Adequate cefazolin therapy for critically ill patients: can we predict active concentrations from given protein-binding data?

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Abstract

Objectives: Therapeutic drug monitoring of β-lactam antibiotics has become an important tool for treatment of severe infections, especially for critically ill patients who often exhibit altered PK/PD. Therapeutic targets are based on MIC, which refers to the active concentration of the drug. Cefazolin, a β-lactam agent used for treating of MSSA bacteraemia, has a protein binding of approximately 80 %. Therefore, a reliable determination of the active, non-protein-bound concentration is required to ensure optimal therapeutic outcome.

Methods: From seven critically ill patients who received an initial dose of 2 g cefazolin, followed by a continuous 24 h infusion, a total of 24 serum samples were obtained. The non-protein-bound concentration was directly measured after ultrafiltration and compared to prediction based total concentrations and protein binding values from the literature. For the analysis, a rapid and reliable LC-MS\textsuperscript{3} based assay was established, offering maximum sensitivity and specificity.

Results: The measured non-protein-bound concentration varied over a wide range (7.6–118 mg/L), with 22 out of 24 samples exhibiting cefazolin levels above the therapeutic target values (8–16 mg/L). Additionally, the observed protein binding ranged from 29 to 78 % (median 66.8 %), which was significantly lower than that reported in the literature. When comparing the measurements to the predictive performance of total concentrations and protein binding values, poor results were obtained.

Conclusions: The results show a high variability in plasma protein binding of cefazolin in critically ill patients.

Introduction

The hospital mortality of methicillin-sensitive \textit{Staphylococcus aureus} (MSSA) bacteraemia is high, ranging from 15 to 40 % [1]. The best clinical outcome in the treatment of MSSA bacteraemia is described for the β-lactam antibiotics flucloxacillin and cefazolin [2]. Cefazolin has a lower risk of adverse drug reactions such as nephrotoxicity and hepatotoxicity compared to flucloxacillin. Therefore, cefazolin is an appealing alternative for the therapy of patients with MSSA bacteraemia. A TDM-controlled optimization of antibiotic therapy is part of the S3 guideline of the German Society for Infectious Diseases on antibiotic stewardship (ABS) [3] and is recommended for an optimal therapeutic outcome [4].

During the last decades a number of HPLC-UV/Vis [5–10] and LC-MS/MS [11–16] methods for the monitoring of cefazolin in various biological matrices were published. Most of these methods apply protein precipitation as a standard procedure for sample preparation [5–9, 11–15]. By precipitating and thus denaturing plasma proteins with organic solvents, salts, or acids, their binding capacity is lost and consequently previously bound antibiotic agents are released. This results in a determination of the total fraction of the investigated analyte. The target serum level of most β-lactam antibiotics is based on minimum inhibitory concentration (MIC) or epidemiological cut-off (ECOFF) values, which, however, refer to the respective non-protein-bound fraction. For antibiotics with a low plasma protein binding (PPB), e.g., meropenem (PPB 2 %) [17], the difference between total and non-protein-bound, active fraction is negligible. For antibiotics with high PPB, such as cefazolin (PPB 78–86 % [18]), the non-protein-bound, “active” serum level is much lower than...
the total concentration. Accordingly, a reasonable therapeu-
tic drug monitoring (TDM) of the non-protein-bound
fraction is appropriate. Although equilibrium dialysis is still
the gold standard, other techniques such as ultrafiltration
(UF), microdialysis (MD), and solid phase extraction (SPE)
for removing plasma proteins including their bound agents,
can be applied to quantify the free concentration of a drug. As an
alternative to these costly and time-consuming procedures,
some studies have tried to calculate the non-protein-bound
fraction based on PPB values published in the literature
[19]. However, this approach is doubtful, as PPB may vary
depending on other factors such as co-medication and
hypoalbuminemia, which often appear in critically ill
patients when optimal antimicrobial therapy is most
necessary [20]. The period of the dosing interval in which the
free (non-protein-bound) part of the agent is above the MIC
of the organism is essential for the efficacy of β-lactam
agents. To optimise the clinical outcome, about four to eight
times the MIC for 100 % of the dosing interval of the non-
protein-bound part of the agent (100 % > four to eight times
MIC) for critically ill patients is recommended [21]. The
epidemiological cut-off value (ECOFF) for MSSA is 2 mg/L for
cefazolin [22]. To achieve the target serum level (100 %
> four to eight times MIC) for critically ill patients is recom-
manded [23]. This study aims to investigate the non-protein-bound fraction of cefa-
zolin in comparison to the protein-bound fraction, taking
into consideration the albumin levels, in order to verify
whether calculating the non-protein-bound fraction from
the total concentration yields satisfactory results.

Materials and methods

Instrumentation

Chromatographic analyses were performed using a Nexera XR series
high performance liquid chromatography system (Shimadzu, Jena,
Germany) with two pumps, vacuum degassers, column oven and a CTC
xt auto sampler (PAL, Zwingen, Switzerland) coupled to a Sciex 5500
QTRAP triple-quadrupole mass spectrometer equipped with electrospray
(ESI) source (Sciex, Darmstadt, Germany). The Peltier sample trays
and oven temperature were set to 8 and 50 °C, respectively. For the
chromatographic separation a reverse phase Luna® 3 µm PFP(2) 100 Å;
50 × 2.0 mm I.D. column (Phenomenex, Aschaffenburg, Germany) with a
nylon pre-filter (Recipe, Germany) was used. Mobile phases A (0.1 %
formic acid, 0.35 mL/min) and B (methanol, 0.15 mL/min) were delivered
through a total flow rate of 0.5 mL/min in an isocratic mode with a total
run time of 2 min. Eluting compounds were detected in a MS3 mode
(MRM3) with electrospray ionization at an ion spray source voltage (IS)
of 4500 V in positive mode. Nebulizer gas (GAS 1) and turbo gas (GAS 2)
were set at 50 psi, curtain gas (CUR) to 40 psi, the collision gas (CAD) to
low flow, and desolvation temperature (TEM) to 400 °C. As 1st precursor
ion m/z 455 was chosen for cefazolin and 458 for ISTD, as 2nd precursor
ion m/z 323 was chosen for cefazolin and 326 for ISTD. For both analytes
the declustering potential (DP) was set to 40 V, the entrance potential
(EP) to 10 V, and the collision energy (CE) to 15 V. The same setting was
used for the detection of CFA and ISTD resulting in a total cycle time of
370 ms (for more details see Table 1). For data acquisition and processing
Analyst software (1.7.0) and Multi Quant Software (3.0.2) were used
(Sciex, Darmstadt, Germany).

Standards and reagents

Water, methanol, acetonitrile, and formic acid (all LC-MS grade) were
supplied by Diagonal (Münster, Germany). Isotope labelled cefazolin
([13C2,15N]-cefazolin) was purchased from Alsachim (Illkirch-Graffen-
stadt, France) and cefazolin-Na from Sigma-Aldrich (Hamburg, Ger-
many). The antibiotic infusion solutions (Cefazolin Hikma 2 g) were
obtained through the dispensary at Klinikum Chemnitz. Fresh frozen
plasma was obtained from a volunteer donor and checked for in-
terferences prior to use.

Ultrafiltration

To determine the free fraction, an ultrafiltration (UF) of the serum
samples was performed to remove proteins and, consequently, the
bound cefazolin. Ultrafiltrates were obtained by filtering serum through
3 kDa UF devices (NanoSep® Centrifugal Devices with Omega™ Mem-
brane 3 K, Pall Corporation, Port Washington, NY, USA). Prior to use, UF
devices were tempered to 37 °C (30 min). Patient samples (300 mL) were
tempered to 37 °C (10 min), loaded on the UF devices and centrifuged at
37 °C (1,250×g; 30 min) for filtration.

Preparation of stock solutions, standards, quality control
samples (QCs), and internal standard solutions

Stock solutions of the antibiotic cefazolin (CFA) were prepared by
diluting the antibiotic infusion solutions and analytical standards of the
individual substances in water. For the preparation of the [13C2,15N]-
cefazolin (ISTD) stock solution 1 mg of ISTD was dissolved in 1 mL of
methanol. All solutions were stored at ~80 °C until further use. Cali-
bration standards (0.25, 1, 4, 20, and 100 mg/L) were obtained by diluting
stock solution from antibiotic infusion solutions with 0.9 % NaCl. For the
preparation of QC samples (2.0 and 40 mg/L) aliquots of plasma and

Table 1: MS3 detection parameters for cefazolin and [13C2,15N]-
cefazolin.

<table>
<thead>
<tr>
<th>QTRAP parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range, Da</td>
<td>154–158</td>
</tr>
<tr>
<td>Scan rate, Da/s</td>
<td>1,000</td>
</tr>
<tr>
<td>Fill time, ms</td>
<td>Dynamic</td>
</tr>
<tr>
<td>Settling time, ms</td>
<td>25</td>
</tr>
<tr>
<td>Excitation time, ms</td>
<td>20</td>
</tr>
<tr>
<td>Q3 entry barrier, V</td>
<td>8</td>
</tr>
<tr>
<td>AF2, V</td>
<td>0.1</td>
</tr>
<tr>
<td>CES, V</td>
<td>0</td>
</tr>
</tbody>
</table>


plasma ultrafiltrates were spiked with 10 % volume of respective analytical standard solution for the preparation of independent matrix IQCs. Before use, calibration standards and IQCs were checked against a commercially available serum calibrator set (Recipe, Munich, Germany), showing a deviation of <10 %. The internal standard solution was prepared by adding the stock solutions of ISTD (25 µL) to a mixture of acetonitrile (5 mL) and methanol (5 mL). The solution was stored at −20 °C until it was needed for further use.

Sample collection

Serum samples were collected between 12/2021 and 12/2022 from adult patients who received an initial dose of cefazolin 2 g infused over 30 min, followed by a continuous infusion of cefazolin 6 g/d (cefazolin 2 g in 0.9 % NaCl 50 mL; 6.3 mL/h, target serum level 8–16 mg/L) until further TDM-based dose adjustments were made. Samples were drawn after at least 24 h at a constant cefazolin infusion rate.

Sample preparation

Patient blood samples were stored and transported under cooled conditions (2–8 °C) until they arrived at the laboratory within 3 h. After centrifugation, the samples were analysed immediately or stored at −20 °C for 1 day at most. For measurements of the free fraction, 50 µL of the prepared internal standard solution was added to 25 µL of ultrafiltrate and mixed thoroughly. Then, 20 µL of this solution was transferred to a glass vial, diluted with 200 µL of a water/methanol (4 + 1) solution, and mixed thoroughly before injection (3 µL) for chromatographic analysis. For measurements of total cefazolin, 50 µL of the prepared internal standard solution was added to 25 µL plasma, vortexed for 30 s, and centrifuged (10,000×g) at ambient temperature for 5 min to remove proteins. Then, 20 µL of the supernatant was transferred into a glass vial, diluted with 500 µL of a water/methanol (4 + 1) solution, and mixed thoroughly before injection (3 µL) for chromatographic analysis.

Determination of albumin and total protein

Serum albumin was determined by a commercial assay (ALB2, Roche Diagnostics, Mannheim, Germany) on a cobas 8000 analyser (Roche Diagnostics). The assay is based on a colorimetric reaction with bromcresol green at a pH of 4.1, resulting in blue-green colour. The colour intensity is directly proportional to the albumin concentration in the sample. It is determined by monitoring the increase in absorbance at 583 nm. The samples were assayed according to the manufacturer’s instructions.

Methods and validation

Calibration: For daily measurements, a five-level-calibration, covering the expected concentration ranges, with an additional blank sample was performed. Calibration curves were calculated by linear regression.

Assay precision and accuracy: Calculations of intra-assay precision and accuracy were performed by conducting 21 replicate measurements of two respectively three quality control samples. Inter-assay precision and accuracy were calculated based on a total of 20 measurements of both QC levels. Calibration was performed before each run, with a maximum of four runs conducted within a 24 h period. Accuracy (in %) was calculated as 100 × concentration observed/nominal concentration. Precision (in %) was described as the relative standard deviation (RSD) of the measured values (see Table 2).

Measuring range: Limits of blank (LOB), limits of detection (LOD), and lower limit of quantitation (LLOQ) were calculated according to the literature [24] (see Table 3).

Recovery: The non-specific binding of filtration membranes was evaluated by comparing the cefazolin concentration in the samples before and after UF. Therefore, UF devices were loaded with CFA spiked ultrafiltrates, containing concentrations of 1.0, 4.0, and 20 mg/L.

Specificity and sample stability: Specificity and sample stability for CFA were determined as described previously by Böhle et al. [25] The blood samples were confirmed to be stable at temperatures of 2–8 °C for up to 3 h and after centrifugation for at least 1 week at −20 °C. Following sample preparation, no loss of concentration was observed after 8 h at ambient conditions (20–30 °C) or after 24 h at 2–8 °C. Furthermore, samples that were frozen at −80 °C for long-term storage were found to be stable for at least six months.

Results

Method development

For an optimal peak shape and signal intensity, cefazolin was measured under acidic conditions, using 0.1 % formic acid and methanol as mobile phases. To establish a high throughput LC-MS method, it is recommended to use an isotope-labelled internal standard to compensate effects of ion suppression. To gain the highest specificity possible, a LC-MS³ method (MRM³) was set up instead of standard MRM mode, eliminating all observed interferences by a two stage fragmentation of cefazolin (455 → 323 → 156) and its internal standard [13C2,15N]-cefazolin (458 → 326 → 156). Under isocratic elution conditions, no column contamination was observed, even at longer test batches (40 samples).

Validation data (Tables 2 and 3)

Table 2: Intra- and inter-assay precision (RSD) and accuracy (bias) for antibiotic drug monitoring of cefazolin with LC-MS³.

<table>
<thead>
<tr>
<th></th>
<th>Ultra-filtrate</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal conc.</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Mean conc.</td>
<td>2.00</td>
<td>2.05</td>
</tr>
<tr>
<td>Bias, %</td>
<td>0.09</td>
<td>1.52</td>
</tr>
<tr>
<td>RSD intra ass.</td>
<td>4.78</td>
<td>3.84</td>
</tr>
<tr>
<td>RSD inter ass.</td>
<td>5.27</td>
<td>5.32</td>
</tr>
</tbody>
</table>
Determination of non-protein-bound and total concentration

To determine the non-protein-bound part of CFA, an UF of the plasma samples was performed, effectively removing proteins and consequently the bound CFA. To maintain physiological conditions and prevent shifts in PPB, patient samples and UF devices were tempered to 37 °C before the filtration process. Since adsorption effects on filtering membranes were previously reported, various UF devices (NanoSep®, Vivacon®, VivaSpin®) were subjected to testing, where only NanoSep® membranes revealed average recoveries of nearly 100 % over the complete investigated concentration range.

Given that serum albumin (46 kDa) is the major transport protein, membranes with a molecular weight cut-offs (MWCO) of 3, 10 and 30 kDa were evaluated for further use. Clouding was occasionally observed in the 10 and 30 kDa MWCO filtrates after the addition of the internal standard solution. Consequently, the 3 kDa MWCO membranes were considered the reasonable and acceptable choice. However, it should be noted that these membranes necessitate longer centrifugation times (approximately 30 min) to attain the minimum required filtrate volume of 50 µL.

Samples for total CFA determination underwent standard protein precipitation with methanol/acetonitrile as a pre-treatment method. This process involves the denaturation of transport proteins, which results in the release of bound drugs from their respective binding sites. A total of 24 plasma samples from seven patients with blood stream or soft tissue infections were analysed (see Table 4). Additionally, three of these patients suffered from kidney failure, and three had a neoplastic disease. CFA monitoring was started after approximately 24 h of antibiotic therapy, when steady state of antibiotic-serum-concentration could be assumed.

Prediction of non-protein-bound concentration from total concentrations

The non-protein-bound portion of CFA is usually calculated from the total concentrations using PPB data [20], which was reported between 76 and 86 % [18]. However, there is doubt about whether this approach is reliable for critically ill patients. For a total number of 24 serum samples from the ICU, albumin, free, and total cefazolin concentrations were determined (see Figure 1). Firstly, a significantly lower PPB of 29–78 % was found (mean PPB 37 %; median PPB 32 %) than reported in the literature. Secondly, there was a linear but unsatisfactory correlation (R=0.667; slope=0.379) between the free and total fraction in the investigated samples. Only 2/24 measured free cefazolin concentrations were in accordance with the expected free concentration calculated from the total fraction (see Figure 2).

As albumin is the main plasma transport protein for cefazolin, the effects of hypoalbuminemia, which occurs in approximately 40 % of critically ill patients [26, 27] should be

Table 3: Limits of blank (LOB), limits of detection (LOD), lower limits of quantification (LLOQ), and upper limits of quantification (ULLOQ) for antibiotic drug monitoring of cefazolin with LC-MS³.

<table>
<thead>
<tr>
<th></th>
<th>LOB, mg/L</th>
<th>LOD, mg/L</th>
<th>LLOQ, mg/L</th>
<th>ULOQ, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra-filtrat</td>
<td>0.04</td>
<td>0.20</td>
<td>0.25</td>
<td>120</td>
</tr>
<tr>
<td>Serum</td>
<td>0.13</td>
<td>0.39</td>
<td>0.80</td>
<td>480</td>
</tr>
</tbody>
</table>

Table 4: Clinical data of the investigated patient cohort.

<p>| | |</p>
<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>7</td>
</tr>
<tr>
<td>Dose cefazolin, g/d</td>
<td>2–8</td>
</tr>
<tr>
<td>Samples</td>
<td>24</td>
</tr>
<tr>
<td>Male sex</td>
<td>4</td>
</tr>
<tr>
<td>Age, years</td>
<td>Mean: 75.4 (±10.0); median: 73</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>Mean: 33.3 (±8.0); median: 33</td>
</tr>
<tr>
<td>Total protein, g/L</td>
<td>Mean: 62.8 (±14.3); median: 60.5</td>
</tr>
<tr>
<td>Total CFA, mg/L</td>
<td>Mean: 156 (±70.5); median: 146.5</td>
</tr>
<tr>
<td>Non-protein-bound CFA, mg/L</td>
<td>Mean: 58.6 (±34.4); median: 52.1</td>
</tr>
<tr>
<td>Non-protein-bound CFA fraction, %</td>
<td>Mean: 36.8 (±8.8); median: 33.2</td>
</tr>
</tbody>
</table>

The ± values given in brackets refer to the standard deviations.

Figure 1: Total, protein-bound, and non-protein-bound cefazolin levels in 24 serum samples of seven patients. The grey bars shows the corresponding albumin concentrations. Between albumin, total cefazolin, and non-protein-bound cefazolin no correlation was found.
considered. Albumin concentrations in the samples ranged from 19 to 51 g/L, with only four samples falling within the reference range (39–50 g/L). Investigations did not reveal a correlation between serum albumin concentration and PPB. One individual with severe hypoalbuminemia (20 g/L) exhibited a cefazolin binding of just 29 %, while in two samples with similar serum albumin (19 and 22 g/L), PPB values of 54 and 67 % were found, respectively. Conversely, samples with normal albumin levels (44 g/L) and low albumin levels (25 g/L) showed PPB within expected range (76 and 86 %). Based on the available data, it is not possible to reliably calculate the free fraction of cefazolin from total plasma cefazolin in critically ill patients with blood stream or soft tissue infections.

Non-protein-bound cefazolin monitoring

The suggested dose adjustments following initial dosing were evaluated with a total of 24 samples from ICU patients, based on the non-protein-bound concentrations only. For CFA, the measured free concentrations ranged from 7.6 to 137 mg/L (median 52.1 mg/L) while the MIC was 2 mg/L (target serum level 8–16 mg/L). Only one sample showed a slightly lower level, representing just 95 % of the desired target value. Out of 24 samples, 22 had higher values than 100 % $f/T>four$ to eight times MIC, indicating dosing of four to eight fold of the ECOFF for MSSA in 7/24 samples. A value lower than 100 % $f/T>four$ to eight times MIC was practically not found in patient cohort observed.

Discussion

Even highly specific detection techniques, such as tandem-mass spectrometry, cannot guarantee the absence of interferences. Therefore, mass spectrometric interference experiments were performed with a panel of 170 test substances after setting up an initial LC-MS/MS method for cefazolin analysis. In the MS$^2$ mode (MRM), an interference for $^{34}$S-cefotaxime with the most intensive mass transition (458 $\rightarrow$ 326; 298; 255; 156; 133; 98) of the internal standard, $^{13}$C$_2^{15}$N-cefazolin was observed. The Sulphur isotope $^{34}$S has a natural abundance of 4.2 % [28], resulting in a 9.1 % occurrence of $^{34}$S-cefotaxime. This is problematic, as co-medications may appear and cefotaxime shows a higher sensitivity under the instrumental conditions applied. Since there was no other commercially available isotope-labelled cefazolin options, we made the decision to use a MS$^3$ mode (MRM$^3$) as a useful tool for interference elimination [29, 30]. The MRM$^3$ transitions 455 $\rightarrow$ 323 $\rightarrow$ 156 for cefazolin and 458 $\rightarrow$ 326 $\rightarrow$ 156 for $^{13}$C$_2^{15}$N-cefazolin exhibited satisfactory signal intensity and no significant interferences for $^{34}$S-cefotaxime or any other investigated drug. Even at high steady state cefotaxime levels of 100 mg/L, there was a residual interference of less than 1 % of the peak area compared to $^{13}$C$_2^{15}$N-cefazolin, which is deemed acceptable (see Figure 3). If cefotaxime can be chromatographically separated from cefazolin, measurements could also be performed in MRM mode. Therefore, an isocratic elution mode with a lower methanol portion (0.35 mL/min MPA + 0.1 mL/min MPB) was applied, resulting in longer run times. Additionally, a significantly reduced linear detection range (0.2–50 mg/L in filtrate and 0.8–200 mg/L in serum) was observed for MRM measurements compared to the dynamic MRM$^3$ trapping method (0.2–120 mg/L in filtrate and 0.8–480 mg/L in serum). Based on this, the determination in MRM$^3$ mode has shown to be superior to the common MRM mode. To ensure the stability of the mobile phase (A: 0.1 % formic acid; B: methanol), the components were stored in separate bottles, as methanol can cause the esterification of formic acid within a few days.

However, based on our studies thus far, it is not possible to reliably calculate the non-protein-bound fraction from the total serum cefazolin in patients with blood stream or soft tissue infections. Although determining the non-protein-bound serum level through UF is more time and material-consuming compared to the commonly used protein precipitation, it seems to be the only applicable procedure. As previously described, filtration results are also dependent on factors such as temperature and filter material [31]. Filtration at ambient temperature (20–25 °C) resulted in lower
filtrate concentrations compared to physiological conditions, as the albumin affinity of CFA increases at lower temperatures. Therefore the ultra-filtration procedure must be carried out at 37 °C to accurately reflect the pharmacological active fraction in vivo. Additionally, filtration devices exhibited significantly different adsorption characteristics for cefazolin depending on the investigated concentrations. According to literature [31], the unbound fraction showed only minor variation in the pH range from 7.4 to 8.5. Thus, no sample buffering was performed. Without further pretreatments, only the NanoSep® filtering membranes (modified polyethersulfone) showed recoveries of over 90 % in our experiments, even at concentrations below 1 mg/L.

A comparison of measured and calculated non-protein-bound CFA showed significantly higher concentrations than expected for in 23/24 cases for the investigated patient cohort. Consideration of albumin, the main transport protein, did not lead to an improved correlation. All attempts to predict the non-protein-bound fractions have been insufficient so far, as PPB varies depending on other factors such as co-medication and hypoalbuminemia, which is often present in critically ill patients. Nevertheless, assuming a PPB of approximately 80 % for calculations in most cases leads to an underestimation of the pharmacological active cefazolin.

**Conclusions**

In the last decade LC-MS methods have resulted in a boost to antibiotic TDM, enabling the development of fast, sensitive, and specific multi-analyte methods. Among these methods, LC-MS³ is the most specific procedure due to its elimination of interferences. In addition to the accessibility of antibiotic concentrations, the question arises regarding the clinical rationale that follows from these measurements. It is commonly accepted that only the free drug fraction mediates biological activity. However, most laboratories will only perform total measurements – especially in routine diagnostics. This difference is particularly crucial for antibiotics with high PPB, such as cefazolin. Current results
demonstrate that a simple calculation of the non-protein-bound cefazolin fraction from the total concentration after protein precipitation is insufficient. Contrary to the reported PPB of approximately 80% for cefazolin, the investigated patient cohort exhibited a much lower binding of approximately 40%, leading to an underestimation of the true free fraction and its antibiotic effects. Despite the disadvantages, direct measurement of the free concentration remains the most effective method for monitoring high-protein-bound antibiotics. Secondly, our investigation did not reveal any values lower than 100% *f/f* > four to eight times MIC for the standard dose in any of the patients. Instead, relatively higher values than 100% *f/f* > four to eight times MIC were found in 22/24 samples. Therefore, TDM is a more useful tool for dose adjustment and optimisation.

**Research ethics:** Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors’ Institutional Review Board or equivalent committee.

**Informed consent:** Informed consent was obtained from all individuals included in this study.

**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Competing interests:** Authors state no conflict of interest.

**Research funding:** None declared.

**Data availability:** The raw data can be obtained on request from the corresponding author.

**References**


