are from patients who are more healthy than septic patients as indicated by the APACHE score and other scoring systems. Thus, for septic patients there are considerable concerns about the value of NCCLS breakpoints, since ‘threshold’ concentrations are based on PKs derived from plasma or on clinical success in mild or moderately ill patients, who hardly reflect the situation in critically ill patients.17

Indeed, in the present study, free piperacillin concentrations in plasma exceeded 64 mg/L and, although not tested, are likely to be able to eradicate pathogens with high MICs (Figure 1). In septic patients, however, free concentrations of antibiotics at the target site are substantially lower compared with plasma concentrations. This has been ascribed to shock and the so-called ‘third spacing’, i.e. a phenomenon that describes leakage of the capillary wall.
because of systemic inflammation and a shift of volume and plasma-proteins from the plasma compartment to the extravascular space.\textsuperscript{8,18} The free piperacillin concentrations in plasma exceeded the MICs for all employed isolates by several-fold during the 8 h of simulation (Figure 1). Irrespective of the MICs (range 4–16 mg/L) for the investigated isolates, no difference in bacterial killing was observed when plasma concentrations of piperacillin were simulated. For S. aureus and P. aeruginosa with respective MICs of up to 8 mg/L, a similar observation was made after simulating concentrations of subcutaneous adipose tissue, although free levels of piperacillin in tissue were only marginally or temporarily above the pathogen’s MIC. This can be attributed to the fact that \( \beta \)-lactams exert ‘time-dependent killing’, i.e. the time that the concentration of the antibiotic exceeds the MIC (\( t > \) MIC) determines the efficacy of bacterial killing.\textsuperscript{19,20} The ratio of the peak concentration to the MIC (\( C_{\text{max}}/\text{MIC} \)) is considered less relevant for \( \beta \)-lactams, provided that the concentration is higher than the pathogen’s MIC. Although maximum concentrations in subcutaneous adipose tissue were close to 16 mg/L for a short period of time, no relevant bacterial growth inhibition was observed for pathogens with MICs of 16 mg/L, confirming current knowledge that the concentrations of antimicrobial agents must exceed the pathogen MIC for distinct time periods.

The presence of subinhibitory concentrations is one possible promoter of resistance in the remaining bacterial population.\textsuperscript{21} For ‘time-dependent’ antibiotics, such as \( \beta \)-lactams, the concentrations in plasma and tissue are strongly recommended to exceed the respective MIC for a period of 35–70% of the dosing interval to avoid regrowth and development of resistance.\textsuperscript{19,22,23} Taking this into consideration, treatment of septic patients with a dose of 4 g of piperacillin three times daily appears rational for strains with MIC < 8 mg/L only (Table 1). Calculating the average steady-state concentrations (average \( C_{\text{ss}} \)) of piperacillin in plasma and subcutaneous adipose tissue by use of the formula: average \( C_{\text{ss}} = \) area under the concentration-versus-time curve from zero to infinity (\( \text{AUC}_{0-\infty} \)) divided by the dosing interval of 8 h, reveals that the average \( C_{\text{ss}} \) of piperacillin will be around 83 ± 52 (range: 30–182) mg/L for plasma and 4.4 ± 3.1 (range: 1.3–8.7) mg/L for subcutis after iv administration of 4 g three times a day. These observations are in line with recent data with levofloxacin obtained in septic patients, showing that high inter-individual variability of tissue concentrations and the subsequent killing of bacteria might exist.\textsuperscript{3} In this condition, modification of the dosing regimen was recommended to help avoid therapeutic failure in individual septic patients. Given the linear PK behaviour of piperacillin, the doubling of the dose to 8 g three times daily should result in average concentrations of 166 ± 104 and 8.8 ± 6.2 mg/L for plasma and subcutaneous adipose tissue, respectively. In order to achieve higher \( t > \) MIC, the infusion time might be increased or the dosing interval shortened, as indicated by a recent study using Monte Carlo simulation for the investigation of different dosing regimens of piperacillin.\textsuperscript{24} Additionally, a loading dose seems advisable in septic patients, since steady-state concentrations could be reached without any marked delay and subinhibitory concentrations could be avoided.

In conclusion, breakpoint concentrations calculated by use of plasma data of piperacillin and probably other \( \beta \)-lactams might overestimate respective drug concentrations and their antimicrobial activity in subcutaneous adipose tissue in septic patients. Our study supports the recommendation of regulatory authorities to evaluate antimicrobial activity by use of \textit{in vivo} PK/\textit{in vitro} PD models based on concentrations derived from the infected site.

### Transparency declarations

We declare that we have no financial or ethical conflicts of interest in connection with this paper.

### References


