Clinica Chimica Acta 488 (2019) 50–60

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Measurement of ceftolozane and tazobactam concentrations in plasma by UHPLC-MS/MS. Clinical application in the management of difficult-to-treat osteoarticular infections

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ARTICLE INFO

Keywords:
Ceftolozane
Osteoarticular infections
Therapeutic drug monitoring
UHPLC-MS/MS
Tazobactam

ABSTRACT

Background: Ceftolozane, in combination with the β-lactamase inhibitor tazobactam, is a new option in the pipeline against multidrug-resistant Gram-negative bacilli. As for other β-lactam antibiotics, optimizing the use of ceftolozane-tazobactam is advisable, especially in difficult-to-treat infections. In this regard, therapeutic drug monitoring would be required to guide the treatment of ceftolozane-tazobactam. Thus, we aimed to develop and validate procedures based on UHPLC-MS/MS for measurement of ceftolozane and tazobactam plasma concentrations in clinical practice.

Material and methods: Analyses were conducted using an Acquity® UPLC® integrated system coupled to an Acquity® TQD® tandem-quadrupole mass spectrometer. Ceftolozane, tazobactam and their internal standards (ceftazidime-D_5 and sulbactam) were detected by electrospray ionization mass spectrometry in positive and negative ion multiple reaction monitoring modes, using transitions of 667.2 → 199.3/139.0 and 551.9 → 467.9 for ceftolozane and ceftazidime-D_5, and 299.0 → 138/254.9 and 232.0 → 140.0 for tazobactam and sulbactam. Measurement procedures developed were used for guiding the treatment and adjusting daily dose of ceftolozane-tazobactam in patients with osteoarticular infections.

Results: Coefficients of variation and absolute relative biases were < 7.9% and 6.5% in all cases. The lower limit of quantification, linearity, normalized-recoveries, normalized-matrix effects and measurement uncertainties for ceftolozane were: 0.97 mg/L, (0.97–125) mg/L, ≤113.6%, ≤108.7%, and ≤ 18.7%, respectively; and for tazobactam: 1.04 mg/L, (1.04–125) mg/L, ≤103.6%, ≤101.9%, and ≤ 20.0%. No interferences and carry-over were observed. Patients plasma concentrations were higher than the recommended 3–4 times the minimal inhibitory concentrations.

Conclusions: Our measurement procedures are suitable for therapeutic drug monitoring of ceftolozane-tazobactam in patients with osteoarticular infections.

Abbreviations: β-LA, β-lactam antibiotic; CLSI, Clinical and Laboratory Standards Institute; CO, Carry-over; *CV*, coefficient of variation; δ_r, Relative bias associated to calibration procedure; Δ_{max}, Maximum permissible root mean square of the relative error of measurement (measurement uncertainty metrological requirement); DMSO, Dimethyl sulfoxide; EMA, European Medicines Agency; ESI, Electrospray ionization; EUROLAB, European Federation of National Associations of Measurement, Testing and Analytical Laboratories; GNB, Gram-negative bacilli; HPLC, High-performance liquid chromatography; IFCC, International Federation of Clinical Chemistry; IUPAC, International Union of Pure and Applied Chemistry; IS, Internal standard; LC, Liquid chromatography; LLOQ, Lower limit of quantification; ME, Matrix effect; MDR, Multidrug-resistant; MIC, Minimal inhibitory concentration; MRM, Multiple reaction monitoring; MS, Mass spectrometer; MS/MS, Tandem mass spectrometry; *m/z*, Mass-to-charge; QC, Quality control; REC, Recovery of extracted samples; SEL, Selectivity; TDM, Therapeutic drug monitoring; UHPLC, Ultra-high performance liquid chromatography; ULOQ, Upper limit of quantification; UV, Ultraviolet; %D, Percent deviation; T > MIC, Time the drug concentration remains above the MIC

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https://doi.org/10.1016/j.cca.2018.10.034
Received 1 September 2018; Received in revised form 11 October 2018; Accepted 28 October 2018
Available online 30 October 2018

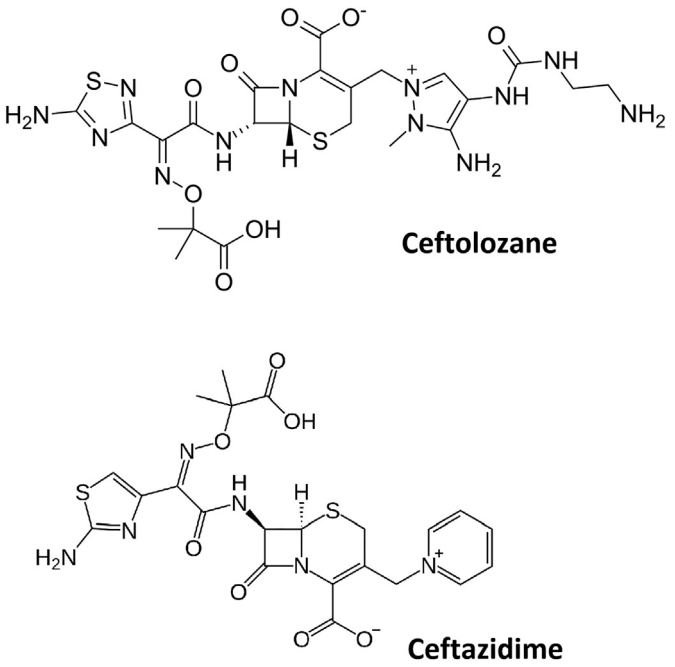

Fig. 1. Chemical structures of ceftolozane and ceftazidime.

1. Introduction

Ceftolozane is a fifth-generation cephalosporin structurally similar to the previous β-lactam antibiotic (β-LA) ceftazidime, but with a modified side chain (Fig. 1). It is administered with the β-lactamase inhibitor tazobactam and this combination is active against several multidrug-resistant (MDR) Gram-negative bacilli (GNB) [1–3]. In the current worldwide concern of infections caused by MDR microorganisms and particularly, when these microorganisms are responsible of difficult-to-treat infections (i.e., biofilm-related osteoarticular infections), an optimized use of antibiotics is advisable [4,5]. In this line, the administration of β-LA in continuous infusion has been used to optimize their pharmacokinetic/pharmacodynamic indices and then to expect an improvement in the efficacy and protection against the emergence of resistance [6–8]. In these cases, therapeutic drug monitoring (TDM) of β-LA to guide this therapy should be considered and thus, this could be also the case for ceftolozane-tazobactam [9].

For the particular measurement of mass concentrations of ceftolozane and tazobactam in plasma, a limited number of studies have applied high-performance liquid chromatography (HPLC) with UV detection procedures [10–12], and only few cases included HPLC coupled to the tandem mass spectrometry (MS/MS) [13–15]. Among these HPLC-MS/MS procedures reported, several methodological limitations can be identified in regard with the lacked information about the selectivity, carry over, matrix effect, dilution integrity, and measurement of uncertainty.

The aim of this work was to develop ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) procedures for the measurement of ceftolozane and tazobactam mass concentrations in plasma, and to validate widely their performance characteristics. Additionally, we aimed to apply these procedures for guiding the treatment of cases of osteoarticular infections.

2. Material and methods

2.1. Chemical, materials and reagents

Reference material of ceftolozane sulfate (pure ceftolozane content of 76.9%) was donated by Merck Sharp & Dohme S.A. (Barcelona, Spain). Certified reference material of tazobactam (purity of 99.6%) was purchased from United States Pharmacopeia (Rockville, MD, USA). Ceftazidime-D_5 (purity of 90.0%, isotopic purity of 98.6%), used as ceftolozane-internal standard (IS), was supplied by Toronto Research Chemicals (Ontario, Canada). Certified reference material of sulbactam (purity of 99.9%; IS for tazobactam) was obtained from European Pharmacopeia (European Directorate for the Quality of Medicines-Council of Europe, Strasburg, France)

LC-MS-grade acetonitrile, dimethyl sulfoxide (DMSO), formic acid, methanol and water were purchased from Sigma-Aldrich Química S.L. (Madrid, Spain).

Ceftolozane/tazobactam-free human plasma (blank plasma) was obtained from blood of patients arrived at Emergency Laboratory of Hospital de Bellvitge. Blood was collected in BD Vacutainer® lithium-heparin tube (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 2000*g* for 10 min at room temperature. Subsequently, the obtained plasma was pooled and stored at (−75 ± 3) °C until use. An aliquot was separated to confirm the absence of ceftolozane and tazobactam using the current UHPLC-MS/MS procedures.

2.2. Preparation of calibration, control and internal standard materials

For ceftolozane and tazobactam, two stock solutions of each one from independent weighing were prepared at a concentration of 2.00 g/L. The stock solutions for ceftolozane were prepared by weighing 5.20 mg of ceftolozane sulfate and dissolving this one in 2 mL water:metanol:DMSO (50:25:25, *v/v/v*); and for tazobactam, weighing 4.02 mg in 2 mL of water:metanol:DMSO (50:25:25, *v/v/v*). For each drug, eight working standards (10.0, 50.0, 100, 250, 450, 750, 1000 and 1250 mg/L) with a volume of 1-mL each one were prepared pipetting, respectively, 5.0, 25, 50, 125, 225, 375, 500, and 625 µL of stock solution and 995, 975, 950, 875, 775, 625, 500, and 375 µL of water. These solutions were stored light-protected at (−75 ± 3) °C. On the day of analysis, 100 µL of calibration materials at 1.00, 5.00, 10.0, 25.0, 45.0, 75.0, 100 and 125 mg/L were prepared pipetting 10 µL of working standard and 90 µL of drug-free plasma. Four plasma quality control (QC) materials were prepared in the same manner as the calibration materials at 3.00, 7.50, 30.0 and 80.0 mg/L.

Stock solution of ceftazidime-D_5 was prepared by diluting 1 mg of ceftazidime-D_5 in 10.0 mL of metanol:DMSO (50:50, *v/v/v*). Sulbactam stock solution was made weighing 2.5 mg in 25 mL of methanol. These solutions were stored at (−75 ± 3) °C. A working solution of IS was prepared freshly for 20 samples analysis by adding 150 µL of each stock solution to 5.70 mL of acetonitrile.

2.3. Instrumentation, measurement procedures and equipment

Analyses were conducted using an Acquity® UPLC® chromatographic system coupled to an Acquity® TQD® tandem-quadrupole mass spectrometer (Waters, Milford, MA, USA).

Given that, at the present time, we use a previously developed and validated UHPLC-MS/MS procedure to measure different β-LA concentrations in plasma [16] in routine practice, for practical reasons, we used the same sample preparation (protein precipitation with acetonitrile and subsequent dilution with water containing 0.1% (*v/v*) formic acid), chromatographic separation conditions (analytical column, mobile phases, flow rate, elution conditions based on gradient mode, injected volume, and autosampler temperature), and generic mass spectrometer parameters (capillary voltage, extractor voltage, RF lens voltage, source temperature, desolvation temperature, desolvation gas flow rate, and collision gas flow).

Ceftolozane and its IS were detected by multiple reaction monitoring (MRM) operating in positive electrospray ionization (ESI+) mode, and using the following transitions of mass-to-charge (*m/z*): ceftolozane, 667.2 → 199.3 (quantifier ion) and 667.2 → 139.0 (qualifier ion); ceftazidime-D_5, 551.9 → 467.9. On the other hand, for

tazobactam and its IS, the mass spectrometer operated in MRM and ESI- modes, and their m/z transitions were 299.0 → 138.0 (quantifier ion) and 299.0 → 254.9 (qualifier ion) for tazobactam, and 232.0 → 140.0 for sulbactam. Cone voltages of 23 V for ceftolozane, 20 V for ceftazidime-D_5 and tazobactam, and 25 V for sulbactam were used. Collision energies were 16/25 eV for ceftolozane and 14/15 eV for tazobactam as quantifier/qualifier ions, as well as, 12 eV for ceftazidime-D_5 and sulbactam. The dwell time was set to 100 ms for every channel.

Also, the following equipment was used: ADA-120/L analytical balance (Adam Equipment, Bletchley, UK), Acura® 825 adjustable 100–1000 µL volume pipette from Socorex Isba (Ecublens, Switzerland) (pipette A), Nichipet® EX II adjustable 10–100 µL (pipette B) and 0.5–10 µL (pipette C) volume pipettes from Nichiryo Co Ltd. (Koshigaya-shi, Saitama, Japan), and 2-mL BLAUBRAND® class A, USP certified bulb pipette with two marks (BRAND GMBH + CO KG, Wertheim, Germany).

2.4. Validation

Validations were carried out according to the European Medicines Agency (EMA) guideline [17], Clinical and Laboratory Standards Institute (CLSI) [18–22] and EUROLAB guidelines [23,24].

2.4.1. Intermediate imprecision and bias associated to calibration procedure

According to CLSI EP05-A3 guideline [18], to estimate intermediate imprecision (within-laboratory coefficient of variation) and bias, plasma QC materials were processed in 20 non-consecutive days over 2 months. Relative bias (δ_r) were calculated using the following equation:

$$\delta_r = \left(\frac{\bar{x} - \mu}{\mu}\right)\cdot 100$$

where $\bar{x}$ is the mean value obtained in the imprecision study for each control material and μ, the conventional values assigned by weighing.

According to the EMA guideline [17], CV should be ≤15% and δ_r should be within the acceptance criteria of ± 15%.

2.4.2. Lower limit of quantification

For each drug, lower limit of quantification (LLOQ) was estimated in the same way than the intermediate imprecision and bias, but processing a plasma sample at concentration of 1.00 mg/L.

According to the EMA acceptance criteria [17], the LLOQ should have a signal-to-noise (S/N) ratio ≥ 5, and an acceptable imprecision (20%) and bias (± 20%).

2.4.3. Selectivity

Selectivity (SEL) studies were performed according to the EMA guideline [17]. Thus, a double blank plasma sample (containing a neither analyte nor IS), a blank plasma sample (spiked only with IS), a sample at LLOQ and 9 drug-free plasma samples from volunteers not treated with ceftolozane-tazobactam but receiving other antibiotics (amikacin, ampicillin, aztreonam, cefepime, ceftazidime, gentamycin, meropenem, tobramycin, and vancomycin) were analyzed for interferences with the analytes. Antibiotic concentrations of the drug-free plasma patient samples were within therapeutic intervals for the aminoglycosids or above 3–5 of the minimal inhibitory concentration (MIC) for β-LA.

Based on the EMA criteria, peak area response of all possible interfering peaks at the retention time of analyte (ceftolozane or tazobactam) should be ≤20% of the LLOQ for the analyte and ≤5% for the IS (ceftazidime-D_5 or sulbactam).

2.4.4. Carry-over

In accordance with the EMA guideline [17], carry-over (CO) studies were assessed by injecting a double blank plasma sample, a sample at LLOQ, and the highest calibration material (125 mg/L) in the following order:

Sample at LLOQ − Highest calibration material

− Double blank plasma sample

The peak are responses obtained in the double blank plasma sample were evaluated at the retention time of ceftolozane, tazobactam and their IS.

Following the EMA criteria [17], carry-over was acceptable if peak area response in the double blank sample was ≤20% of the analyte peak area response at the LLOQ, and ≤5% of the peak area response of their IS.

2.4.5. Calibration curve

Eight plasma calibration samples were prepared and daily calibration curves for each batch were assessed. Integration of smoothed peak areas, calibration curves and calculation of ceftolozane or tazobactam concentrations were performed using the TargetLynx™ v4.1 software (Waters, Milford, MA, USA). Plasma calibration level 1 (1.00 mg/L) served as the LLOQ and calibration level 8 (125 mg/L) as the upper limit of quantification (ULOQ).

The calibration curves were generated by linear fit of the analyte/IS area response ratio multiplied by IS concentration vs. analyte concentration (1/X weighting; excluding the option to force through the point of origin). According to the EMA guideline [17], calculated concentrations of the calibration standards should be all within ± 15% of the nominal value (± 20% for the LLOQ).

2.4.6. Linearity

According to the CLSI EP06-A guidelines [19], to verify statistically the linearity interval of the measurement procedure for concentrations of ceftolozane and tazobactam in plasma, the highest calibrator material (125 mg/L), was either diluted with the lowest calibrator material (LLOQ, 1.00 mg/L) at ratios of 0:5 (dilution factor 1), 1:4 (dilution factor 2), 2:3 (dilution factor 3), 3:2 (dilution factor 4), 4:1 (dilution factor 5), and 5:0 (dilution factor 6). Dilution samples were randomly processed in triplicate. The measured values obtained were plotted on the y-axis versus the dilution factor on the x-axis. Using the Analyse-it® v5.10 statistical software (Analyse-it Software, Ltd., Leeds, UK), the polynomial regression method was used in order to evaluate the non-linearity. Briefly, this method consists of two parts. The first part examines whether a nonlinear polynomial fits the data better than a linear one. The second part, performed in cases when a nonlinear polynomial fits the data better than a linear one, assesses whether the difference between the best-fitting nonlinear and linear polynomial is less than a previously predefined bias requirement of the measurement procedure (in our case 15%, which was established from EMA [17]). Also, taking into account that the random variability can lead to poor ability to detect nonlinearity, imprecision values obtained for each dilution should be lower than the imprecision requirement previously predefined of the measurement procedure (in our case 15%, which was established from EMA [17]).

2.4.7. Dilution integrity

According to the EMA guideline [17], for ceftolozane and tazobactam, the dilution integrity experiment was carried out analyzing six replicates of a plasma sample prepared at two times the ULOQ, and subsequently diluted 1/10 with drug-free plasma. According to the EMA guideline, imprecision should be ≤15% and bias should be within ± 15%.

2.4.8. Recovery of extracted samples and matrix effect

According to the EMA, the CLSI-IFCC C50-A and the C62-A guidelines [17,20,21] and Viswanathan et al. [25], to examine the recovery of extracted samples (REC) and the matrix effect (ME), 4 concentrations

of ceftolozane and tazobactam (3.00, 7.50, 30.0 and 80.0 mg/L) and 1 concentration of their IS (2.5 mg/L) were tested. Recovery and ME (based on estimation of matrix factor) were calculated according to the following equations:

$$\text{REC (\%)} = \frac{\text{Peak Area response of extracted sample (with analyte)}}{\text{Peak Area response of post} - \text{extracted spiked sample}} \cdot 100$$

$$\text{ME (\%)} = \left(\frac{\text{Peak Area response of post} - \text{extracted spiked sample}}{\text{Peak Area response of analyte in pure solution}} \right) \cdot 100$$

Six different batches of plasma matrix samples were analyzed.

Considering the REC and ME of the IS, IS-normalized REC and IS-normalized matrix factors were also calculated by dividing the REC or ME of each analyte by the REC or ME of its IS.

According to the EMA [17] or CLSI [20,21] criteria, the variation in recoveries and matrix effects among all concentrations should be ≤15%.

2.4.9. Stability

Stability studies included working standard and control solutions stabilities of ceftolozane and tazobactam, IS-stock solutions, extracted samples in-autosampler stability and short- and long-term stabilities for concentration of ceftolozane and tazobactam. These studies were performed following the EMA guideline recommendations [17].

For stability of working standard, and control material solutions, these one were freshly prepared, diluted 10-fold with water, and processed. Afterwards, these solutions were kept at (−75 ± 3) °C until their respective analyses either 6 months later.

For stability IS-stock solutions, solutions were freshly prepared, diluted 10-fold with metanol:DMSO (50:50, *v/v/v*) for ceftazidime-D_5 and with methanol for sulbactam, and processed at the day of preparation and 6 months later, after a storage at (−75 ± 3) °C.

Stability of extracted samples in the autosampler was tested by processing the extracted samples and reinjecting them after 6 h, 12 h and 24 h storage at (4 ± 1) °C into autosampler.

Stability studies of concentration of analytes in the studied matrix (plasma in our case) were performed using the four plasma QC materials (3.00, 7.50, 30.0 and 80.0 mg/L). Three different batches of plasma QC materials were freshly prepared and processed, and left on the bench-top at room temperature, (5 ± 3) °C and at (−75 ± 3) °C until their respective analyses at different times. For short-term stability, the batch plasma QC stored at room temperature were processed 2, 4, 8 and 12 h later, and the batch plasma QC stored at (5 ± 3) °C, were processed 1, 2, 3 and 5 days later. On the other hand, for the long-term stability, the batch plasma QC stored at (−75 ± 3) °C were processed 6 months later.

All solutions, extracted samples and plasma QC materials were analyzed against a freshly prepared calibration curve and using ten replicates of each one. In all cases, to estimate the stability, percent deviations (%D) from the nominal concentrations (areas in the case of IS), i.e. biases, were calculated as:

$$\text{\%D} = \left(\frac{\text{Mean value of the 10 replicates} - \text{Nominal concentration}}{\text{Nominal concentration}} \right) \cdot 100$$

According to the EMA criteria [15], the %D should be within ± 15%.

2.4.10. Measurement uncertainty

Measurement uncertainties of ceftolozane and tazobactam concentration in plasma values were estimated using the top-down approach called *single-validation approach*, according to different guidelines [22–24]. Fig. 2. shows a *cause and effect* diagram used to identify the different sources of uncertainty. The main uncertainty sources considered were related with calibrator's-assigned values, the intermediate precision and the bias of the measurement system. These standard uncertainties were estimated using information from certified reference materials manufacturers (certificates of analysis), mass and volumetric equipment calibration certificates, and the performance characteristic results obtained from the current measurement procedures. Once the standard uncertainties were quantified, they were combined to give a combined uncertainty for, finally, to estimate an expanded uncertainty using a coverage factor of 2 ($k = 2$).

Uncertainties associated with the assigned values of calibration materials (u_{cal}) were due to the ceftolozane sulfate and tazobactam certified reference materials mass weighted into the balance, the different pipetted volumes used to prepare calibration materials, and the stability of working standards.

The measuring mass uncertainty from analytical balance (u_{bal}) was indicated in the certificate of external calibration as (5.00 ± 0.17) mg ($k = 2$).

According to the manufacturer's data, the 2-mL bulb pipette accuracy is 0.006 mL. To estimate its uncertainty (u_{bulb}) a type-B estimation using a triangular distribution was used.

The uncertainty associated to the measuring volume from pipetting (u_{pip}) was: (1000.0 ± 2.6) µL ($k = 2$), (500.0 ± 1.8) µL ($k = 2$) and (100.0 ± 0.4) µL ($k = 2$) for pipette A; (10.00 ± 0.18) µL ($k = 2$), (50.00 ± 0.22) µL ($k = 2$) and (100.0 ± 0.4) µL ($k = 2$) for pipette B; and (0.500 ± 0.02) µL ($k = 2$), (5.00 ± 0.08) µL ($k = 2$) and (10.00 ± 0.16) µL ($k = 2$) for pipette C.

Additionally, u_{bulb} and u_{pip} include the uncertainty related to the temperature change (u_{temp}) associated to the volume. By assuming a rectangular distribution, the uncertainty for the temperature variation for 2 mL bulb pipette and pipettes were calculated using the following equation:

$$u_{temp} = \frac{V_x \cdot \alpha \cdot \Delta T}{\sqrt{3}}$$

where V_x is the volume of the bulb pipette or the volume pipetted; α, the volume expansion coefficient for water ($2.14 \cdot 10^{-4}$ °C^{-1}); and ΔT, the difference between the actual laboratory temperature and the calibration temperature indicated in the bulb pipette ($\Delta T = 5$ °C) or the temperature during the calibration of the pipettes ($\Delta T = 2$ °C).

Uncertainty related to the stability of working standards (u_{stab}) was estimated as [26]:

$$u_{stab} = \frac{L_s}{\sqrt{18}}$$

where L_s is the absolute %D value obtained in the stability study.

Taking into account the procedure to prepare each calibration material described above, the relative u_{cal} was obtained as:

$$u_{cal} = \sqrt{u_{bal}^2 + u_{bulb}^2 + u_{pip,stock}^2 + u_{pip,water}^2 + u_{pip,ws}^2 + u_{pip,plasma}^2 + u_{stab}^2}$$

where $u_{pip,\ stock}$ is the relative uncertainty related to the stock solution volume pipetted for the preparation of the working standards; $u_{pip,water}$, the relative uncertainty associated to the water volume pipetted for the preparation of the working standards; $u_{pip,ws}$, the relative uncertainty related to the working standard solution volume pipetted for the preparation of the calibration materials; and $u_{pip,plasma}$, the relative uncertainty associated to the blank plasma volume pipetted for the preparation of the calibration materials.

The relative uncertainties associated to intermediate imprecision (u_p) were the *CV* obtained in the present study.

The different possible sources of bias considered were: calibration procedure (δ_r), REC, ME, CO and SEL. The REC and the ME biases are corrected by using the IS, whereas the other sources remain uncorrected. Treatment of corrected and uncorrected biases was performed according to Magnusson et al. recommendations [27].

Since there are no high-order reference materials to be used, we used the control materials as reference to estimate the δ_r as described above. The relative uncertainties related to the δ_r (u_δ) were calculated as follows:

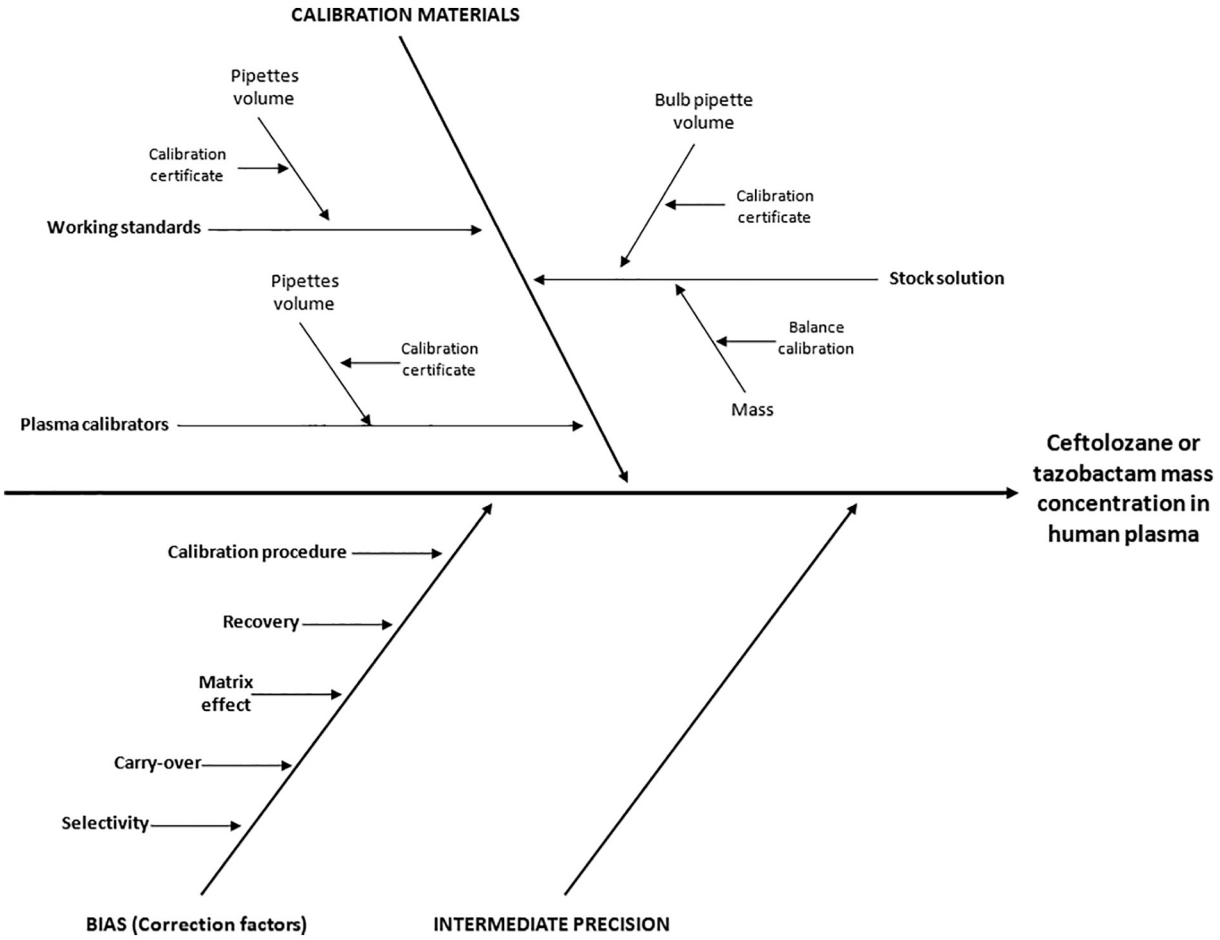

Fig. 2. Cause and effect diagram of the most relevant measurement uncertainty sources of ceftolozane or tazobactam mass concentration in human plasma using the single laboratory approach.

$$u_\delta = \sqrt{\delta_r^2 + \left(\frac{u_p}{n}\right)^2 + u_\mu^2}$$

where n, the number of reference materials processed ($n = 80$ in our case); u_μ, the relative uncertainty associated with the assigned value of the reference material estimated in the same manner than the u_{cal} described above but for the control materials.

Biases related to the REC, ME, CO and SEL were estimated using the following equations:

$$\delta_{REC} = \frac{1}{n} \cdot \sum_{i=1}^{n} (REC_i - \mu_{REC})$$

$$\delta_{ME} = \frac{1}{n} \cdot \sum_{i=1}^{n} (ME_i - \mu_{ME})$$

$$\delta_{CO} = CO - \mu_{CO}$$

$$\delta_{SEL} = \frac{1}{n} \cdot \sum_{i=1}^{n} (SEL_i - \mu_{SEL})$$

where n is the number of samples used to perform the REC, ME and SEL studies ($n = 12$ for REC and ME; and $n = 9$ for SEL); REC_i is the normalized REC in % (100·REC sample/REC internal standard) value obtained for the sample i; μ_{REC}, the REC reference value assigned as 100% (indicating 100% of REC); ME_i is the normalized ME in % (100·ME sample/ME internal standard) value obtained for the sample i; μ_{ME}, the ME reference value assigned as 100% (indicating that no ME exist); CO, the carry-over value in %; μ_{CO}, the CO reference value assigned as 0% (indicating that no CO exist); SEL_i, the selectivity value in % for the possible interference i; μ_{SEL}, the SEL reference value assigned as 0% (indicating that no interference exist).

The relative uncertainty associated to the REC (u_{REC}) and the ME (u_{ME}) were their respective coefficient of variations obtained divided by the root square of their number of samples used to perform the REC and ME studies. The relative uncertainties related to CO (u_{CO}) and the SEL (u_{SEL}) were calculated as:

$$u_{CO} = \sqrt{\delta_{CO}^2 + u_{s-CO}^2}$$

$$u_{SEL} = \sqrt{\delta_{SEL}^2 + \sum_{i=1}^{n} (u_{s-SEL})_i^2}$$

where u_{s-CO} and u_{s-SEL} were estimated using a right-angled triangle distribution (type-B approach) as:

$$u_{s-CO} \text{ or } u_{s-SEL} = \sqrt{\frac{(b-a)^2}{18}}$$

where a and b are, respectively, the lower and upper limits of the interval; being $a = 0\%$ in our case for CO and SEL; and b the CO value or the mean SEL value of all interferences considered.

All uncertainty bias sources were combined to obtain the uncertainty related to the bias (u_{bias}):

$$u_{bias} = \sqrt{u_\delta^2 + u_{REC}^2 + u_{ME}^2 + u_{CO}^2 + u_{SEL}^2}$$

Once the individual contribution of uncertainty sources was estimated, we combined them to give a relative combined standard uncertainty (u_c) according to the following equation:

Table 1
Characteristics of patients and the plasmatic concentrations of ceftolozane-tazobactam obtained in each case.

Patient	Age (years)	Weight (kg)	GFR (mL/min)	MIC (mg/L)	Ceftolozane-tazobactam dosage/frequency (g/h)*	Steady-state ceftolozane concentration (mg/L)	Steady-state tazobactam concentration (mg/L)
1	73	70	> 90	1.5	6–3/24	38.1	7.5
2	69	120	86	1.0	2–1/24	6.60	1.3
3	71	75	> 90	2.0	2–1/24	11.4	2.3
4**	39	77	> 90	4.0	4–2/24	18.5	2.7
					6–3/24	21.8	3.4
					6–3/24	32.9	4.2

In all cases, patients suffered for osteoarticular infections caused by multidrug-resistant *Pseudomonas aeruginosa*.
GFR, Glomerular filtration rate estimated using the CKD-EPI formula.
MIC, Minimum inhibitory concentration of ceftolozane-tazobactam for each *P. aeruginosa* strain.
* Ceftolozane-tazobactam was administered in continuous infusion in all cases.
** For the particular Case 4, three different samples are represented, which were obtained at different time during the treatment.

Table 2
Intermediate imprecision and bias values obtained in the UHPLC-MS/MS measurement system for ceftolozane and tazobactam mass concentrations in plasma.

Quantity	Material	n	$\bar{x}$ (mg/L)	CV (%)	μ (mg/L)	δ_r (%)
P—Ceftolozane; mass c.	LLOQ	80	0.97	12.5	1.00	−3.0
	QC1	80	3.05	7.2	3.00	1.7
	QC2	80	7.71	5.4	7.50	2.8
	QC3	80	31.5	3.6	30.0	5.0
	QC4	80	85.2	3.1	80.0	6.5
P—Tazobactam; mass c.	LLOQ	80	1.04	15.9	1.00	4.0
	QC1	80	2.99	7.9	3.00	−0.3
	QC2	80	7.28	6.6	7.50	−2.9
	QC3	80	28.6	4.9	30.0	−4.7
	QC4	80	75.4	3.8	80.0	−5.8

LLOQ, lower limit of quantification; QC1, control material 1; QC2, control material 2; QC3, control material 3; QC3, control material 4; n, number of materials processed; $\bar{x}$, mean value; CV, intermediate coefficient of variation; μ, reference value (conventional value); δ_r, intermediate relative bias.
Quantities are described according to the IFCC and IUPAC recommendations [30]. P, plasma; mass c., mass concentration.

$$u_c = \sqrt{u_{cal}^2 + u_p^2 + u_{bias}^2}$$

Relative expanded uncertainty (U) was obtained by multiplying u_c by a coverage factor of 2 [22].

Finally, the U values obtained were compared with the uncertainty requirement, whose was established based on the maximum permissible root mean square of the relative error of measurement (Δ_{max}) concept, in accordance to the guideline of the German Medical Association on quality assurance in medical laboratory testing [28]. The Δ_{max} value used was 21% and obtained as:

$$\Delta_{max} = \sqrt{\delta_{max}^2 + CV_{max}^2}$$

being δ_{max} and CV_{max}, the EMA relative bias and imprecision requirements (15%), respectively [17].

2.5. Application to biological samples

The current UHPLC-MS/MS procedures as well as another previously published by our group [16] have been developed to be introduced into an institutional antimicrobial stewardship program approved by our hospital. This program included the optimized use of antibiotics in difficult-to-treat infections, such as those occurring in critically-ill-patients and osteoarticular infections. Particularly, the administration of β-LA in continuous infusion or extended infusion against these difficult-to-treat infections is considered a routine clinical practice in our hospital.

2.5.1. Patients and sample collection

We evaluated the applicability of the UHPLC-MS/MS procedures by processing plasma samples from patients treated with ceftolozane-tazobactam admitted in Infectious Diseases Department. All these patients suffered osteoarticular infections.

Blood samples were obtained at least 72 h after the beginning of ceftolozane-tazobactam in order to assure that it represented

Table 3
Recoveries, internal standard-normalized recoveries, matrix factors, and internal standard-normalized matrix factors obtained in the UHPLC-MS/MS measurement system for ceftolozane and tazobactam mass concentrations in plasma.

Value	Ceftazidime-D_5 recovery (%)	Ceftolozane recovery (%)	IS-normalized recovery (%)	Ceftazidime-D_5 matrix factor (%)	Ceftolozane matrix factor (%)	IS-normalized matrix factor (%)
2.5 mg/L	75.4 (11.5)	–	–	112.4 (10.5)	–	–
3.0 mg/L	–	75.2 (5.9)	100.5 (9.4)	–	112.9 (6.9)	100.9 (7.9)
7.5 mg/L	–	78.4 (4.8)	104.7 (9.1)	–	113.9 (4.3)	102.3 (12.0)
30.0 mg/L	–	81.5 (2.6)	109.3 (11.9)	–	117.6 (2.5)	105.5 (10.3)
80.0 mg/L	–	85.1 (4.6)	113.6 (8.1)	–	121.1 (4.6)	108.7 (11.4)

Value	Sulbactam recovery (%)	Tazobactam recovery (%)	IS-normalized recovery (%)	Sulbactam matrix factor (%)	Tazobactam matrix factor (%)	IS-normalized matrix factor (%)
2.5 mg/L	80.9 (10.9)	–	–	105.9 (11.2)	–	–
3.0 mg/L	–	81.0 (13.5)	100.1 (12.4)	–	102.4 (9.9)	96.7 (10.2)
7.5 mg/L	–	81.7 (13.1)	101.0 (12.1)	–	104.0 (10.1)	98.2 (10.6)
30.0 mg/L	–	82.0 (13.9)	101.4 (12.8)	–	106.5 (7.3)	100.6 (9.7)
80.0 mg/L	–	83.4 (9.8)	103.6 (10.7)	–	107.9 (5.9)	101.9 (7.8)

Coefficients of variation (in %) between patients are indicated in brackets.
IS, internal standard.

Table 4
Measurement uncertainty budget for the measurement of ceftolozane and tazobactam mass concentrations in human plasma using the *single laboratory validation* approach.

Quantity	Material	Value (mg/L)	u_{cal} (%)	u_p (%)	u_δ (%)	u_{REC} (%)	u_{ME} (%)	u_{SEL} (%)	u_{CO} (%)	u_{bias} (%)	u_c (%)	U (%)	U (mg/L)
P—Ceftolozane; mass c.	QC1	3.05	2.65	7.20	3.29	2.71	2.28	2.06	3.39	5.32	9.34	18.67	0.57
	QC2	7.71	2.65	5.40	3.92	2.63	3.46	2.06	3.39	5.88	8.41	16.83	1.30
	QC3	31.5	2.65	3.60	5.67	3.43	2.97	2.06	3.39	6.03	7.51	15.0	4.7
	QC4	85.2	2.65	3.10	7.00	2.34	3.29	2.06	3.39	5.66	6.98	14.0	11.9
P—Tazobactam; mass c.	QC1	2.99	3.16	7.9	3.36	3.58	2.94	1.52	2.05	5.30	10.02	20.04	0.60
	QC2	7.28	3.16	6.6	4.39	3.49	3.06	1.52	2.05	5.30	9.04	18.07	1.32
	QC3	28.6	3.16	4.9	5.52	3.70	2.80	1.52	2.05	5.30	7.88	15.8	4.5
	QC4	75.4	3.16	3.8	6.59	3.09	2.25	1.52	2.05	4.60	6.75	13.5	10.2

QC1, control material 1; QC2, control material 2; QC3, control material 3; QC4, control material 4; u_{cal} (%), relative uncertainty associated with the assigned value of the calibrator materials; u_p (%), relative uncertainty related to the intermediate precision; u_δ (%), relative uncertainty related to the bias associated to the calibration procedure; u_{REC}, relative uncertainty associated to the bias related to the recovery of the extracted samples; u_{ME}, relative uncertainty related to the bias associated to the matrix effect; u_{SEL}, relative uncertainty associated to the bias related to the selectivity; u_{CO}, uncertainty related to the bias associated to carry-over; u_{bias} (%), relative uncertainty related to the bias; u_c (%), relative combined uncertainty; U (%), relative expanded uncertainty; U (mg/L), expanded uncertainty in mg/L units. Quantities are described according to the IFCC and IUPAC recommendations [30]. P, plasma; mass c., mass concentration.

concentrations at the steady-state condition. Samples were collected in BD Vacutainer® lithium-heparin tube (Becton Dickinson, Franklin Lakes, NJ, USA) and immediately refrigerated at (2–8) °C. Finally, they were then centrifuged at 2000*g* for 10 min at (4 ± 1) °C, aliquoted, and stored at (−75 ± 3) °C until analysis.

2.5.2. Microbiological studies

The isolation of the microorganisms was carried out by microbiological conventional procedures. Identification was performed with the MALDI-TOF Biotyper® system (Bruker, Billerica, MA, USA).

Antibiotic susceptibility was performed using the MicroScan® automated microdilution system (Beckman Coulter Inc., Brea, CA, USA). In addition, exact MIC values for ceftolozano-tazobactam was determined by *E*-test® method (bioMérieux, Marcy-l'Étoile, France). Minimal inhibitory concentration clinical breakpoints were defined according to the CLSI criteria [29].

3. Results

3.1. Intermediate imprecision and bias

Data for intermediate imprecision and relative bias are summarized in Table 2. The imprecision values ranged from 3.1% to 7.9% whereas relative bias values ranged between −5.8% and 6.5%. The imprecision and absolute relative bias values obtained were within the EMA criteria.

3.2. Lower limit of quantification

Lower limits of quantification were 0.97 mg/L (signal-to-noise ratio of 15.2) and 1.04 mg/L (signal-to-noise ratio of 8.9) for ceftolozane and tazobactam, respectively. Data for intermediate imprecision and relative bias at LLOQ are summarized in Table 2. All LLOQ obtained were in accordance to the EMA criteria.

3.3. Selectivity

Ceftolozane, tazobactam and their IS were clearly separated from endogenous peaks originating from the blank matrix.

No significant endogenous area response peaks were observed at the retention time of ceftolozane, tazobactam and their IS. For amikacin, ampicillin, aztreonam, cefepime, ceftazidime, gentamycin, meropenem, tobramycin, and vancomycin plasma batches, the peak area responses observed at ceftolozane retention times were 1.4%, 2.2%, 1.8%, 2.6%, 2.9%, 1.9%, 2.7%, 1.3%, and 1.6% of the LLOQ of ceftolozane, respectively, being 0.4%, 0.3%, 0.5%, 0.6%, 0.7%, 0.5%, 0.8%, 0.3%, and 0.6% at ceftazidime-D_5 retention time. Further, the peak area responses observed at tazbactam retention times were 1.7%, 1.1%, 2.7%, 1.4%, 1.3%, 2.1%, 1.8%, 0.9%, and 0.7% of the LLOQ of tazobactam, respectively; and being 0.3%, 0.4%, 1.0%, 0.5%, 0.4%, 0.8%, 0.5%, 0.4%, and 0.3% at sulbactam retention time. All peak area responses obtained were within the EMA criteria.

3.4. Carry-over

No significant area response peaks at the same retention time of ceftolozane, tazobactam and their IS were observed in the chromatogram of double blank plasma extract immediately after injection of highest calibration material. Peak areas responses observed in the double blank plasma sample after measurement of the highest calibration sample were 3.3% of the peak area response at LLOQ of ceftolozane, and 2.0% of the peak area response at LLOQ of tazobactam. On the other hand, peak area responses were 0.9% and 0.6% of the peak area responses of ceftazidime-D_5 and sulbactam, respectively. Peak area response obtained accomplished the EMA acceptance criteria.

3.5. Calibration curve

Typical calibration curve equations were $y = 0.7016 \cdot x - 1.061$ ($r^2 = 0.9957$) and $y = 0.3028 \cdot x + 0.1262$ ($r^2 = 0.9948$) for ceftolozane and tazobactam, respectively; where y is the analyte/IS standard area response ratio multiplied by the IS concentration, and x is the nominal concentration of analyte. The deviations of the calculated concentrations from their nominal values at LLOQ ranged from 5.6% to 12.2% for ceftolozane and − 10.4% to −3.5% for tazobactam. Calibration materials other than LLOQ were within (3.2–7.9)% and (1.9–9.3)% for ceftolozane and tazobactam, respectively. All these values accomplished the EMA criteria.

3.6. Linearity

For ceftolozane, mean values ± standard deviation obtained for each dilution were: (0.94 ± 0.08) mg/L, (25.2 ± 0.85) mg/L, (48.9 ± 1.27) mg/L, (74.6 ± 1.59) mg/L, (99.7 ± 2.08) mg/L, and (122 ± 1.53) mg/L. In the linearity analysis, no second or third order polynomial fit was statistically better than the linear fit at the 5% significant level, indicating that between the LLOQ and the ULOQ a linearity interval exists. Dilution integrities of all samples achieved the EMA acceptance criteria for imprecision and bias.

For tazobactam, mean values ± standard deviation obtained for each dilution were: (1.02 ± 0.09) mg/L, (25.2 ± 0.95) mg/L, (50.9 ± 1.53) mg/L, (74.3 ± 2.10) mg/L, (104 ± 2.08) mg/L, and (122 ± 2.08) mg/L. The linearity analysis showed a nonlinear

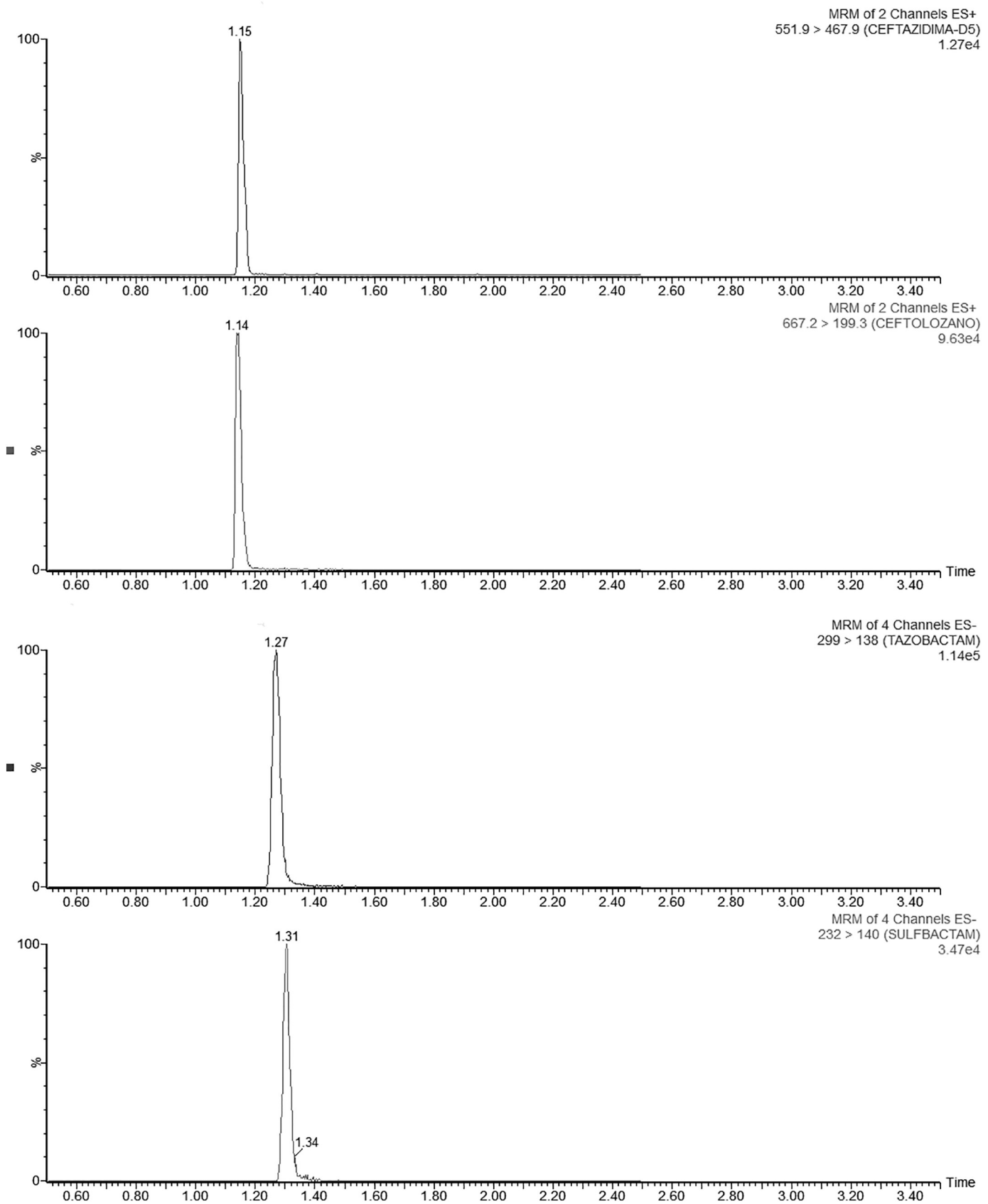

Fig. 3. Multiple reaction monitoring chromatograms for ceftolozane, tazobactam, and their internal standards (ceftazidime-D_5 and sulfactam) for a control sample at 3.00 mg/L.

response. The third-order term in the third-degree polynomial was significant ($p = 0.0399$) and the nonlinear terms in the second-order model were not significant ($p = 0.2363$). The standard errors also showed that the third-order model fitted better than the first- or second-order models. Percentage differences in predicted values between the first- and third-order models were within −1,7 and 9.4%. Taking into account that none of these differences exceeded the linearity (bias) criterion of 15%, as well as the imprecision requirement (15%), the measurement procedure was considered linear between the LLOQ and the ULOQ.

3.7. Dilution Integrity

Imprecision and bias values for dilution integrity at ten-fold dilution for ceftolozane were found to be 6.4 and −5.2%, respectively; and 7.8 and −7.3% for tazobactam, being lower than the EMA requirements.

3.8. Recovery of extracted samples and matrix effect

Values for REC, IS-normalized REC, matrix factor, IS-normalized matrix factor and variabilities of REC and ME of ceftolozane and tazobactam at different concentrations are showed in Table 3. Evaluation of the matrix effect showed ion enhancement for ceftolozane, tazobactam well compensated by their IS. The variation in recoveries and matrix effects among all concentrations accomplished with the EMA or CLSI criteria.

3.9. Stability

Working standard and control solutions (10.0, 50.0, 100, 250, 450, 750, 1000 and 1250 mg/L; and 30.0, 75.0, 300 and 800 mg/L) were stable during storage at (−75 ± 3) °C for at least 6 months with absolute %D values of 8.2%, 8.1%, 7.8%, 7.9%, 7.7%, 7.4%, 7.6%, 7.9%, 8.0%, 7.6%, 8.3%, and 7.2%, respectively for ceftolozane; and 9.7%, 9.3%, 8.9%, 7.5%, 10.1%, 9.4%, 9.3%, 8.2%, 9.8%, 9.1%, 8.8%, and 8.9% for tazobactam. Internal standard-stock solutions stored at (−75 ± 3) °C was stable for 6 months (absolute %D value of 9.2% for ceftolozane and 10.9% for tazobactam). On the other hand, ceftolozane and tazobactam concentrations in extracted samples were stable in the autosampler at (4 ± 1) °C for 12 h (absolute %D values ≤13.4% and ≤14.2% for ceftolozane and tazobactam, respectively). Furthermore, ceftolozane and tazobactam concentrations in plasma were stable during storage at (5 ± 3) °C for a period of 2 days (absolute %D ≤ 13.9% for ceftolozane and ≤14.5% for tazobactam), at room temperature for 8 h (absolute %D ≤ 12.9% and ≤13.3% for ceftolozane and tazobactam, respectively), and at (−75 ± 3) °C for at least 6 months (absolute %D ≤ 14.2% for ceftolozane and ≤14.7% for tazobactam).

All percent deviations were negative, indicating a decomposition or degradation of ceftolozane and tazobactam.

3.10. Measurement uncertainty

Table 4 shows the measurement uncertainty budget containing all main uncertainty sources, as well as the combined and expanded uncertainties. The *U* values obtained were lower than the maximum permissible root mean square of the relative error of measurement (21%).

3.11. Clinical application

Ceftolozane and tazobactam concentrations in plasma as well as clinical and microbiological information of selected cases are shown in Table 1. In all cases, ceftolozane-tazobactam was administered in continuous infusion. Plasma drug concentrations were above the MIC all the time (T > MIC 100%) and they were also higher than the recommended 3–4 times the MIC value for optimizing the pharmacodynamic parameters of these antibiotics [1–3,12–15].

4. Discusion

The UHPLC-MS/MS procedures were developed and validated for measurement of ceftolozane and tazobactam concentrations in plasma. These procedures were applied in the management of patients with osteoarticular infections caused by MDR *P. aeruginosa* to monitor the treatment with ceftolozane-tazobactam in continuous infusion.

Chromatographic separation and generic mass spectrometer conditions, and sample preparation were previously reported by our group as adequate for the analysis of different β-LA concentrations in plasma [16]. Taking into account that we use the reported measurement procedure for TDM of β-LA in routine practice, for practical reasons, we decided to use those conditions for measurement of ceftolozane and tazobactam concentrations in plasma. Under these chromatographic separation conditions, ceftolozane eluted at retention time of 1.14 min, ceftazidime-D_5 at 1.15, tazobactam at 1.27 min, and sulbactam at 1.31 min. Typical MRM chromatograms for the lowest control material (3.00 mg/L) are shown in Fig. 3. The UHPLC-MS/MS run times were 3.5 min, being shorter than other procedures previously reported [10–12]. On the other hand, setting the generic MS parameters described by our group [16], specific MS parameters as *m/z* precursor and product ions, cone voltage and collision energy were optimized injecting 10.0 mg/L of each drug and IS solution in a mixture of water:-acetonitrile 50:50 *v/v* containing 0.1% formic acid at a flow rate of 10 μL/min. The most abundant ions obtained were the $[M-H]^+$ adducts in ESI+ for ceftolozane, and ceftazidime-D_5, and the $[M-H]^-$ adducts in ESI- for tazobactam and sulbactam. Furthermore, because ceftolozane and ceftazidime-D_5 detected in ESI+ presented similar elution times than tazobactam and sulbactam in ESI-, we preferred not use the polarity switching mode option. Therefore, two injections were carried out, one to monitor ceftolozane and ceftazidime-D_5 and the second one to trace tazobactam and sulbactam. Furthermore, taking into account the REC and ME results obtained in this study, extraction procedure previously reported by our group [16] (based on protein precipitation with acetonitrile), besides simplifying the extraction procedures published by other groups [10–15], can also be applied for measurement of ceftazolane and tazobactam concentration.

Regarding to the measurement procedure performance characteristics, we evaluated intermediate imprecision, bias, selectivity, carry-over, calibration curve, linearity, dilution integrity, recovery of extracted samples, matrix effect, stability, and measurement of uncertainty. Of all of them, selectivity, carry-over, matrix effect and measurement uncertainty have not been evaluated by other groups [10–15], although the importance that these metrological characteristics have for obtaining accurate ceftolozane and tazobactam concentration results, and that these pharmacological quantities are measured for TDM or to perform pharmacokinetic/pharmacodynamic studies.

The imprecision, bias and LLOQ results obtained for the proposed UHPLC-MS/MS procedures were similar or better to those of previous publications [10–15]. These results indicate acceptable precisions and trueness. Also, we considered that the LLOQ were low enough given that ceftolozane-tazobactam MIC's for many multidrug-resistant Gram-negative bacilli are higher than 1.00 mg/L [28,31], and because patients receiving a continuous infusion administration rarely will have low concentrations of ceftolozane and tazobactam in plasma.

Selectivity data obtained showed that no endogenous interferences exist. Carry-over values obtained were negligible, indicating that there are no necessary include blank samples between patient samples to prevent the CO.

For each analyte, a calibration curve consisting of 8 plasma calibrators was prepared with a LLOQ and an ULOQ based on available literature and *in-house* experience. The calibration curves generated showed that linear regression with a weighting scheme of 1/X can

describe the data set generated in the intervals of (0.97–125) mg/L for ceftolozane, and (1.04–125) mg/L for tazobactam. Also, statistical linearity studies were performed to verify the linearities between the LLOQ and the ULOQ, indicating that the linear regression model used for the calibration curve were valid.

According to the results obtained in the dilution integrity study, for those samples with a concentration above the ULOQ, a 1/10 dilution can be applied. Higher dilution factors were not investigated because of the wide calibration range.

We showed steady REC and ME values, given that the use of the ceftazidime-D_5 and sulbactam as IS well compensated the lack of REC and the ME observed in the measurements of ceftolozane and tazobactam concentrations. For ceftolozane, we did not use a ceftolozane-labeled compound due to its high price, but we used a labeled-cephalosporin analogue structurally similar to the ceftolozane, the ceftazidime-D_5, which elutes simultaneously with ceftolozane. For tazobactam, we used a chemical structural analogue, sulbactam, as IS due to problems of availability at the moment of purchase of its stable labeled compound.

Ceftolozane and tazobactam working standard and control solutions, as well as the IS-stock solutions were stable during storage at (−75 ± 3) °C for at least 6 months. Extracted samples were stable in autosampler at (4 ± 1) °C for 12 h. The storage capabilities of plasma samples were investigated and deemed to be acceptable for a minimum of 8 h at room temperature, 2 days at (5 ± 3) °C and 6 months at (−75 ± 3) °C. Despite this, taking into account that the stability of concentration of β-LA in plasma at room temperature or refrigerated is low [32,33], mainly in sample extracts, we recommend process a maximum run of 30 samples and using a refrigerated autosampler temperature to prevent the gradual antibiotic decomposition.

Finally, in the validation process we included a detailed procedure to estimate a “new” performance characteristic: the measurement uncertainty, considering that it is essential to evaluate the reliability of β-LA concentration results facilitated by clinical laboratories and it is being required in accredited clinical laboratories [34].

5. Conclusions

In conclusion, we developed simple UHPLC-MS/MS procedures to measure concentrations of ceftolozane and tazobactam in plasma, and validated them following international guidelines. The mentioned procedures were useful for monitoring the treatment of difficult-to-treat osteoarticular infections caused by MDR *P. aeruginosa*. Overall, we believe our measurement procedures can be applied in the daily routine of the clinical laboratory, considering the performance characteristics obtained.

Funding

This study was supported by a research grant from the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III (PI14/00511) and funded in part by Merck Sharp & Dohme S.A (Merck Investigator Studies Program, IISP 54711).

Conflict of interest

The funders of the study did not play any role in the design, analysis or reporting of the results.

The authors declare that they have no potential conflict of interest.

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