Journal of Mass Spectrometry and Advances in the Clinical Lab 33 (2024) 43–48

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Research Article

Isotope-dilution-LC-MS/MS candidate reference measurement procedure for cefepime in human serum

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ARTICLE INFO

Keywords:
Cefepime
Isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)
Reference measurement procedures
Metrological traceability
Standardization

ABSTRACT

Background: Reference measurement procedures are an essential element in the standardization and comparability of analytical measurement results in laboratory medicine. No LC-MS/MS-based reference measurement procedure for cefepime in serum has been published previously.

Materials and methods: An isotope-dilution based two-dimensional LC-MS/MS reference measurement procedure for cefepime concentrations in human serum was developed and tested. The value assignment of unknown samples is based on a defined measurement series validation. Six unknown samples can be measured per series. Pass criteria for the run and the samples were determined empirically based on a performance evaluation. For this purpose, a between-run determination of five runs of the defined measurement series with six cefepime samples was carried out and evaluated. The goal was to define rigorous, realistic target limits and minimize measurement uncertainty. The final defined target limits are used for series-based validation and value assignment. The results for the six unknown samples are provided with the associated measurement uncertainty for this series.

Results: The developed and extensively studied measurement procedure for the quantification of cefepime in serum was found to be practicable and fit for its purpose. The between-run mean imprecision of the six cefepime samples was $\leq$ 2.0 %, for the QCs it was $\leq$ 2.3 % and the between-run mean inaccuracy of the QCs was within $\pm$ 1.1 %.

Conclusion: The novel isotope-dilution-LC-MS/MS measurement procedure in accordance to ISO 15193 can be recommended as candidate reference measurement procedure for the value assignment of cefepime concentrations in human serum.

1. Introduction

Reference measurement procedures (RMPs) play a crucial yet not widely known role in laboratory medicine. They are an important link in an unbroken metrological traceability chain to ensure a traceable value assignment of manufacturer calibration material and controls, as well as interlaboratory test material, to a generally accepted higher order standard, ideally an SI unit. This enables standardization, comparability, and reliability of the results of different analytical methods [1–3]. Only with standardized values, evidence-based therapeutic target ranges for certain drugs can be correctly analyzed, defined, and monitored. Drugs for which standardized values are of particular and increasing relevance are antibiotics. Therapeutic drug monitoring (TDM) of antibiotics for critically ill patients is gaining increasing attention as an important and strategic tool to significantly improve the treatment of serious bacterial infections and to slow down the progressive development and spread of antibiotic resistance [4–6]. This reveals the significant status and need for RMPs in general and especially for antibiotics. There are currently only two RMPs for antibiotics, vancomycin and gentamicin, published and listed in the database of *Joint Committee for Traceability in Laboratory Medicine* (*JCTLM*) [7–8].

A relevant and frequently used antibiotic for the intensive care

Abbreviations: ACN, acetonitrile; Cal, calibrator; Conc., concentration; DI, dilution integrity; ID-LC-MS/MS, isotope dilution-liquid chromatography-tandem mass spectrometry; IRB, Institutional Review Board; IS, internal standard; ISO, International Standardization Organization; JCTLM, Joint Committee for Traceability in Laboratory Medicine; MU, measurement uncertainty; QC, quality control; NaCl, sodium chloride; OSPE, online solid phase extraction; RMP, reference measurement procedure; SST, system suitability test sample; TDM, therapeutic drug monitoring.

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https://doi.org/10.1016/j.jmsacl.2024.08.001
Received 23 April 2024; Received in revised form 22 July 2024; Accepted 5 August 2024
Available online 8 August 2024

treatment of bacterial infections, such as life-threatening sepsis or pneumonia, is the second-line antibiotic cefepime [9–11]. As a broad-spectrum cephalosporin, cefepime belongs to the group of beta-lactam antibiotics for which time-dependent antimicrobial activity is crucial for effective treatment and to avoid resistance. Therefore, the time during which free cefepime concentration remains above the minimum inhibitory concentration of the pathogen is a critical parameter [12–13]. On the other hand, due to nephro- and neurotoxic side effects, excessive serum levels should be avoided [14–15]. Thus, TDM is recommended for relevant patient groups, for whom target levels are difficult to achieve. This concerns especially critically ill patients, patients with or suspected renal dysfunction, and those with suspected risk for neurotoxicity [16–18].

Elaborate high-performance liquid chromatography-based methods, particularly isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS), are nowadays preferred for the TDM of antibiotics due to their high sensitivity and specificity [19–21]. Likewise, ID-LC-MS/MS is increasingly used as the basis for the RMPs of small molecular analytes [7–8,22].

The aim of the study was to investigate and establish an ID-LC-MS/MS candidate RMP for the value assignment of cefepime concentrations in human serum.

2. Materials and methods

A previously published novel approach with a standard protocol for value assignment in the context of ID-LC-MS/MS reference measurement procedures based on a spreadsheet tool (RMP-Processing-Excel-Tool) was applied [22]. The RMP-Processing-Excel-Tool used for this work is provided in Supplemental file 1 and recommended as basis for series-based validation and value assignment of serum cefepime concentrations measured with this RMP.

Accordingly, the template of the method description, as well as the analytical structure of the standardized measurement series of this protocol, were used.

2.1. General method description in brief

A comprehensive and very detailed method description (preparation process, LC-MS/MS condition, material, LOT and manufacturer information, etc.) is provided in Supplemental file 2.

2.1.1. Pre-sample preparation

Cefepime hydrochloride [USP Reference Standard from Sigma-Aldrich Chemie GmbH] was weighed out for the stock solutions for the two highest calibrators (Cals; Cal 7, Cal 6), the highest quality control (QC; QC D) and the upper control using an ultra-micro balance [Satorius Cubis® II Ultra micro balance Type MSA 2 from Satorius AG]. The working solutions for the Cals 1–5, QCs A-C and the lower control were prepared from the stock solutions using a direct displacement pipette and tips [Eppendorf Multipipette® E3, Eppendorf Combitips® advanced from Eppendorf AG]. For the preparation of the stock and working solutions, 0.9 % sodium chloride (NaCl) solution and certified 20 mL volumetric flasks [BLAUBRAND® glass grade A with USP individual certification from Brand GmbH+Co KG] were used. Spiking with human serum (for matrix samples: Cals, QCs, upper and lower control) was performed at a ratio of 1 + 9, followed by incubation (light protected, 4 °C, 30 min). Spiking in 0.9 % NaCl solution for the Cals in solvent was carried out in the same way. The dilution integrity (DI) samples were prepared by diluting the ready-made upper control in a ratio of 1 + 1 and 1 + 4 with serum. Separate aliquots of Cal 1/Cal 7/blank in matrix were used as system suitability test samples (SSTs) 1/2/3.

2.1.2. Sample preparation

50 µL of internal standard (IS) [cefepime-$^{13}C,^{2}H_3$-sulfate from Alsachim] working solution was added to a sample volume of 50 µL, vortexed and incubated (light protected, 4 °C, 15 min). Protein precipitation was carried out at a ratio of 1 + 4 by adding 400 µL acetonitrile (ACN) followed by vortexing, incubation (light protected, 4 °C, 15 min) and centrifugation (light protected, 4 °C, 15 min). Afterwards, 50 µL supernatant was diluted with 550 µL 0.1 % formic acid (FA) in a ratio 1 + 11, vortexed and placed in the autosampler (lights off, 8 °C). All pipetting steps were performed with direct displacement pipette and tips.

The target concentrations of the final prepared samples were:

Cals 1–7: 1.0 mg/L – 2.0 mg/L – 4.0 mg/L – 6.3 mg/L – 13 mg/L – 25 mg/L – 50 mg/L
QCs A-D: 1.2 mg/L – 3.0 mg/L – 9.3 mg/L – 37 mg/L
Lower and upper control, DI samples: 0.80 mg/L, 60 mg/L, 12 mg/L, 30 mg/L
SSTs 1–3: 1.0 mg/L – 50 mg/L – 0 mg/L

2.1.3. Online solid phase extraction (OSPE) and LC-MS/MS analysis

The measurement was carried out with an ultra-high performance liquid chromatography instrument [Acquity UPLC from Waters] with a binary pump-system coupled with a tandem mass spectrometer [Xevo TQ-S from Waters]. Two-dimensional chromatography was performed via OSPE with a hydrophilic-lipophilic balance extraction column [Oasis HLB Direct Connect HP, 20 µm, 2.1 x 30 mm (copolymer, reversed phase) from Waters] and with a biphenyl column [Raptor Biphenyl, 2.7 µm, 2.1 x 100 mm (superficially porous particles, reversed phase) from Restek] as the analytical column. For the 11-minute run, 8 µL per sample were injected. 0.1 % FA and ACN were used as LC-MS-grade mobile phase for OSPE, 0.1 % FA and 0.1 % FA in ACN were used for gradient elution with the analytical column at 40 °C. An electrospray ionization and a triple stage quadrupole were used for ionization and manipulation.

2.1.4. Quantification

Four mass transitions were measured using multiple reaction monitoring. Quantification of cefepime concentrations was made by averaging two mass transitions [23] of cefepime as one averaging pair and two transitions of IS cefepime-$^{13}C,^{2}H_3$-sulfate as the other averaging pair.

2.2. Standardized measurement series

This structure includes a specific, fixed sample sequence and defined analytical metadata monitored and recorded during the measurement of the series. The sample sequence includes the following samples: SSTs, Cals (solvent and matrix-based), blanks (with and without IS), the unknown samples, QCs, lower and upper controls covering the ± 20 % of the calibration range, and DI samples. The monitored data include inaccuracy, nominal and calculated averaged concentrations, as well as signal-to-noise, which is monitored for both transitions of cefepime. Furthermore, data that are additionally monitored for the IS, such as ion ratio, retention time, and other chromatographic parameters (peak area, peak asymmetry, full width of the peak at half maximum, height-to-area ratio), which are each monitored for both transitions of cefepime and the IS.

The structure of the standardized measurement series is schematically depicted in Fig. 1.

2.3. Data acquirement

The acquired measurement data was exported from Mass Lynx™/Target Lynx™ software and transferred to the assigned fields of the specific format of the spreadsheet tool. The calculated results of the run, including the QCs and of the unknown samples, as well as the pass/fail rating of the criteria, are then automatically generated based on links in

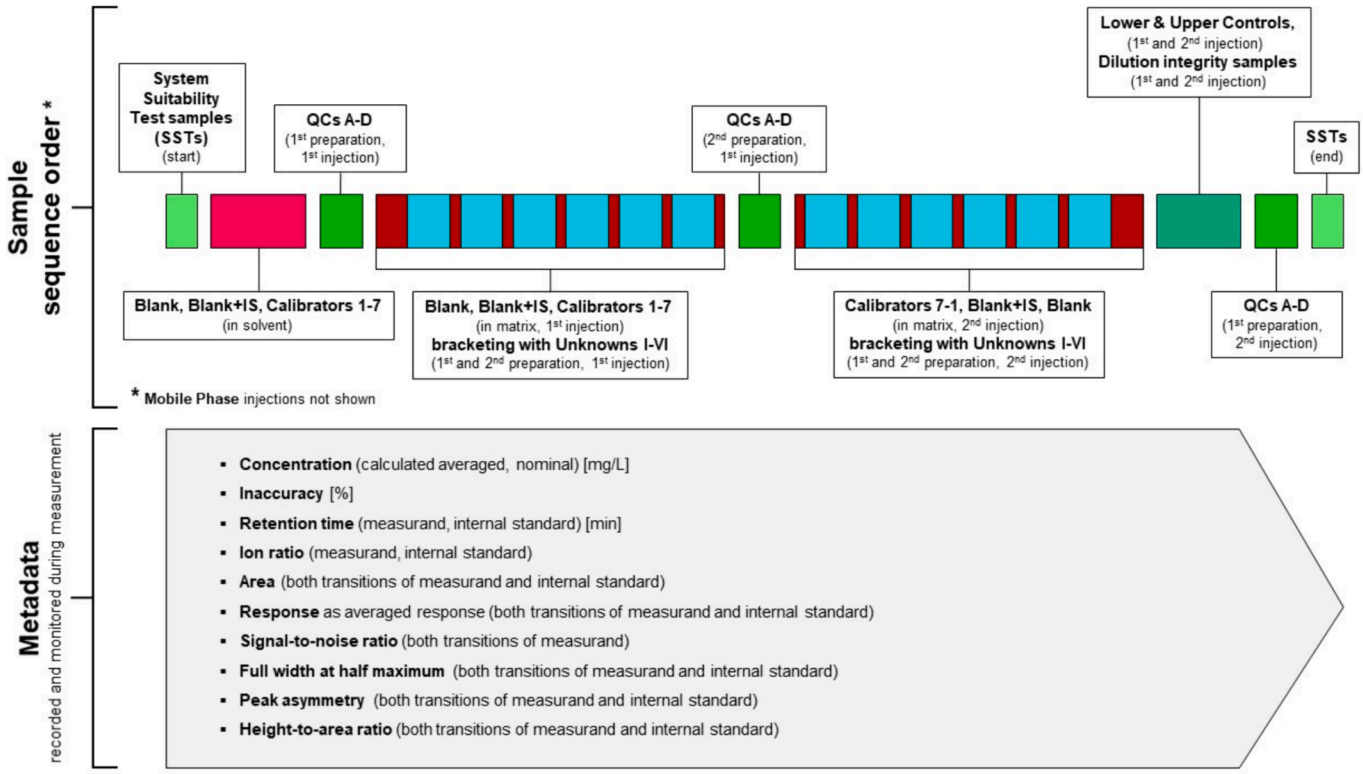

Fig. 1. Structure of the standardized measurement series.

the spreadsheet. Furthermore, the final result (interval) for unknown samples with the associated measurement uncertainty (MU) and the plots for the IS area are generated in the same way.

2.4. Performance evaluation

A performance evaluation was conducted to study inaccuracy and imprecision of between-run reproducibility and to determine the technically feasible and realistic target limits. For this purpose, five measurement series with QCs and six cefepime samples were analyzed, which were measured on different days over a period of six weeks. For each analysis of one measurement series, calibrators and controls were freshly prepared, including weighing of the substance for the stock solutions, and then directly measured on the same day according to the defined measurement series. The duration of the measurement series was approximately 27 h.

Anonymized residual sample material from intensive care patients treated with cefepime from the Institute's routine diagnostic services and external quality samples were used as unknown samples. As only fully anonymized patient samples were used that were not obtained specifically for use in this study through an interaction or intervention with living individuals, neither informed consent nor Institutional Review Board (IRB) review were required. The use of anonymized residual sample material for analytical research purposes is waived by the Ethics Committee of the Ludwig-Maximilians-University.

Reconstituted lyophilized proficiency test samples from *Rili-BAEK* (guideline of the German Medical Association for quality assurance in medical laboratories) approved EQA providers (*RfB* (Bonn, Germany) and *INSTAND e.V.* (Düsseldorf, Germany)) were used as external quality samples.

The unknown samples were aliquoted immediately after receipt and according to Zander et al. [24] stored at −80 °C.

The performance targets for the run (run-pass criteria) and unknown samples (sample-pass criteria) were derived and defined analyte- and method-specifically for the cefepime RMP based on a critical review and assessment of the performance evaluation results. They were then transferred to the required fields in the spreadsheet tool. Table 1 provides a general overview of the objectives addressed in the criteria for the run and the unknown samples.

In parentheses: the corresponding outline numbers of the RMP-Processing-Excel-Tool are shown.

An exemplarily, representative measurement series of these five measured series is shown in the Supplemental file 3.

3. Results

The results of between-run inaccuracy and imprecision of the performance evaluation is shown in Table 2.

Table 1
Objectives of run- and sample-pass criteria.

Run-pass criteria	Sample-pass criteria
System suitability check at start and end (*1.*)	Calculated concentrations and imprecision (*1.*)
Calibration in matrix and solvent (calibration parametes, inaccuracy) (*2., 3.1*)	Conformity of ionization of analyte and IS (in relation to calibration and QCs in matrix) (*2., 3.*)
Specificity and potential matrix effects on ionization of analyte and IS in matrix versus solvent (*3.2, 3.3, 3.4*)	Conformity of chromatography, selectivity (in relation to calibration and QCs in matrix) (*4.*)
Inaccuracy, Imprecision and RRMSE (*4.*)	
Integrity of ± 20 % of calibration range (*5.*)	
Dilution integrity (*5.*)	
Sensitivity (*6.*)	
Selectivity, analytical specificity and potential interferences (*7.*)	
Consistency of ionization of analyte and IS (*8., 9.*)	
Consistency of chromatography and selectivity (*10.*)	

Table 2
Quantification of cefepime concentration (conc.) in serum, performance evaluation results – between-run study.

Between-run study		Run 1		Run 2		Run 3		Run 4		Run 5		Between-mean	
QCs	Target conc. [mg/L]	Inaccuracy	Imprecision	Inaccuracy	Imprecision	Inaccuracy	Imprecision	Inaccuracy	Imprecision	Inaccuracy	Imprecision	Inaccuracy	Imprecision
A	1.2	−1.6 %	2.7 %	−2.5 %	2.2 %	1.6 %	1.4 %	1.8 %	3.6 %	−0.47 %	1.6 %	−0.26 %	2.3 %
B	3.0	−0.53 %	2.3 %	0.015 %	2.5 %	−0.78 %	2.1 %	2.2 %	1.7 %	0.048 %	2.3 %	0.19 %	2.2 %
C	9.3	−1.0 %	1.1 %	−1.6 %	0.55 %	0.25 %	1.3 %	−1.0 %	1.1 %	−1.2 %	2.1 %	−0.91 %	1.2 %
D	37	1.0 %	0.86 %	−0.33 %	2.2 %	−1.6 %	0.23 %	1.0 %	0.70 %	−1.0 %	1.6 %	−0.19 %	1.1 %
Within-mean		−0.56 %	1.7 %	−1.1 %	1.9 %	−0.13 %	1.3 %	1.0 %	1.8 %	−0.64 %	1.9 %		

Samples	Mean conc. [mg/L]	Imprecision	Imprecision	Imprecision	Imprecision	Imprecision	Imprecision (CV %)
1	2.65	1.9 %	1.0 %	2.1 %	2.4 %	2.4 %	2.0 %
2	4.19	1.6 %	0.91 %	1.7 %	0.55 %	1.3 %	1.2 %
3	6.27	0.42 %	1.4 %	1.9 %	1.1 %	0.41 %	1.0 %
4	11.0	1.8 %	1.0 %	0.93 %	1.7 %	1.4 %	1.3 %
5	16.8	1.0 %	0.49 %	0.51 %	1.2 %	1.8 %	1.0 %
6	36.6	2.3 %	0.94 %	0.87 %	1.1 %	1.0 %	1.2 %
Within-mean		1.5 %	1.0 %	1.3 %	1.3 %	1.4 %	

QCs: quantification in 3-fold determination, freshly prepared.
Samples: quantification in 4-fold determination, freshly prepared.

The inaccuracy of the QCs was between −2.5 % and 2.2 % with a total mean inaccuracy of −0.29 %. The imprecision of the QCs was between 0.23 % and 3.6 % with a total mean imprecision of 1.7 %. For the six unknown samples, imprecision was between 0.41 % and 2.4 %, while the total mean imprecision was 1.3 %.

The evaluation of the external quality samples (proficiency test samples) showed a between-run inaccuracy between −7.7 % and −1.2 % (total mean inaccuracy: −4.6 %), compared to the provider's stated target values.

The measurement results of the representative example series are shown in Supplemental file 3 and a multiple reaction monitoring chromatograms for calibrator 4 of cefepime and the IS are depicted in Fig. 2.

The finally defined performance targets for this RMP and the results for the exemplary series validation are presented in the following reports: the report for the run in Supplemental file 4, for the unknown samples in the Supplemental file 5, for the measurement result (interval) with associated MU of these samples in Supplemental file 6 and the IS area plots in the Supplemental file 7.

4. Discussion

In this article, we present a new candidate RMP for value assignment of cefepime concentrations in human serum.

General, non-method-specific requirements for RMPs are addressed in the international standard ISO 15193 [25]. In this context, ISO 17511 [26] contains the requirements for the metrological traceability of values. The proposal of a protocol as a method-specific concretization for the standardization of ID-LC-MS/MS measurements in the context of RMPs of small molecules was applied in this work. .

Significant efforts have been made to optimize the RMP in terms of measurement inaccuracy, imprecision and uncertainty. This includes in particular: fresh preparation (weighing) of the stock solutions (two for the calibrators, one for the QCs) and respective working solutions for each measurement series; traceability through suitable material selection (e.g., reference standard, direct displacement pipettes, certified volumetric flasks, ultra-micro balance – see Supplemental 2 for more details); substance specific treatment through elaborate sample extraction and ID-LC-MS/MS analysis (e.g., incubation with IS, protein precipitation, two-dimensional chromatography with online solid phase extraction and ultra-high performance chromatography, extended gradient elution, carryover assessment, specific corresponding mass transitions for measurand and stable isotope labeled IS); quantification by averaging two specific corresponding mass transitions for the measurand and for the IS; defined structure of the measurement series as shown in Fig. 1, which includes a specific sample sequence (e.g., double bracketing of calibrators and individual unknown samples with calibrators in descending and ascending order, 2-,3- and 4-fold determinations of the samples through multiple injections and sample preparations – depending on sample type, systematically interspersed QCs over the run, system suitability test samples at the beginning and end to assess continuous performance) and a specific set of recorded metadata of both transitions of the measurand and IS (e.g., ion ratio, retention time, signal-to-noise-ratio, height/area); assessment of analytical metadata to evaluate the reliability within the entire measurement series.

The series-based validation concept used in this work is based on the value assignment of unknown samples by validating individual analytical, specific series. For each series it is assessed and confirmed that the specified analytical performance targets are met for the current conditions (considering technical equipment and material, chain of traceability, dynamic performance variation of the LC-MS/MS systems).

An initial performance evaluation of the method was carried out to establish analytical performance targets that could realistically be achieved in the long-term application of the RMP. The performance evaluation demonstrated excellent results in terms of between-run imprecision (<2.3 %) and between-run inaccuracy (<1%). The

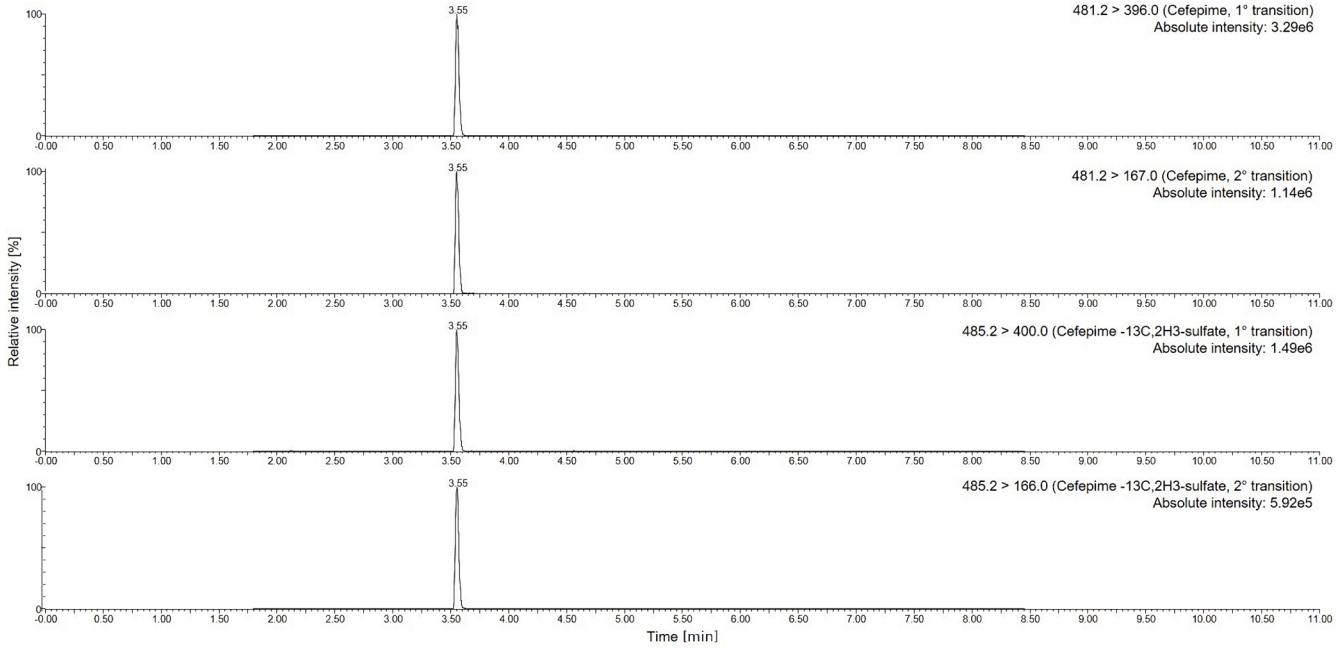

Fig. 2. Multiple reaction monitoring chromatograms for calibrator 4 of cefepime and the IS.

measurement of the proficiency test samples also yielded very satisfactory results.

5. Conclusion

An innovative transparent, traceable and reliable ID-LC-MS/MS candidate RMP for the quantification of cefepime concentrations in human serum, including a spreadsheet tool for automatic value calculation, has been developed and described for the first time. It can be recommended for the value assignment with associated MU of reference samples, inter-laboratory studies and calibration material.

Its implementation can contribute significantly to establishing the standardization and comparability of values obtained from different methods and can be of great benefit beyond laboratory medicine. In intensive care medicine, especially, it can improve the definition of clinical decision limits and therapeutic, evidence-based, target ranges in the context of prospective and observational studies.

CRediT authorship contribution statement

Judith Schäffler: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michael Vogeser:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Katharina Habler:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank the German Federal Ministry of Education and Research (BMBF) for funding the program “KMU-innovativ: Medizintechnik” (FKZ 13GW0354 A-F) as part of a collaborative project “Diagnostic test for rational antibiotic therapy control”.

Funding Support

This research was funded by the German Federal Ministry of Education and Research (BMBF) in the funding program “KMU-innovativ: Medizintechnik” (grant number: FKZ 13GW0354 A-F) as part of a collaborative project “Diagnostic test for rational antibiotic therapy control”.

Ethics Statement

The measurement of cefepime samples involved the analysis of residual sample materials. Residual sample materials of intensive care patients from the Institute’s routine diagnostic services were no longer required for the requested routine diagnostics. The use of anonymized residual sample material for analytical research purposes is waived by the Ethics Committee of the Ludwig-Maximilians-University.

As only fully anonymized patient samples were used that were not obtained specifically for use in this study through an interaction or intervention with living individuals, neither informed consent nor IRB review were required.

Disclaimer:

The procedures and files provided with this submission are not intended for application in clinical diagnostic procedures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.08.001.

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