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Development and Validation of a High-Performance Liquid Chromatography–Ultraviolet Spectrometry Method for Ampicillin and Its Application in Routine Therapeutic Drug Monitoring of Intensive Care Patients

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Background: Ampicillin/sulbactam, a combination of a β-lactam and β-lactamase inhibitor, is widely used in clinical settings. However, therapeutic drug monitoring (TDM) of ampicillin is not commonly performed, particularly in intensive care units (ICUs). The purpose of this study was to develop and validate a rapid and cost-effective high-performance liquid chromatography (HPLC)–ultraviolet spectrometry method to quantify ampicillin in human serum and evaluate its clinical application in ICU patients.

Methods: Sample cleanup included a protein precipitation protocol, followed by chromatographic separation on a C18 reverse-phase HPLC column within 12.5 minutes using gradient elution of the mobile phase. The assay was validated according to the German Society of Toxicology and Forensic Chemistry criteria. Clinical applications involved the retrospective analysis of TDM data from ICU patients receiving continuous infusion of ampicillin/sulbactam, including the attainment of target ranges and individual predicted and observed pharmacokinetics.

Results: The method was robust, with linear relations between the peak area responses and drug concentrations in the range of 2–128 mg/L. The coefficient of variation for precision and the bias for accuracy (both interday and intraday) were less than 10%. Clinical application revealed variable pharmacokinetics of ampicillin in ICU patients (clearance of 0.5–31.2 L/h). TDM-guided dose adjustments achieved good therapeutic drug exposure, with 92.9% of the samples being within the optimal (16–32 mg/L) or quasioptimal (8–48 mg/L) range.

Conclusions: This method provides a practical solution for the routine TDM of ampicillin, facilitating individualized dosing strategies to ensure adequate therapeutic drug exposure. Given its simplicity, cost-effectiveness, and clinical relevance, HPLC–ultraviolet spectrometry holds promise for broad implementation in hospital pharmacies and clinical laboratories.

Key Words: ampicillin, pharmacokinetic/pharmacodynamics, therapeutic drug monitoring, high-performance liquid chromatography–ultraviolet spectrometry, continuous infusion

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INTRODUCTION

Ampicillin/sulbactam is a well-established combination of a β-lactam antibiotic and a β-lactamase inhibitor, exhibiting antibacterial activity against gram-negative, gram-positive, and anaerobic pathogens.[1,2] As a β-lactam, ampicillin demonstrates time-dependent bactericidal activity in which the reduction in bacterial load is directly related to the time that the free drug concentration is above the minimum inhibitory concentration (MIC) of the pathogen during the dosing interval. To take advantage of these bactericidal properties, prolonged (extended and continuous) infusion of intravenous β-lactams can be used to maintain concentrations above the MIC.[3] Recent studies have shown favorable outcomes associated with prolonged administration of β-lactams compared

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This study was approved by the local Ethics Committee (No. 137/19, amendment June 2021).

The requirement for signed informed consent was waived due to the retrospective and observational nature of the investigation, according to hospital agreements.

Data are available upon reasonable request.

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with those of intermittent dosing.[4] Indeed, ampicillin/sulbactam is a valuable alternative for susceptible pathogens, allowing de-escalation of antimicrobial stewardship programs, such as in pneumonia, provided adequate antibiotic exposure is guaranteed.[5,6]

Therapeutic drug monitoring (TDM) is increasingly used to ensure antibiotic concentrations and is endorsed by specialized medical societies, including the German Society of Intensive Care Medicine.[7–9] This is particularly pertinent for patients with variable pharmacokinetics, such as those in critical care settings, to address the variability in exposure observed during critical illness.[10]

Although concentration measurement is commonly established for vancomycin and aminoglycosides to balance effective drug concentrations and potential toxicities,[11,12] TDM for β-lactam antibiotics is not widely implemented.[12] Moreover, if β-lactam antibiotics are measured, this most often covers broad-spectrum and reserve antibiotics, for instance, piperacillin or meropenem.[12] However, for substances, such as ampicillin, TDM approaches do not appear to be readily implemented in routine care.[12] Developing a simple and rapid quantification method for ampicillin that can be easily integrated into practice-oriented TDM programs may enhance effective de-escalation strategies, particularly in critically ill patients.

Therefore, our objective was to validate a simple, rapid, and cost-effective high-performance liquid chromatography–ultraviolet (HPLC-UV) spectrometry method for routine TDM of ampicillin in human serum and evaluate its applicability in intensive care unit (ICU) patients in routine care.

MATERIALS AND METHODS

Reagents and Chemicals, and Chromatographic Setup

All solvents used were of HPLC or equivalent quality, and all reagents were of analytical grade. An overview of the reagents and chemicals employed in the developed method can be found in the **Supplemental Digital Content 1** (see **Supplement**, http://links.lww.com/TDM/A785).

Chromatographic analysis was performed using a reversed phase column Shim-pack XR-ODS III with 2.2-μm particle size (150 × 2 mm, Shimadzu, Duisburg, Germany) in combination with a column guard [Shim-pack GISS-H (G) C18, 3 μm; 10 × 2.1 mm, Shimadzu]. An aliquot of 20-μL was injected onto the HPLC-UV system (Nexera-I 3D plus; Shimadzu) equipped with a diode array detector (SPD-M20A, Shimadzu). The column temperature was kept at 50°C. Eluent A was prepared by diluting formic acid in water to a final concentration of 0.1% formic acid. Eluent B was prepared by diluting formic acid in acetonitrile to a final concentration of 0.1% formic acid. The detailed gradient elution program is presented in the **Supplemental Digital Content 2** (see **Table**, http://links.lww.com/TDM/A786, which demonstrates the elution gradient program). Ampicillin was monitored at a wavelength of 210 nm with a retention time of 3.6 minutes.

For peak identification, ampicillin (16 mg/L in water) was analyzed and assessed for intensity (area), shape, and retention time (see **Figure S1**, **Supplemental Digital Content 3**, http://links.lww.com/TDM/A787). Furthermore, chromatograms of a blank human serum sample, a sample with ampicillin 16 mg/L and internal standard (IS) in human serum, as well as a sample with ampicillin, IS and other antibiotics in human serum are depicted in the **Supplemental Digital Content 3** (see **Figures S2–S4**, http://links.lww.com/TDM/A787).

Preparation of Calibration Standards, Quality Controls, and IS

Stock solutions for calibration standards and quality controls (QCs) for measuring ampicillin were prepared by reconstituting 2000 mg of ampicillin in 1000 mL of ultrapure water (final concentration of 2000 mg/L ampicillin). Seven calibration standards were prepared using blank human serum at concentrations of 2, 4, 6, 16, 32, 64, and 128 mg/L. Three QC standards were prepared using blank human serum: low concentration (LQC) of 2 mg/L, medium concentration of 16 mg/L, and high concentration (HQC) of 128 mg/L. Two stability standards were prepared in blank human serum at concentrations of 20 and 40 mg/L. All solutions were aliquoted as 100 μL in polypropylene Eppendorf tubes and stored at −80°C, thawed just before use. For the IS, caffeine was dissolved in an acetonitrile:methanol (1:1) mixture to obtain a concentration of 50 mg/L.

Patient Sample Preparation

To determine ampicillin in serum, 100 μL of serum was mixed with 200 μL of IS solution. The mixture was vortexed for 10 seconds and centrifuged at ×8000*g* for 3 minutes. One hundred microliters of the supernatant were diluted 1:6 with water and vortexed for 10 seconds.

Validation Procedures

The method was validated according to Valistat 2.0 (ARVECON, Walldorf, Germany) as required by the German Society of Toxicology and Forensic Chemistry.[13] All validation procedures are described subsequently.

Linearity and Lower Limit of Quantification

The linearity of ampicillin was determined through a calibration curve of serum calibration standards, with each standard analyzed 6 times and evaluated by peak area and peak height versus target concentration, with an acceptable correlation coefficient of >0.95. The signal measured in the linearity and lower limit of quantification (LLOQ) samples should be at least 6 times the signal measured in a blank sample, with 6 blank samples prepared as described for QC, analysis, and comparison with the LLOQ.

Accuracy and Precision

Accuracy and precision were assessed by applying the developed method to measure QC (LQC, medium concentration, and HQC) concentrations 3 times in 3 runs on the same day and 3 runs on 6 different days. Accuracy was evaluated by calculating the bias (%) for each concentration. Assay

precision was expressed in terms of the coefficient of variation (%) and calculated for both between-run (reproducibility) and within-run (repeatability) variations. The maximum accepted value for both accuracy and precision was ±15%.

Recovery and Selectivity

Recovery was evaluated by measuring the spiked serum samples and spiked aqueous solutions (LQC and HQC) 6 times. Absolute recoveries (%) were calculated by comparing the peak areas of ampicillin in spiked human serum samples extracted as described previously with those of spiked aqueous solutions at the same concentration levels without extraction.

Selectivity was assessed by comparing the response of the blank serum of 6 individuals not receiving ampicillin with the corresponding blank serum spiked at the LLOQ to determine whether the detection of ampicillin or IS was affected by endogenous substances. Furthermore, interference with other drugs was assessed for interfering peaks at a retention time of 3.6 minutes and a detection wavelength of 210 nm by cross-examination of 50 chromatograms of patients not receiving ampicillin but other anti-infectives and other intensive care–specific drugs. A complete UV spectrum was obtained for peak purity and identification.

Stability

The stability of ampicillin in human serum was assessed under different conditions, including at room temperature (20–25°C), 2–8°C, and at freezing (−20°C and −80°C). For stability testing, the 4 samples at freezing conditions were thawed and analyzed. Stability was defined as the duration for which samples maintained a concentration of 90%–110% of their baseline levels.

Clinical Application

Data Collection and Patient Sample

This method was developed and validated for routine ampicillin TDM in ICU patients receiving ampicillin/sulbactam as continuous infusion. Therefore, we performed a retrospective analysis to evaluate its clinical application. Anonymized, routinely collected data on the TDM process in routine care at Heidenheim General Hospital from June 2018 to June 2021 were used. The analysis included all eligible ICU patients treated with ampicillin/sulbactam, excluding those with missing information (such as dose or plasma creatinine levels) and patients undergoing intermittent dialysis. This study was approved by the Ethics Committee of Ulm (No. 137/19, amendment June 2021). The requirement for informed consent was waived due to the retrospective nature of this study.

TDM Procedures

Blood samples were obtained from serum tubes 6 hours after therapy initiation. Treatment with ampicillin/sulbactam was initiated according to the internal protocol (see **Figure**, **Supplemental Digital Content 4**, http://links.lww.com/TDM/A788, which demonstrates the internal protocol for ampicillin initial dosing and TDM), with a loading dose (1000-mg ampicillin for 15 minutes), followed by a maintenance dose administered as a continuous infusion. The initial maintenance dose was selected by the responsible physician based on the Calculator to Approximate Drug-Dosing in Dialysis program using creatinine clearance and, if applicable, the respective dialysis settings.[14,15] Further dose adjustments were supervised by trained clinical pharmacists according to the measured ampicillin concentration (c), pharmacokinetic/pharmacodynamic (PK/PD) target, and patient's clinical condition.

Assessment of Therapeutic Drug Exposure

The assessment was conducted in accordance with the established clinical objectives of the routine TDM program. The *f*c:MIC ratio was selected as the PK/PD parameter that best described the time-dependent efficacy of ampicillin. Optimal therapeutic drug exposure was defined as an *f*c:MIC ratio in the range of 4–8 times the European Committee on Antimicrobial Susceptibility Testing break point of ampicillin/sulbactam for *Enterococcus* spp. and anaerobic bacteria (4 mg/L).[16,17] Consequently, the therapeutic drug exposure was classified as optimal for c between 16 and 32 mg/L, quasioptimal for c between 8 and 16 mg/L or between 32 and 48 mg/L, and supraoptimal for c exceeding 48 mg/L. Suboptimal therapeutic drug exposure was defined as $c < 8$ mg/L, considering the break point of Enterobacterales (8 mg/L), intermediate susceptible *Enterococcus* spp., and anaerobic bacteria (8 mg/L).[16,17]

To assess the suitability of the initial dosing, c was stratified by the time of observation, and the prediction of initial dosing was evaluated by comparing the predicted clearance calculated using the Calculator to Approximate Drug-Dosing in Dialysis program with the observed clearance (CLobs). CLobs was calculated using the following equation: $\mathrm{CLobs} = \frac{[\text{maintenance dose (mg)}]}{24\ \text{hours}} \cdot c^{-1}\left(\frac{\text{mg}}{\text{L}}\right)$.

Statistics

Descriptive statistics were used, as appropriate. Continuous data were presented as mean (±SD) or median (interquartile range), whereas categorical variables were expressed as a count and percentage. Statistical analyses were performed using R/RStudio (version 4.3.2; CRAN.R-project.org). Plots were generated using the "ggplot2" package (version 3.4.4).

RESULTS

Method Validation

The calibration curve was linear over the concentration range of 2–128 mg/L with a determination coefficient (R^2) of >0.99 (see **Figure** and **Table**, **Supplemental Digital Content 5**, http://links.lww.com/TDM/A789, which demonstrates the calibration curve and the calibration values of ampicillin calibration standards). The LLOQ was determined as 2 mg/L. The signals measured in the blank serum samples were well below the LLOQ.

TABLE 1. Accuracy and Precision of the High-Performance Liquid Chromatography–Ultraviolet Spectrometry Method for the QC Samples (n = 3) of Ampicillin in Human Serum

	Mean Concentration (mg/L)	Intraday CV (%)	Interday CV (%)	Accuracy Bias (%)
LQC	2.03	0.17	2.85	+1.40
MQC	15.34	0.60	3.67	−4.11
HQC	116.95	0.09	3.40	−8.63

CV, coefficient of variation; MQC, medium concentration.

Accuracy and precision indicated good assay performance, with a relative SD of 0.09%–3.67% for interday and intraday precision and a bias accuracy range between −8.63% and 1.4% (Table 1).

No significant endogenous interference was observed, indicating acceptable selectivity (see **Table**, **Supplemental Digital Content 6**, http://links.lww.com/TDM/A790, which demonstrates the recovery rate).

The results of the stability tests under different conditions are presented in the **Supplemental Digital Content 7** (see **Table**, http://links.lww.com/TDM/A791, which demonstrates the stability of ampicillin in human serum). The QC samples remained stable at room temperature for up to 6 hours. Long-term stability at −80°C was within predefined limits for at least 6 months. Representative chromatograms are shown in the **Supplemental Digital Content 3** (see **Figures S1–S4**, http://links.lww.com/TDM/A787, which demonstrates the chromatograms of ampicillin in water, blank human serum, caffeine and ampicillin in human serum, and other antibiotics in human serum).

Clinical Application

This method was successfully applied for the analysis of routine TDM serum samples from ICU patients. A total of 336 serum samples from 152 patients in the ICU were assessed. The demographic and clinical characteristics of the patients are shown in Table 2.

The median initial daily dose of ampicillin before concentration measurement was 5800 (3175–6000) mg, with a range of 1200–12,000 mg. Recommendations for dose adjustment based on measured concentrations were available on average 6.33 (±1.86) hours after blood sampling as part of the morning routine. The median dose was reduced to 4000 mg (3000–6000 mg). The CLobs ranged from 0.5 to 31.2 L/h, which explains the variability of the required dose (see **Figure**, **Supplemental Digital Content 8**, http://links.lww.com/TDM/A792, which describes the distribution of the observed ampicillin clearance). A good correlation between predicted clearance and CLobs was observed, supporting the administration of variable maintenance doses at the start of the therapy (Fig. 1).

Following the initial dosing, the median serum concentration was 26.8 (17.2–38.0) mg/L and was further adjusted through TDM to a median serum concentration of 22.5 (17.0–29.3) mg/L (Fig. 2). Only 1 measured concentration of the 336 samples was outside (ie, below) the validated concentration range. Of the 336 samples, 58.9% were within the optimal concentration range of 16–32 mg/L and nearly all of the samples (92.9%) were within the optimal or quasioptimal concentration range of 8–48 mg/L. Only 25 (7.4%) ampicillin concentrations exceeded the defined threshold. Considering only concentration measurements after the initial dosing, 50.7% of the samples were already within the optimal concentration range of 16–32 mg/L, and nearly all of the samples (87.5%) were within the optimal or quasioptimal concentration range of 8–48 mg/L. The details of the concentration data are presented in Table 3.

DISCUSSION

This study presents a rapid and robust HPLC-UV protocol for ampicillin detection in human serum and demonstrates its effective application in routine TDM. This method was successfully validated with a high degree of accuracy and precision in the calibration range of 2–128 mg/L, which spans the expected pharmacokinetic concentration range in clinical practice. After a rapid sample cleanup, analytes were separated on a C18 reversed-phase HPLC column within 12.5 minutes and quantified at a wavelength of 210 nm using UV spectrometry, resulting in a total turnaround time of approximately 6 hours from patient blood sampling to result communication. Given that the concentrations of ampicillin in serum under continuous infusion are

TABLE 2. Demographic and Clinical Characteristics of the Study Population at Admission

	Median (IQR), No. (%)
Sex, men	105 (69%)
Age, yr	69 (60–78)
Body mass index, kg/m^2	27.4 (24.2–31.3)
Weight, kg	81 (71–95)
Height, cm	172 (165–180)
Serum creatinine, mg/dL	1.0 (0.7–1.7)
CrCL, mL/min	65.3 (39.6–94.9)
CVVHD	5 (3.3%)
CRP, mg/dL	176 (95.3–253)
White blood cell count, cells/μL	11.6 (8.73–14.8)
Site of infection	
CAP	150 (98.7%)
Endocarditis	1 (0.7%)
CNS Infection	1 (0.7%)

CAP, community-acquired pneumonia; CNS, central nervous system; CrCL, creatinine clearance calculated using the Cockcroft–Gault equation; CRP, C-reactive protein; CVVHD, continuous venovenous hemodialysis; IQR, interquartile range.

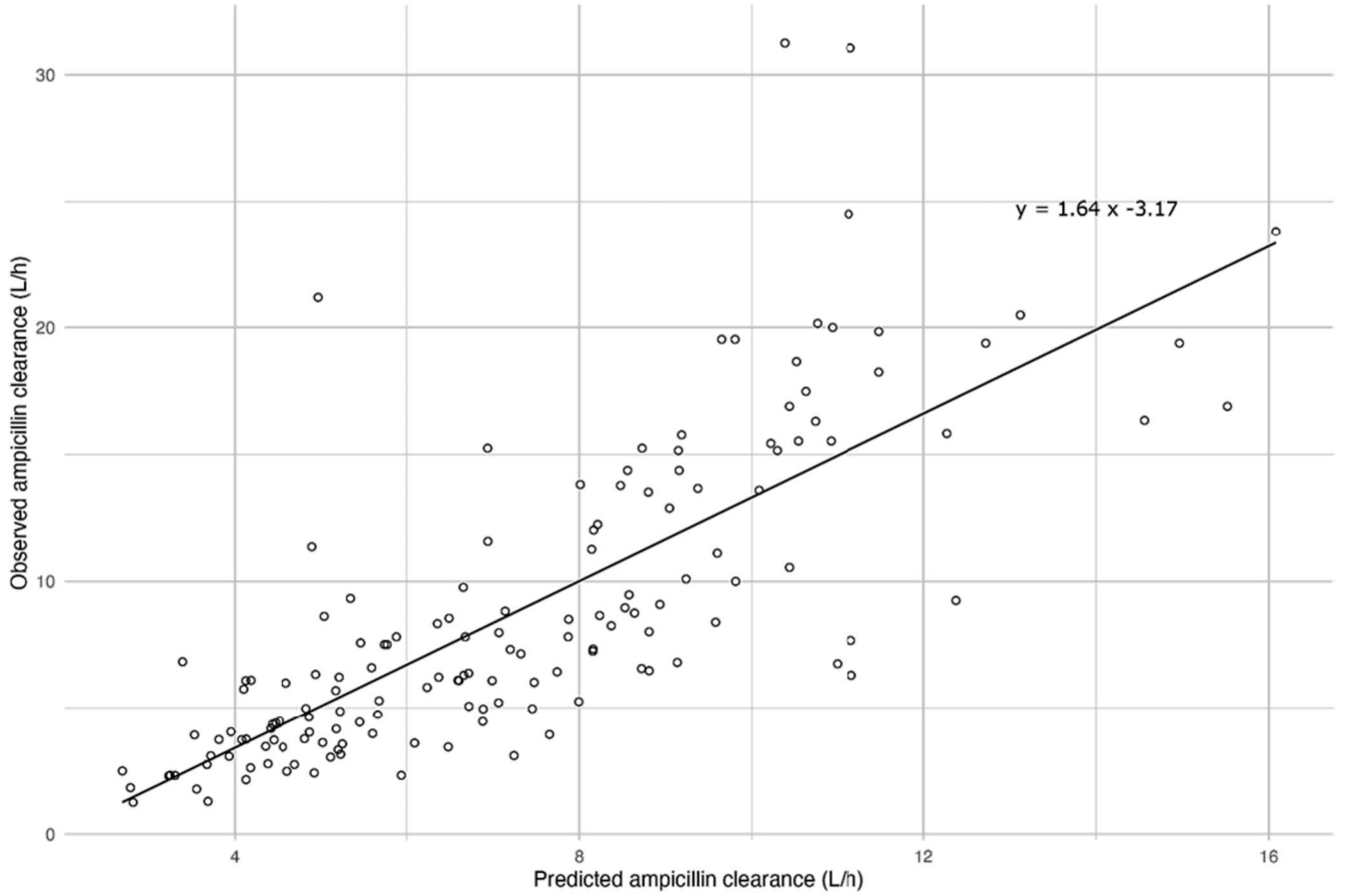

FIGURE 1. Predicted ampicillin clearance (CLpred) versus observed ampicillin clearance (CLobs). (R^2 = 0.67, Pearson correlation coefficient = 0.82). The solid black line indicates the linear regression of the fit.

rather high, the quantification is suitable from an analytical perspective with an LLOQ of 2 mg/L. No interference was detected at the retention times or wavelengths specific to ampicillin. Thus, this method allows the determination of ampicillin in the presence of other coadministered drugs.

Previously, HPLC-UV[18,19] and LC with tandem mass spectrometry–mass spectrometry[20–22] methods have been used for the quantification of ampicillin. However, these methods do not consider the linear range required for TDM in routine care. By contrast, more recent assays[20–22] have addressed this critical aspect, presenting excellent methods for analyzing ampicillin in plasma. These updated assays have been successfully applied in patients.[20–22] In comparison to these published assays, the UV method described in this study offers a more cost-effective analysis than that of LC with tandem mass spectrometry–mass spectrometry methods, considering instrument purchase price and maintenance costs. The simple instrumentation setup used in the HPLC-UV method, combined with the short turnaround time, allows for daily and reliable antibiotic drug monitoring in clinical practice. Therefore, this method appears to be of interest to hospital pharmacies and small laboratories where staffing and workload constraints might necessitate the prioritization of measurements and where HPLC-UV is usually already available. This is of particular interest as access to β-lactam TDM is still limited and, when available, the time to results and dosage adjustments is often too long, posing a significant barrier to implementation.[23,24]

As many antibiotics, particularly β-lactams, are chemically unstable once dissolved, preparing stock solutions, calibrators, and QCs becomes a critical step.[25] In that respect, it is essential to freeze patient samples as quickly as possible. Although serum storage at −80°C is recommended, our stability studies revealed that samples can also be stored for up to 7 days at −20°C without any significant reduction in antibiotic concentrations.

The analysis of the clinical application of ampicillin showed variable pharmacokinetics in ICU patients, which is well-known from a large number of previous studies.[26–30] Using an individualized dosing approach, ampicillin concentrations above 16 mg/L (4-fold MIC) were attained in 83.3% of all measurements. TDM-guided dose adjustments increased the target attainment of optimal therapeutic drug exposure (16–32 mg/L) from 50.7% to 66.3%. Furthermore, 92.9% of the samples fell within the optimal and quasioptimal ranges. None of the patients had concentrations below the break point of ampicillin/sulbactam for *Enterococcus* spp. or anaerobic bacteria at 4 mg/L.

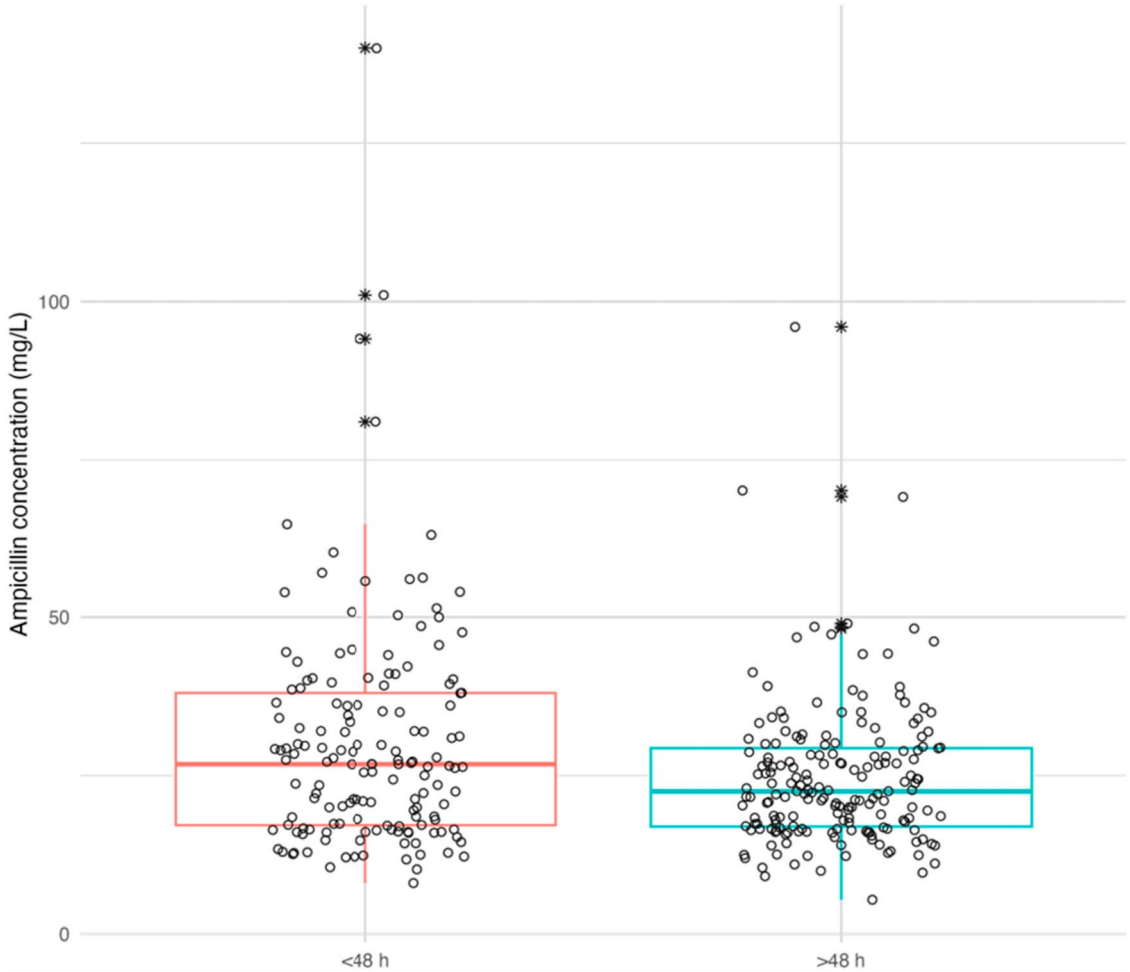

FIGURE 2. Ampicillin concentrations (c) stratified by the time of observation. Boxes: interquartile range (IQR), horizontal line: median, whiskers: range below first or above third quartile to 1.5-fold the IQR, stars: concentrations outside whiskers, points: measured ampicillin concentrations.

It is commonly acknowledged that traditional dosing of β-lactams has repeatedly failed to attain PK/PD targets in previous studies involving critically ill patients, which is associated with poorer chances of clinical cure and bacteriological eradication. In the Defining Antibiotic Levels in Intensive care unit patients trial,[28] a prospective multinational point prevalence study on β-lactam levels in critically ill patients, only 67% of the patients achieved 100% $fT > MIC$. This may have consequences for de-escalation strategies or the treatment of community-acquired pneumonia, as potential therapeutic failure may be more closely associated with inadequate exposure rather than inadequate coverage of narrow-spectrum ampicillin.[23] Extending the infusion time (prolonged and continuous infusion) can address this issue as it promises higher β-lactam concentrations at the end of the dosing interval. Recent studies have reported a significant reduction in mortality and improved clinical cures in critically ill patients.[4] A small study with prolonged infusion of ampicillin/sulbactam in patients with sepsis also demonstrated favorable outcomes.[31] However, it is currently unclear, which subgroups benefit most from prolonged or continuous administration.[4]

TABLE 3. Therapeutic Drug Exposure of Ampicillin Stratified by the Time of Observation

c (mg/L)	<8	8–16	16–32	32–48	>48
All (n = 336)	2 (0.6%)	54 (16.1%)	199 (58.9%)	57 (16.9%)	24 (7.2%)
<48 h (n = 152)	1 (0.7%)	24 (15.8%)	77 (50.7%)	32 (21.1%)	18 (11.8%)
>48 h (n = 184)	1 (0.5%)	30 (16.3%)	122 (66.3%)	25 (13.6%)	6 (3.3%)

Values are expressed as absolute numbers (n) and relative incidence (%).
c, ampicillin concentration.

Thus far, extended modes of administration have become mandatory, especially in critically ill patients with good renal or augmented renal clearance.[32,33] Indeed, in a recent study of 147 ICU patients, target nonattainment of β-lactam antibiotics was significantly associated with an estimated glomerular filtration rate >90 mL/min/1.73 m^2, whereas attainment of 100% $fT > MIC$ was associated with a decreased length of ICU stay.[33]

This study had several limitations. First, the validated concentration range was defined as 2–128 mg/L, which potentially limited the applicability of measuring ampicillin concentrations during bolus administration. However, as the estimated target concentration range for continuous infusion is 16–32 mg/L, concentrations above 128 mg/L or below 2 mg/L are relatively unlikely and, if present, would most likely be due to medication errors, such as overdosing in severe renal failure, incorrect dosage adjustments, or improper timing of sampling. Second, sulbactam was not analyzed within this study because it was not selectively detectable or quantifiable in critically ill patients. However, ampicillin and sulbactam exhibit comparable pharmacokinetic properties with regard to the volume of distribution and clearance.[34] Therefore, ampicillin appears to be a potential surrogate for estimating the adequacy of sulbactam exposure in practice, which needs to be explored in future studies. Moreover, this was a retrospective analysis of serum concentrations measured as total drug concentrations, which should be interpreted carefully because biasing factors may have been present. However, these results are consistent with those of previously published studies reporting pharmacokinetic variability in ICU patients.[26–30]

CONCLUSIONS

The proposed method was successfully used for TDM and showed good performance in monitoring ampicillin levels in intensive care patients treated with ampicillin. Owing to its practical design, cost-effective sampling, and short turnaround times, this method appears to be particularly relevant for small laboratories or hospital pharmacies, providing β-lactam measurements in routine clinical practice. Furthermore, ampicillin showed a relevant pharmacokinetic variability, highlighting the particular importance of individual β-lactam dosing. This method enables the implementation of a feasible TDM approach for ampicillin, consisting of individual starting doses, continuous administration, and subsequent dosage adjustments to ensure target attainment in critically ill patients.

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