Protein Binding of Beta-Lactam Antibiotics in Critically Ill Patients: Can we successfully predict unbound concentrations?

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Keywords: pharmacokinetics, pharmacodynamics, therapeutic drug monitoring, TDM, intensive care, ICU

Word counts: Abstract 258; Main Body 2463

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The use of therapeutic drug monitoring (TDM) to optimize beta-lactam dosing in critically ill patients is growing in popularity, although there is limited data describing the potential impact of altered protein binding on achievement of target concentrations. The aim of this study was to compare the measured unbound concentration with the unbound concentration predicted from published protein binding values for seven beta-lactams using data from blood samples obtained from critically ill patients. From 161 eligible patients, we obtained 228 and 221 plasma samples at mid-dosing interval and trough respectively for ceftriaxone, cefazolin, meropenem, piperacillin, ampicillin, benzylpenicillin, and flucloxacillin. The total and unbound beta-lactam concentrations were measured using validated methods. Variability in both unbound and total concentrations were marked for all antibiotics, with significant differences present between measured and predicted unbound concentrations for ceftriaxone, and for flucloxacillin at mid-dosing interval (p<0.05). The predictive performance for calculating unbound concentrations using published protein binding values was poor with bias for over-prediction of unbound concentrations for ceftriaxone (83.3%), flucloxacillin (56.8%), and benzylpenicillin (25%) and under-prediction for meropenem (12.1%). Linear correlations between the measured total and unbound concentrations were observed for all beta-lactams ($R^2 = 0.81 – 1.00$, $p<0.05$) except ceftriaxone and flucloxacillin. The percentage protein binding of flucloxacillin and plasma albumin concentration were also found to be linearly correlated ($R^2 = 0.776$, $p<0.01$). In conclusion, significant differences between measured and predicted unbound drug concentrations were found only for the highly protein bound beta-lactams ceftriaxone and flucloxacillin. However, direct measurement of unbound drug in research and clinical practice is suggested for selected beta-lactams.
INTRODUCTION

Infections are common in intensive care units (ICU), with over 50% of patients considered infected at any one time (1). Morbidity and mortality remains high among critically ill patients with infection and antibiotics are the single most effective intervention for reducing mortality rates (2-7). Complicating the likelihood of achieving effective antibiotic therapy are the effects of the complex disease processes that critically ill patients undergo and the associated significant effects on antibiotic pharmacokinetics (PK). For the commonly used family of antibiotics, the beta-lactams, these PK changes, including those relating to altered protein binding, have been shown in numerous studies to result in high proportions of critically ill patients manifesting sub-therapeutic or toxic concentrations when standard dosing approaches are used (7-12). Given the association between therapeutic antibiotic exposure and improved patient outcomes (13, 14), the use of therapeutic drug monitoring (TDM) to optimize beta-lactam exposures has been proposed as potentially useful for dose optimization in critically ill patients (8, 15-18).

The existing reports for beta-lactam TDM have all used total antibiotic concentrations for determining the need for dose adjustment which is problematic given the unbound concentration of antibiotic is responsible for bacterial killing. The accuracy of such an approach is unclear. Given that hypoalbuminaemia occurs in approximately 40% of critically ill patients (19, 20), the potentially negative effects on altering protein binding of beta-lactam antibiotics may be common (21-24). It is likely that direct measurement of unbound antibiotic concentrations should be preferred to calculating unbound concentrations from published protein binding values because such calculations may not reflect the unbound beta-lactam plasma concentration in a critically ill patient and therefore reducing the likelihood of achieving optimized therapeutic dosing. Understanding the accuracy of protein binding of beta-lactams in this challenging patient population is essential to ensure optimal clinical dose adjustment.
Given the uncertainty regarding protein binding changes in critically ill patients, the aim of this study was to compare the measured unbound concentration with the unbound concentration predicted from published protein binding values for seven beta-lactam antibiotics (ceftriaxone, cefazolin, meropenem, piperacillin, ampicillin, benzylpenicillin, and flucloxacillin).

**MATERIALS AND METHODS**

*Patient selection*

This observational study was conducted as part of a beta-lactam TDM program in critically ill patients at a 27-bed tertiary referral ICU. Beta-lactam TDM is provided as a part of routine clinical care in this unit. Approval to collect this data was granted by the local institutional review board (Royal Brisbane and Women's Hospital, Human Research Ethics Committee).

Ten antibiotics are included in the routine TDM service: ampicillin, benzylpenicillin, dicloxacillin, flucloxacillin, piperacillin, ceftriaxone, cephalothin, cephazolin, meropenem, and ertapenem (27). Patients were eligible for inclusion in this analysis if they were >18 years old, receiving one of the selected study antibiotic(s) and expected to remain on the treatment for the next 24 hours. The empiric dosing regimen and subsequent dose adjustment was undertaken by the treating physician in consultation with the clinical pharmacist.

Various demographic and clinical data were collected to describe the patient sample including age, gender, weight, plasma albumin and creatinine concentrations. Creatinine clearance on the sampling day was also collected, which was estimated from plasma creatinine concentrations using the Cockcroft-Gault equation (28).
Sampling

As per the TDM protocol, blood samples were obtained at assumed PK 'steady state', defined as sampling after administration of at least four prior doses. For intermittent dosing, two plasma samples were obtained, firstly at the mid-point of the dosing interval and secondly, immediately prior to re-dosing (trough concentration). For continuous infusion, plasma samples were taken after at least four half-lives. Patients treated with more than one study antibiotic were eligible to provide more than one set of blood samples on the same day.

Beta-lactam assays

Plasma total and unbound concentration of beta-lactams were determined using two different validated high-performance liquid chromatography (HPLC) assays, which have been published previously (27, 29). Briefly, to measure total beta-lactam concentrations, plasma samples were extracted with a known amount of internal standard and de-proteinated with acetonitrile. The supernatant was added to chloroform and the aqueous phase was analyzed using HPLC. The concentration ranges of the standard curves were 1 – 500 mg/L for all antibiotics (except meropenem 1 – 250 mg/L). The coefficients of variation for inter-assay and intra-assay precision were <10%, and the accuracy was within 6% for all antibiotics. To measure unbound drug concentration, plasma sample were filtered by an Amicon Ultra-0.5 mL 30,000 molecular weight cut-off centrifugal filter device. The ultrafiltrate were mixed with MES buffer (pH 6.6) and analyzed using HPLC. The concentration ranges of the standard curves were 0.1 - 50 mg/L for all antibiotics (except piperacillin 0.1 – 100 mg/L). The coefficients of variation for inter-assay and intra-assay precision were <10%, and the accuracy was within 10% for all antibiotics.

Calculation of unbound concentrations from total concentrations
Unbound concentrations of antibiotics were calculated from total concentrations using published percentage binding data as shown in Table 1. In view of the saturable concentration-dependent protein binding kinetics of ceftriaxone, unbound concentrations of ceftriaxone were calculated from total concentrations ($C_{\text{tot}}$) using the following equation (30):

$$C_{\text{free}} = \frac{1}{2} \left[ -(nP + \frac{1}{k_{\text{eff}}} - C_{\text{tot}}) + \sqrt{(nP + \frac{1}{k_{\text{eff}}} - C_{\text{tot}})^2 + \frac{4C_{\text{tot}}}{k_{\text{eff}}}} \right]$$

where capacity constant ($nP$) = 517 mol/L, binding affinity constant ($K_{\text{aff}}$) = 0.0367 L*/mol

Statistics

All continuous data were reported as means and standard deviations, or medians and interquartile ranges as appropriate. Continuous data were analyzed using the Student’s t-test. Correlation between total and unbound antibiotic concentrations, and co-variations between continuous demographic and clinical variables with antibiotic concentrations and percentages of protein binding were determined using linear regression. Bland-Altman plots were constructed with GraphPad (version 6.0a, Graphpad Software Inc) to assess the agreement between calculated unbound beta-lactams concentrations and measured unbound concentrations. Percentage transformation was performed to increase the normality of the data for the constructions of Bland-Altman plots, as evaluated by Spearman’s rank correlation coefficient ($\rho$). Bias, 95% limits of agreement and corresponding 95% confidence intervals were calculated as previously described (31). $P$ values < 0.05 were considered significant.

RESULTS

One hundred and sixty one patients were eligible for analysis and their demographic and clinical characteristics are shown in Table 2. Patients were typically male, older than 50 years old, with a serum albumin concentration below the normal range on the day of sampling. Renal
function, as described by plasma creatinine concentrations, was highly variable. Forty-two patients had TDM performed on more than one occasion and one patient received two beta-lactams simultaneously. Two hundred and twenty eight and 220 mid-dosing and trough samples were assayed. Due to limited sensitivity of the assay for total concentrations below 1 mg/L, 3 mid-dosing interval concentrations and 23 trough concentrations reported as <1 mg/L were excluded from the analysis. The total number of samples included in the below analyses was 422. Seventeen patients received continuous renal replacement therapy (CRRT) on the day of sampling.

The dosage ranges for the prescribed antibiotics are shown in Table 3. Variabilities in dosing regimen and resultant concentrations are observed, with marked deviation of predicted concentrations from the line of identity in particular for piperacillin and benzylpenicillin at both sampling times, and for trough concentrations of meropenem, ceftriaxone (Fig 1a) and flucloxacillin. Measured unbound concentrations tend to be higher than predicted for ceftriaxone at all concentrations, and for piperacillin, benzylpenicillin and flucloxacillin especially at higher concentrations. However, measured unbound concentrations were only significantly higher \( (p<0.05) \) than predicted for ceftriaxone at both sampling times, and for flucloxacillin at mid-dosing interval. The unbound fraction of piperacillin was significantly higher at the trough time point in patients receiving CRRT (82.5% versus 69.6% in non-CRRT patients, \( p<0.01 \)). No significant relationship was observed for other studied beta-lactams.

Linear correlation between the measured and predicted unbound concentrations was established for all studied beta-lactams except ceftriaxone and flucloxacillin. Linear regression correlations between total and unbound concentrations described using an \( R^2 \) value were between 0.81 and 1.00 for beta-lactams \( (p<0.001 \) for all except cefazolin (Fig 1b), \( p=0.003 \)). Non-linear correlation between the measured and predicted unbound concentrations was observed for
The predictive performance of the calculated unbound beta-lactams trough concentrations was assessed by Bland-Altman plots as shown in Figure 2 and Table 4. Bias in calculated unbound concentrations were observed for ceftriaxone, flucloxacillin and benzylpenicillin, where actual (measured) unbound concentrations were under-predicted. For meropenem and cefazolin, the calculated unbound concentrations biased for over-predicting unbound concentrations. The 95% limits of agreement for calculated unbound concentrations as a predictor of measured unbound concentrations were wide for the majority of studied beta-lactams.

No significant associations were found between percentage of binding and albumin concentrations for any of the beta-lactams studied except flucloxacillin ($R^2=0.76$, $p<0.01$). For cefazolin, the relationship between percentage of protein binding and albumin concentrations was not analyzed due to inadequate albumin concentration data available for those subjects.

**DISCUSSION**

In this study of critically ill patients, we compared the observed unbound concentration of beta-lactam antibiotics with the unbound concentration predicted using published protein binding values. This data confirms the high variability and in some cases unpredictability of unbound beta-lactam concentrations in critically ill patients. The present work is unique in terms of the number of antibiotics studied and the evaluation of predictive performance for calculating unbound beta-lactams concentrations using published protein binding values in critically ill patients.

The efficacy of beta-lactams has been well defined according to the time that the unbound (or free) concentration exceeds the minimum inhibitory concentration ($T_{\text{MIC}}$) of the bacterial
pathogen. Traditionally, most assays used in PK studies in critically ill patients measure total beta-lactam antibiotic concentrations and subsequently, calculate unbound concentrations from published plasma protein binding data that has been obtained from non-critically ill patient groups.

Since variation in protein binding and prevalence of hypoalbuminaemia among the critically ill has been observed in other studies (21, 26, 27), there is increasing concern of the accuracy of this estimation especially for high protein bound drugs in the critically ill. Since the time course of unbound beta-lactam concentrations is more relevant than total concentration, direct measurement of the unbound fraction has been suggested to have potential advantage in antibiotic dose optimization in the critically ill. In this study, we utilized a rapid and inexpensive assay for measurement of unbound beta-lactam concentrations in clinical practice. The data presented again demonstrate severely altered PK of beta-lactams in the critically ill.

As expected, in our cohort of critically ill patients with low plasma albumin concentration (mean 24.5 g/L), significant differences between predicted (from total concentration) and measured unbound concentrations were found for the highly protein bound antibiotics, ceftriaxone and flucloxacillin. The mean percentage of protein binding for ceftriaxone (87.3 – 87.7%) determined in this study lies within the lower limits (83 - 96%) found in healthy volunteers (32), yet is similar to that found in a group of surgical critically ill patients (85.5 - 91.5%) (33). However, when we used a saturable model of ceftriaxone protein binding with published binding parameters to predict unbound concentrations from our total concentrations data, percentage of protein binding was approximated to be around 95%, suggesting over estimation of protein binding by the model when applied to our patient cohort. Nevertheless, no significant correlation between albumin concentrations and the unbound fraction of ceftriaxone was found in this study. On the other hand, correlation between albumin concentrations and the unbound fraction was found for flucloxacillin
(Fig. 2), as observed previously in a cohort of septic neonates and adult critically ill patients whom both had lower than normal plasma albumin concentrations (26, 34). Of note, significant differences between measured and calculated unbound flucloxacillin concentrations were found only at the higher concentrations at the mid-dosing time point but not at the trough time point. This may reflect the non-linearity of protein binding at high concentrations, and thus poor prediction of unbound values at this range.

A reduction of unbound fraction for meropenem from 98% in healthy volunteers to a median of below 90% was observed among our patients. Despite this fact, there was no significant difference between measured and predicted unbound meropenem concentrations, possibly due to its relative low fraction of protein binding, where a small change in percentage of binding would only minimally alter the unbound drug concentrations (35). Our data demonstrate that plasma protein binding of beta-lactams in critically ill patients is highly variable and correlation with albumin concentration only exists for selected agents. Although linear correlations between total and unbound concentrations exist for some of the studied beta-lactams, the predictive performance of calculated unbound concentrations was of concern in terms of under-dosing especially for piperacillin at low concentrations (<50 mg/L) and for meropenem, where unbound concentrations were consistently under-predicted of a limited but considerable magnitude. Another important finding from this analysis was that for ceftriaxone, benzylpenicillin and flucloxacillin, the observed unbound concentration was higher than the predicted concentration meaning that dose adjustments based on the low predicted unbound concentrations may not always be required. Dose adjustments for possible concentration-related adverse events may also not be accurate in this context. The unbound concentration assay used in this study is an inexpensive and convenient means to overcome the limitation of predicting unbound drug concentrations under these circumstances.
Limitations of the study

Firstly, the variability of clinical conditions and interventions that can vary beta-lactams PK in the critically ill, as well as the small cohort of patients and samples available for the analysis of some antibiotics (namely cefazolin, benzylpenicillin and flucloxacillin) could be considered a limitation of this study. Nevertheless, this is the largest dataset of unbound measurement of these drugs.

Secondly, the assay used in this study has limited sensitivity for total beta-lactam concentrations less than 1 mg/L, such that conversion from total to unbound concentrations was not established for this low concentration range. Lastly, the 95% limits of agreement as determined by Bland-Altman plots depend on the assumption that the differences between the two measurement methods are constant throughout the range of measurements and follow a Gaussian distribution. Although percentage transformation (or logarithmic transformation, data not shown) improved the distribution of our data, the percentage differences between predicted and measured unbound concentrations still significantly varied with the mean of the two measurements for benzylpenicillin, flucloxacillin, piperacillin and cefazolin. However, the analysis used provides sufficient information on bias and precision of calculating unbound concentrations from total measured concentrations to conclude whether the predictive performance of the calculated unbound concentrations is adequate in the clinical context.

In summary, this is the first paper that directly compares measured total and unbound beta-lactam antibiotic concentrations in critically ill patients. We found a high variability in beta-lactam concentrations and plasma protein binding in a cohort of critically ill patients. A correlation between percentage protein binding and plasma albumin concentrations was only observed in flucloxacillin. Given the variability of unbound beta-lactam concentrations in critically ill patients and the clinical importance of unbound drug concentrations, utilization of an inexpensive and convenient assay for unbound drug in research and clinical practice is suggested.
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401 Simulation to the Development of Susceptibility Breakpoints for Neisseria meningitidis.
Table 1. Published percentages of protein binding for the study antibiotics.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Published Average (range)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>30%</td>
<td>Anonymous (Pfizer) (36)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>2%</td>
<td>Craig (37)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>89.5% (83 - 96%)</td>
<td>Adnan et al. (38)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20% (15 - 25%)</td>
<td>Burgess et al. (39)</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>80% (74 - 86%)</td>
<td>van Kralingen et al. (40)</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>65%</td>
<td>Petri (41)</td>
</tr>
<tr>
<td>Flucloxacillin</td>
<td>93%</td>
<td>Chambers (42)</td>
</tr>
</tbody>
</table>
Table 2. Demographics and clinical characteristics of the studied patients. Data are described as mean ± SD, or median (IQR).

* Plasma creatinine and plasma albumin concentrations were measured on the day of sampling;
other parameters were measured upon admission.

** Creatinine clearance was estimated using the Cockcroft-Gault formula.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (% male)</td>
<td>59.6%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52 ± 17</td>
</tr>
<tr>
<td>Plasma creatinine concentration (μmol/L)</td>
<td>69 (30 – 381)</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)**</td>
<td>131.9 ± 75.4</td>
</tr>
<tr>
<td>Plasma albumin concentration (g/L)</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>80.6 ± 22.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.6 ± 7.4</td>
</tr>
</tbody>
</table>
Table 3. Dosage ranges for studied beta-lactams.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Dosage range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>500 mg 12-hourly – 2000 mg 6-hourly</td>
</tr>
<tr>
<td>Piperacillin/ tazobactam</td>
<td>4500mg 12-hourly – 4500mg 6-hourly</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1000 mg 8-hourly – 1000 mg 6-hourly</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>1000 mg 12-hourly – 2000 mg 8-hourly</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1000 mg 8-hourly – 2000 mg 8-hourly</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>1200 mg 4-hourly – 2400 mg 4-hourly</td>
</tr>
<tr>
<td>Flucloxacillin</td>
<td>1000 mg 4-hourly – 2000 mg 4-hourly</td>
</tr>
</tbody>
</table>
Table 4. Performance evaluation of calculated unbound concentrations as a predictor of measured unbound concentrations using bias, 95% limits of agreement and associated confidence intervals (CIs) as determined by Bland-Altman plots.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Bias</th>
<th>95% CI (bias)</th>
<th>95% limits of agreement</th>
<th>95% CI (lower limit)</th>
<th>95% CI (upper limit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piperacillin</td>
<td>0.43%</td>
<td>-6.6% to 7.4%</td>
<td>(-34.7%, 39.6%)</td>
<td>-41.7% to -27.7%</td>
<td>33.6% to 46.6%</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>-5.08%</td>
<td>-15.5% to 5.4%</td>
<td>(-22.2%, 12.0%)</td>
<td>-32.7% to -11.7%</td>
<td>1.5% to 22.5%</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>-25.00%</td>
<td>-57.4% to 7.4%</td>
<td>(-86.97%, 37.0%)</td>
<td>-119.3% to -54.6%</td>
<td>4.6% to 69.4%</td>
</tr>
<tr>
<td>Meropenem</td>
<td>12.08%</td>
<td>6.6% to 17.6%</td>
<td>(-10.2%, 34.4%)</td>
<td>-15.7% to -4.7%</td>
<td>28.8% to 39.9%</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>-83.30%</td>
<td>-117.2% to -49.4%</td>
<td>(-168.5%, 1.9%)</td>
<td>-202.4% to -134.6%</td>
<td>-32.0% to 35.8%</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>9.33%</td>
<td>-41.1% to 59.7%</td>
<td>(-55.7%, 74.4%)</td>
<td>-106.1% to -5.3%</td>
<td>24.0% to 124.8%</td>
</tr>
<tr>
<td>Flucloxacillin</td>
<td>-56.80%</td>
<td>-130.1% to 16.5%</td>
<td>(-197.2%, 83.6%)</td>
<td>-270.5% to -123.9%</td>
<td>10.3% to 156.9%</td>
</tr>
</tbody>
</table>
Figure 1. Linear correlation between measured and predicted unbound trough concentrations of a) ceftriaxone and b) cefazolin ($R^2 = 0.96$, $p=0.003$). The x=y plots is shown as the grey dashed line.

Figure 2. Bland-Altman plots of relative difference (percentage of measured unbound concentrations) against mean of predicted and measured unbound concentrations for a) piperacillin ($n=94$, $\rho = -0.51$, $p<0.01$); b) ampicillin ($n=8$, $\rho = 0.42$, $p=0.30$); c) benzylpenicillin ($n=11$, $\rho = -0.92$, $p<0.01$); d) meropenem ($n=49$, $\rho = 0.02$, $p=0.91$); e) ceftriaxone ($n=19$, $\rho = -0.43$, $p=0.07$); f) cefazolin ($n=5$, $\rho = -0.80$, $p<0.01$), and g) flucloxacillin ($n=11$, $\rho = -0.81$, $p<0.01$). The bias and 95% limits of agreement were shown in solid (-----) and broken (------) horizontal lines respectively.