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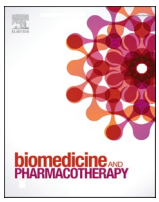

Analytical validation of a novel UHPLC-MS/MS method for 19 antibiotics quantification in plasma: Implementation in a LC-MS/MS Kit

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ABSTRACT

Background: Therapeutic drug monitoring (TDM) for antibiotic drugs represents a consolidated practice to optimize the effectiveness and to limit the toxicity of specific drugs by guiding dosage adjustments. The comparison of TDM results with drug-specific pharmacokinetic/pharmacodynamic (PK/PD) parameters, based on killing dynamics and bacterial susceptibility, increases the probability of therapeutic success.
Purpose: The aim of this study was the analytical validation of a new UHPLC-MS/MS assay for the quantification of 19 antibiotics divided in two different sets considering their chemical/pharmacological properties. This method has been implemented in an analytical LC-MS/MS Kit System by CoQua Lab s.r.l (Turin).
Methods: The analytical validation is developed in accordance with "ICH Harmonized Guideline M10 on bioanalytical method validation and study sample analysis" and "Guidelines for regulatory auditing of quality management system of medical device manufacturers". Method suitability in the clinical context was tested by analysing clinical samples from patients treated with antibiotic drugs.
Results: This method allows for simultaneous TDM of the following molecules: dalbavancin, daptomycin, linezolid, tedizolid, levofloxacin, moxifloxacin, meropenem, ertapenem, vaborbactam, avibactam, sulbactam, tazobactam, ceftazidime, ceftriaxone, ceftolozane, ceftobiprole, cefiderocol, ceftaroline and piperacillin. These drugs were quantified showing analytical performance parameters compliant with guidelines in terms of repeatability, reproducibility, robustness, bias, LOD, LOQ and linearity. The method was capable to successfully monitor drug concentrations in 65 samples from 52 patients undergoing treatment.
Conclusion: The UHPLC-MS/MS method described in this work can be useful for TDM of the reported antimicrobial agents. The analytical protocol is rapid and suitable to be used in routine analysis.

Abbreviations: IVDDs, in vitro diagnostic devices; ATB, antibiotics; UHPLC, ultra-high performance liquid chromatography; TDM, therapeutic Drug monitoring; MIC, minimum inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic; LC-MS, liquid chromatography-mass spectrometry; DBV, Dalbavancin hydrochloride; DPT, daptomycin; LZD, linezolid; TDZ, tedizolid; MOX, moxifloxacin; LEV, levofloxacin; ERT, ertapenem sodium; AVI, avibactam sodium salt; CFDCOL, cefiderocol; CFZD, ceftazidime; CFTRX, ceftriaxone sodium; CFBPL, ceftobiprole; SUL, sulbactam; TAZ, tazobactam; VAB, vaborbactam; PIP, piperacillin sodium salt; MEM, meropenem trihydrate; CEFTRL, ceftaroline ditrifluoroacetate salt; CEFTLZ, ceftolozane trifluoroacetate salt; IS, internal standard; QCs, quality control samples; ULOQ, upper level of quantification; LLOQ, lower level of quantification; ESI, electrospray ionization interface; LOD, limit of detection; LOQ, limit of quantitation; EQA, external quality assessment; RF, response factor; ICH, International Council of Harmonisation.

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1. Introduction

The increased use of in vitro diagnostic devices (IVDDs) has also applied to the clinical field of the "personalized medicine", including pharmacogenetics and therapeutic drug monitoring (TDM). This latter practice consists of measuring the active drugs in biological matrices (most often plasma) at defined timings, to control if their concentrations fall within therapeutic ranges to obtain optimal treatment effectiveness and tolerability [1]. This practice can be extremely useful to guide therapeutic adjustments for drugs (particularly those with narrow therapeutic indexes), in case of polypharmacy (when high risk of drug-drug interactions is expected) and in case of peculiar pathophysiological conditions (e.g., hepatic or renal insufficiency, pregnancy, intensive care unit patients, etc.). Moreover, it can be even useful for the evaluation of patient's compliance and to prevent resistance phenomena by optimizing drug exposure to exclude resistance phenomena [1,2].

TDM of antibiotics represents a consolidated practice in several contexts: in particular, this strategy has been applied to limit toxicity of specific classes of antimicrobial agents, like oxazolidinones and fluoroquinolones [3]. Moreover, the current global increase in antimicrobial resistance is leading to a gradual increase in the minimum inhibitory concentrations (MIC) for several drugs against many bacterial strains, making the achievement of adequate drug concentrations particularly critical to reach therapeutic success [4].

Furthermore, antimicrobial agents are the most frequently administered drugs to the critically ill patients, who show altered physiological profile and great intra- and inter-individual pharmacokinetic variability [3,4]. For these reasons, the comparison of TDM results with drug-specific pharmacokinetic/pharmacodynamic (PK/PD) parameters, based on killing dynamics of the drug (time vs concentration dependent killing) and bacterial susceptibility, greatly increases the probability of therapeutic success. [5].

These evidences have extended the use of TDM to many classes of antibiotic agents, which do not possess narrow therapeutic index, such as β–lactams, and have contributed to a wider application of this practice, avoiding toxicity and maximizing therapeutic efficacy [6].

Currently, the gold-standard for the TDM is considered liquid chromatography, usually coupled with tandem mass spectrometry (LC-MS/MS) [7].

Nevertheless, antibiotic drugs comprehend several classes and a lot of compounds, thus the development of an LC method for such a wide panel of molecules, maintaining great precision and standardization, involves many technical challenges. Among these, analytical interference between compounds (eg. matrix effect, cross-talk) must be explored and avoided, the chromatographic separation must be efficient and rapid, in order to obtain both high analytical performance (eg. accuracy and precision) and acceptable runtime and, finally, all these parameters have to be evaluated through a thorough analytical validation, following specific guidelines.

Considering these and other aspects, laboratories have started relying on industrial kit for antibiotics quantification. Commercially available analytical kits are being used also in the context of clinical pharmacokinetic trials, pointing out useful information, for example, about reduced antibiotic concentrations in critically ill patients undergoing Extracorporeal Membrane Oxygenation [8]. Nevertheless, current commercially available kits present several aspects which can be improved; in general, they fail to group a comprehensive panel of antibiotics, primarily due to different chemical/physical properties of the analytes and the challenge of developing simple and rapid analytical method still maintaining acceptable accuracy and precision. In this scenario, the aim of this work is to present the analytical validation of a UHPLC-MS/MS method for 19 antibiotics determination in plasma according to reference guidelines, to be considered for implementation in a new dedicated UHPLC-MS/MS Kit.

Table 1
SET A and SET B distribution of analytes and their corresponding IS.

SET A	SET B
Avibactam→ External standard	Dalbavancin → Dalbavancin IS
Cefiderocol → Cefiderocol IS	Daptomycin → External standard
Ceftaroline → Ceftaroline IS	Levofloxacin → Levofloxacin IS
Ceftazidime → Ceftazidime IS	Linezolid → Linezolid IS
Ceftobiprole → External standard	Meropenem → Meropenem IS
Ceftolozane → Ceftolozane IS	Ertapenem → Jolly IS
Ceftriaxone → Ceftriaxone IS	Moxifloxacin → Moxifloxacin IS
Piperacillin → Piperacillin IS	Tedizolid → Tedizolid IS
Sulbactam → External standard	
Tazobactam → External standard	
Vaborbactam → Vaborbactam IS	

2. Material and methods

The analytical method comprehends 19 antibiotics and 13 stable isotopic-labeled internal standard (IS). These molecules have been distributed, accordingly to their chemical properties and the most frequently administered combination of drugs in the clinical practice, in two SETs: SET A and SET B.

For some analytes IS normalization is not required and calibration with external standard is sufficient; an additional IS, named "IS Jolly", has been included in both SETs: being a non-endogenous and not particularly reactive molecule, IS Jolly could serve on different type of detectors to correct for analytical errors and its presence in constant quantity (in both SETs) comes in handy for performance evaluation in the context of KIT production. Each SET is composed of a 6 points (plus blank sample) calibration curve and two levels of quality controls. The same asset has been retained in the KIT design phase.

2.1. SET A and SET B analytes and IS distribution of the kit

The SET A includes: cefiderocol (CFDCOL), ceftazidime (CFZD), ceftriaxone (CFTRX), ceftobiprole (CFBPL), sulbactam (SUL), tazobactam (TAZ), vaborbactam (VAB), piperacillin (PIP), avibactam (AVI), ceftaroline (CEFTRL) and ceftolozane (CEFTLZ); CFDCOL-IS, CFZD-IS, CFTRX-IS, CEFTRL-IS, CEFTLZ-IS, PIP-IS, VAB-IS and IS Jolly were considered as IS to correct for analytical errors. (See Table 1).

The SET B groups: Dalbavancin (DBV), daptomycin (DPT), linezolid (LZD), tedizolid (TDZ), moxifloxacin (MOX), levofloxacin (LEV), ertapenem (ERT), meropenem (MEM); DBV-IS, LZD-IS, TDZ-IS, MOX-IS, LEV-IS, MEM-IS and IS Jolly were used for IS normalization. (See Table 1).

2.2. Standard, quality control and IS working solution

Stock solutions were prepared dissolving all powders according to each specific certificate of analysis and were used to independently spike blank plasma to obtain six levels of calibration and two quality control samples (QCs): low and high (QC 1 and QC2, respectively). Calibration curve levels and QCs concentrations in μg/mL for SET A and SET B are reported in Table 2 and Table 3 respectively. Calibration curve has been designed to allow for description of PK/PD target of attainment, considering antibiotics expected ranges and plasma cut-off values reported in the literature [9,10]. IS working solutions for both SETs were prepared at a concentration corresponding to the L3 of the calibration curve.

2.3. Standards, QCs and patients' samples extraction

After equilibration at room temperature, the following protocol was applied for each sample: 10 μl IS-working solution was mixed with 100 μl of samples, standards, and QCs and then, extraction from matrix was obtained by addition of 250 μl extraction solution. Following, vortex-mixing for 15 s, samples were kept at − 20 °C for 10 min to maximize

Table 2
SET A: Analytes concentration in the calibration curve levels and QCs (μg/mL).

	L0	L1	L2	L3	L4	L5	L6	QC 1	QC 2
AVIBACTAM	0.000	2.500	5.000	10.000	20.000	40.000	80.000	6.000	60.000
CEFIDEROCOL	0.000	3.750	7.500	15.000	30.000	60.000	120.000	9.000	90.000
CEFTAROLINE	0.000	1.875	3.750	7.500	15.000	30.000	60.000	4.500	45.000
CEFTAZIDIME	0.000	3.750	7.500	15.000	30.000	60.000	120.000	9.000	90.000
CEFTRIAXONE	0.000	3.750	7.500	15.000	30.000	60.000	120.000	9.000	90.000
CEFTOBIPROLE	0.000	1.875	3.750	7.500	15.000	30.000	60.000	4.500	45.000
CEFTOLOZANE	0.000	3.125	6.250	12.500	25.000	50.000	100.000	7.500	75.000
PIPERACILLIN	0.000	4.688	9.375	18.750	37.500	75.000	150.000	11.250	112.500
SULBACTAM	0.000	1.563	3.125	6.250	12.500	25.000	50.000	3.750	37.500
TAZOBACTAM	0.000	1.563	3.125	6.250	12.500	25.000	50.000	3.750	37.500
VABORBACTAM	0.000	1.563	3.125	6.250	12.500	25.000	50.000	3.750	37.700

Table 3
SET B: Analytes concentrations in the calibration curve levels and QCs (μg/mL).

	L0	L1	L2	L3	L4	L5	L6	QC 1	QC 2
DALBAVANCIN	0.000	6.250	12.500	25.000	50.000	100.000	200.000	15.000	150.000
DAPTOMYCIN	0.000	3.750	7.500	15.000	30.000	60.000	120.000	9.000	90.000
TEDIZOLID	0.000	0.625	1.250	2.500	5.000	10.000	20.000	1.500	15.000
LINEZOLID	0.000	0.625	1.250	2.500	5.000	10.000	20.000	1.500	15.000
MEROPENEM	0.000	2.500	5.000	10.000	20.000	40.000	80.000	6.000	60.000
ERTAPENEM	0.000	2.500	5.000	10.000	20.000	40.000	80.000	6.000	60.000
MOXIFLOXACIN	0.000	0.313	0.625	1.250	2.500	5.000	10.000	0.750	7.500
LEVOFLOXACIN	0.000	0.313	0.625	1.250	2.500	5.000	10.000	0.750	7.500

Table 4
Chromatographic elution: gradient phases concentration (%v/v).

Time (min)	Phase A (%)	Phase B (%)
0.0	100.0	0.0
0.70	100.0	0.0
1.80	88.0	12
2.50	76.0	24
3.90	76.0	24
5.50	48.0	52.0
6.50	48.0	52.0
7.00	25.0	75.0
7.50	25.0	75.0
8.50	0.0	100.0
9.00	0.0	100.0
9.20	100.0	0.0
10.0	100.0	0.0

protein precipitation.

Subsequently, all samples were centrifuged at 10000 rpm for 10 min at RT.

50 μl supernatant were then diluted with 100 μl of diluent solution (specific for SET A and SET B), transferred in total recovery vials and 10 μl was injected in the chromatographic system.

2.4. Chromatographic conditions

The chromatographic system used for validation was an Acquity H-Class PLUS ® (Waters), with a Sample Manager FTN-H® auto-sampler and a column manager Acquity UPLC® column oven. The chromatographic separation was performed on KIT column at 40 °C. The flow rate was maintained constant at 0.4 mL/min; chromatographic separation was optimized in gradient elution, as reported in Table 4.

The total run time was 10 min. SET A and SET B are designed to run in the same chromatographic session. The temperature of the sample manager was set at 10 °C.

The separation efficiency was evaluated considering Van Deemter model through N (number of theorical plates) and HETP (height equivalent to a theoretical plate) calculus, as follow: $N = 16 \bullet \left(\frac{t_R}{W_b}\right)^2$ and $H = \frac{L}{N}$ where t_R was retention time expressed in minutes, W_b was the width calculated at the peak base and L was the column length in millimetres.

2.5. Mass spectrometry conditions

Analytical determination was performed on a tandem mass spectrometry XEVO TQ-S micro, Waters® (Milan, Italy), with an electrospray ionization (ESI) interface. Most analytes and corresponding IS were detected in ESI positive ionization mode (ESI+), while SUL, VAB and VAB-IS were optimized in negative ionization (ESI-). (As reported in Table 5 and Table 6 for SET A and SET B, respectively).

Optimization of the MS conditions has been performed by infusion of reference standards of each compound and corresponding IS (concentration:1 ppm in water/methanol 50% v/v) at 5.0 μl/min into the mass spectrometer, in combination with the flow from the chromatographic system at medium concentrations phases (Phase A and Phase B 50%v/v).

Nitrogen (>99.9%) from a Nitrogen LCMS 40–1 nitrogen generator (Claind, Lenno, CO, Italy) was used as nebulizer and heating gas, while argon was used as collision gas.

Electrospray voltage was set at 3.5 kV; source temperature at 550 °C; nebuliser gas flow at 1000 L/h as general conditions.

2.6. Method validation

Analytical validation was performed in compliance with EMA, FDA and ICH Harmonised Guideline for bioanalytical method validation [11–14] for the following parameters: specificity and selectivity, linearity range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, intra-day and inter-day precision, robustness and matrix-effect. All used solvent were LC-MS/MS grade and purity for all powders was ≥ 95%.

2.6.1. Analytical selectivity and specificity

Analytical selectivity was considered as "the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behavior" [15] and was assessed analyzing blank sample (plasma sample without addition of analyte or IS) obtained from six different lots of

Table 5
SET A: Mass transition of the analytes and the IS measured with ESI + /- mode.

Analyte/IS, ESI+ /-	Ion QUANTIFIER MRM [m/z]	Ion QUALIFIER MRM [m/z]	Cone Voltage [V]	Collision Energy [V]
AVIBACTAM (+)	266.0 → 154.2		35	15
		266.0 → 124.2	35	15
CEFIDEROCOL (+)	752.2 → 285.0		35	15
		752.2 → 468.0	35	18
CEFTAROLINE (+)	605.2 → 208.0		35	30
		605.2 → 262.0	35	25
CEFTAZIDIME (+)	547.1 → 468.0		35	10
		547.1 → 440.0	35	15
CEFTOBIPROLE (+)	535.2 → 203.0		35	28
		535.2 → 264.0	35	21
CEFTOLOZANE (+)	334.2 → 199.1		35	10
		334.2 →166.9	35	15
CEFTRIAXONE (+)	555.2 → 396.0		35	12
		555.2 → 324.0	35	15
PIPERACILLIN (+)	518.3 → 359.2		35	8
		518.3 → 302.1	35	10
TAZOBACTAM (+)	301.0 → 207.0		35	15
		301.0 → 188.0	35	10
SULBACTAM (-)	232.0 → 140.0		-35	-12
		232.0 → 188.0	-35	-12
VABORBACTAM (-)	296.3 → 234.0		-35	-18
		296.3 → 278.0	-35	-12
CEFIDEROCOL IS (+)	760.2 → 293.0		35	15
		760.2 → 468.0	35	18
CEFTAROLINE IS (+)	609.2 → 212.0		35	30
		609.2 → 266.0	35	25
CEFTAZIDIME IS (+)	553.0 → 474.0		35	10
		553.0 → 402.0	35	16
CEFTOLOZANE IS (+)	337.2 → 205.1		35	10
		337.2 → 139.0	35	20
CEFTRIAXONE IS (+)	559.3 → 400.0		35	12
		559.3 → 328.0	35	15
PIPERACILLIN IS (+)	523.2 → 364.0		35	10
		523.2 → 307.0	35	8
VABORBACTAM IS (-)	301.0 → 239.0		-35	-18
		301.0 → 283.0	-35	-12
JOLLY IS (+)	313.1 → 102.0		35	60
		313.1 → 153.0	35	60

plasma. The instrumental response was evaluated through percent deviation from LLOQ concentration level: acceptability criteria in terms of absolute value were gap $< 20\%$ and $\leq 5\%$ for analytes and IS responses, respectively.

2.6.2. Calibration curve and range

The calibration curve comprehended six concentration levels in addition to blank sample (LV0). The interpolation of two points represented by Area/Area IS and concentration was calculated by least square method. The acceptability criteria for linearity were represented by correlation factor (R^2) > 0.995 and linearity test by Olivieri [16]. The percent deviation of single concentration level was calculated as follows: $\Delta\% = \frac{(C_c - C_t)}{C_t} \bullet 100$. The $\Delta\%$ for each concentration level was accepted with values between $\pm$ 20% for LLOQ and $\pm$ 15% for other concentration levels [15].

Hubaux-Vos algorithm [17] algorithm was used for estimating the limit of detection (LOD) and the limit of quantification (LOQ) was defined theoretically as three times the LOD and considering a signal to noise ratio (S/N ratio) > 10 as requested by EMA and FDA guidelines [18].

The evaluation of instrumental deviation in linearity range of calibration curve was carried out through the response factor (RF) calculated as follow: $RF_i = \frac{A_i}{C_i}$, where A_i was the single analyte area and C_i was the related concentration. For those analytes were IS was used, the numerator for the equation was obtained by ratio of Analyte Area/Is Area. The deviation in each concentration level for each analyte was evaluated with percent difference of RF ($\Delta RF\%$) as follows: $\Delta RF\% = \frac{RF_i - \overline{RF}}{\overline{RF}} \bullet 100$, where the mean RF was calculated by relation $\overline{RF} = \frac{\sum_{i=1}^{n} RF_i}{n}$ and $n = 6$ was the number of concentration levels of calibration curve.

2.6.3. Repeatability and reproducibility

The repeatability and reproducibility were evaluated through ten repeated measures at four levels of concentration in three independent analytical sessions. The statistical analysis of this data was executed at 97.5% level of confidence of t-student distribution.

In this context, the intra-laboratory precision was established with limit repeatability calculus as follow: $r = \sqrt{2} \bullet t \bullet s_r$ where t was represented the t-student at $(1 - \alpha) = 0.975$ with $\nu = 9$ (degrees of freedom for ten repetition of experiment intra-day), then s_r was the standard deviation in repeatability conditions.

Precision evaluation in repeatability and reproducibility conditions was assessed through relative standard deviation $RSD\% = \frac{s}{\bar{x}} \bullet 100$, where s represented standard deviation in repeatability or reproducibility conditions, $\bar{x}$ is the mean value of ten measures executed by single operator on the same sample. The repeatability measures were conducted on the same day while the reproducibility measures on three different days, by two different operators. Inaccuracy has been calculated through the percent deviation as follows: $\Delta(\%) = \frac{\bar{x} - x_{ref}}{x_{ref}} \bullet 100$where $\bar{x}$ is the mean of the results and x_{ref} is the nominal value of each concentration level.

2.6.4. Accuracy, precision, recovery, uncertainty

The method's efficiency of extraction, identification and quantification of analytes was demonstrated by spiking plasma samples with

Table 6
SET B: Mass transition of the analytes and the IS measured with ESI + /- mode.

Analyte/IS, ESI+	Ion QUANTIFIER MRM [m/z]	Ion QUALIFIER MRM [m/z]	Cone Voltage [V]	Collision Energy [V]
DALBAVANCIN (+)	909.5 → 158.0		35	40
		909.5 → 634.2	35	27
DAPTOMYCIN (+)	811.1 → 313.2		35	30
		811.1 → 641.0	35	18
LEVOFLOXACIN (+)	362.2 → 318.2		35	18
		362.2 → 261.0	35	26
MOXIFLOXACIN (+)	402.4 → 261.0		35	22
		402.4 → 341.5	35	30
MEROPENEM (+)	384.2 → 298.1		35	15
		384.2 → 141.0	35	12
ERTAPENEM (+)	476.2 → 432.2		35	8
		476.2 → 233.1	35	14
TEDIZOLID (+)	371.2 → 343.0		35	18
		371.2 → 288.0	35	30
LINEZOLID (+)	338.2 → 296.2		35	16
		338.2 → 195.0	35	20
DALBAVANCIN IS (+)	911.9 → 340.2		35	20
		911.9 → 358.2	35	22
LEVOFLOXACIN IS (+)	366.2 → 322.5		35	18
		366.2 → 348.2	35	26
MOXIFLOXACIN IS (+)	407.3 → 266.0		35	22
		407.3 → 389.2	35	21
MEROPENEM IS (+)	390.2 → 304.1		35	15
		390.2 → 147.0	35	10
TEDIZOLID IS (+)	375.2 → 273.2		35	30
		375.2 → 289.1	35	30
LINEZOLID IS (+)	346.25 → 203.1		35	20
		346.25 → 243.5	35	20
JOLLY IS (+)	313.1 → 102.0		35	60
		313.1 → 153.0	35	60

standard solutions at four levels of concentrations: LLOQ, 12.5% of ULOQ, 50% of ULOQ and 75% of ULOQ. These samples were analyzed as ten replicates on three different days by two different operators to evaluate precision. Acceptability conditions for coefficient of variation (CV)% were: ± 15% for each level, except for the LLOQ, where ± 20% was considered.

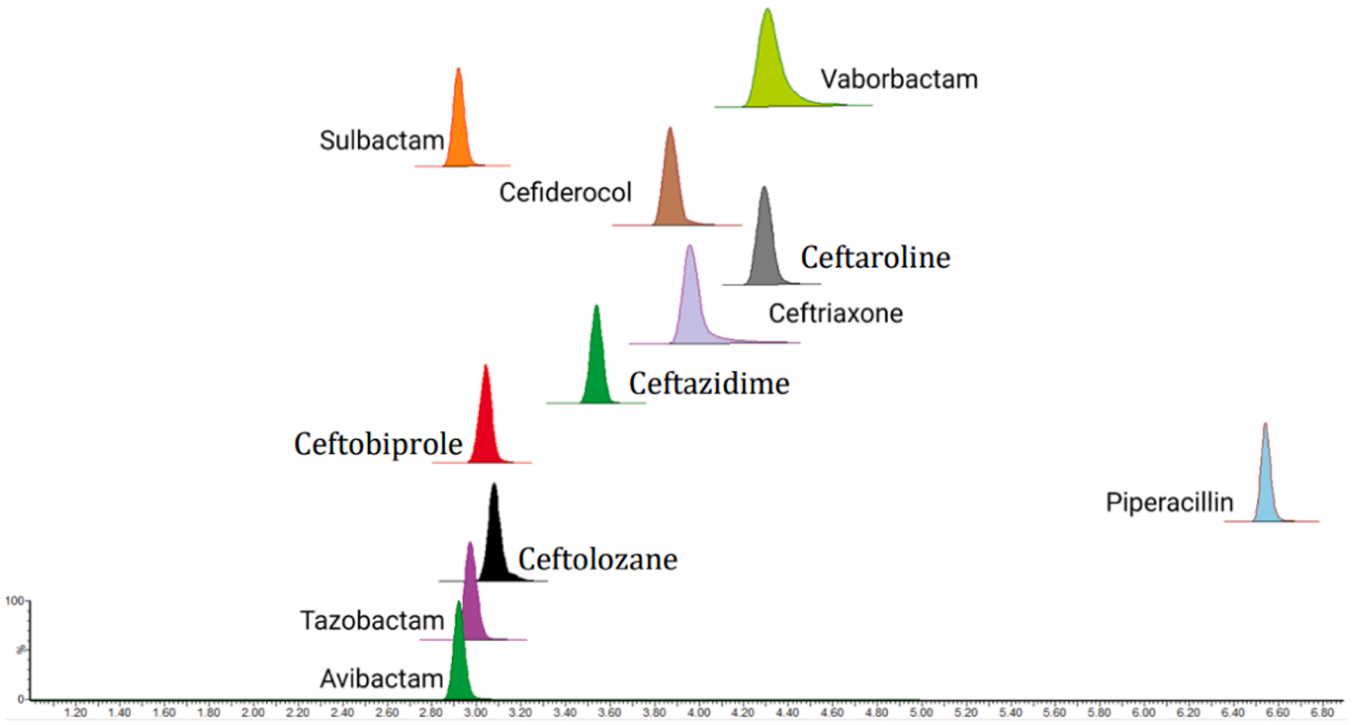


Fig. 1. Chromatogram of analytes related to SET A (standard in plasma matrix at concentration L3 ref. at Table 2). On the x-axis is reported time (minutes) while peaks, reported in y-axis, are normalized with relative percentage signal, referred to an absolute maximum intensity of 7.5×10^6.

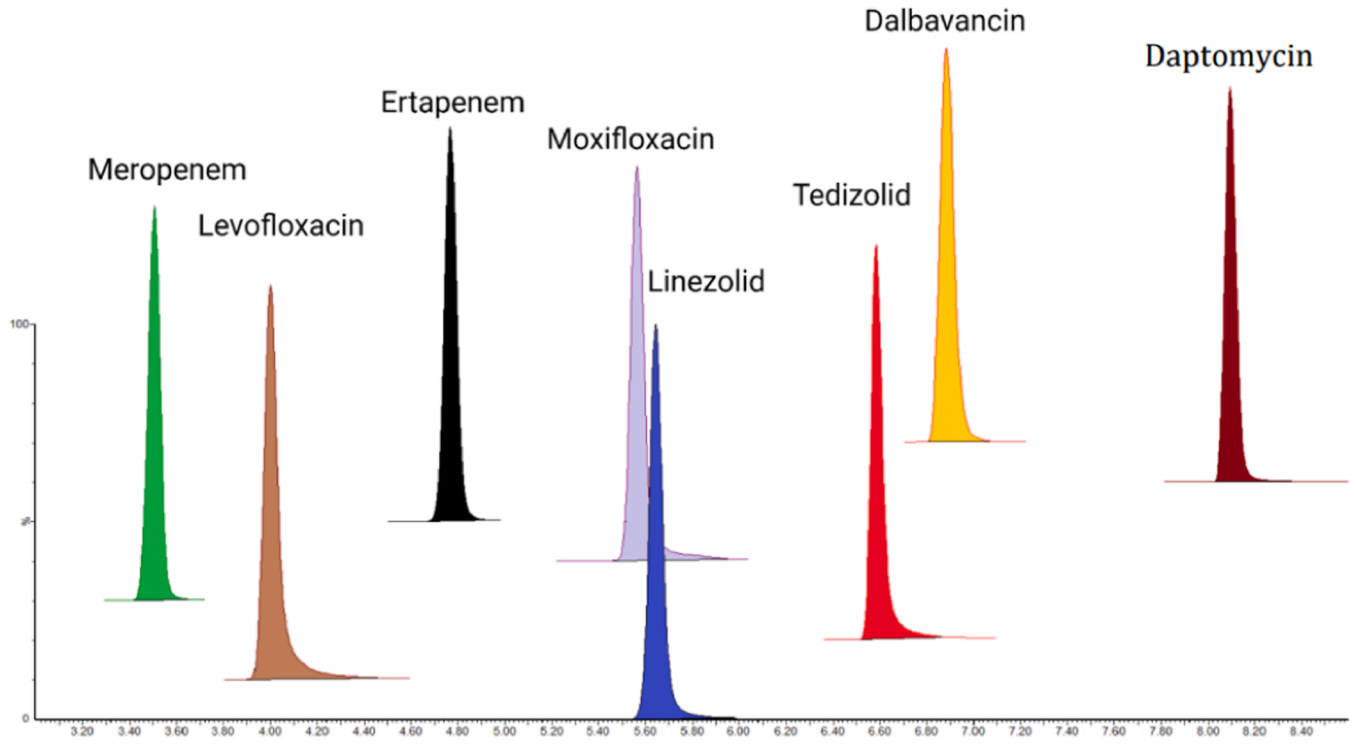


Fig. 2. Chromatogram of analytes related to SET B (standard in plasma matrix at concentration level n.3 ref. at Table 9). On the x-axis is reported time (minutes) while peaks, reported in y-axis, are normalized with relative percentage signal, referred to an absolute maximum intensity of 7.5×10^6.

Relative recovery percentage is described as $R(\%) = \frac{C_f - C_s}{C_a} \bullet 100$ where C_f is the mean concentration of spiked QCs at 4 levels of concentration, C_s is the mean concentration of not spiked QCs at 4 levels of concentration and C_a is the concentration of QCs at 4 levels of concentration; acceptability conditions were the same adopted for precision evaluation.

According to ICH guidelines [11], accuracy evaluation is performed through Bias % calculus as $b(\%) = \frac{\overline{x} - x_{ref}}{x_{ref}} \bullet 100$ where $\overline{x}$ is the mean of the results and x_{ref} is the reference value obtained by proficiency test specimens (EQA, external quality assessment); for those analytes without available EQA, accuracy has been calculated on nominal values in accordance with guidelines.

Horowitz heuristic model was used for estimating the measurement uncertainty. The conditions of applicability of Horwitz equation were verified on data distribution as follow: *ratio* between s_r and sR was to be comprised between 0.50 and 0.67. The sR (standard deviation in reproducibility conditions extrapolated with Horwitz equation) was calculated as follows: $sR = \frac{C}{100} \bullet 2^{(1-0.5\mathrm{Log}C)}$ where C was the concentration level of single analytes, reported as mass ratio.

2.6.5. Method robustness ad matrix effect

Method robustness has been assessed performing drugs quantification in QCs at 4 levels of concentrations (LLOQ, 12.5% of ULOQ, 50% of ULOQ and 75% of ULOQ) in three different plasma conditions: normal, haemolytic and lipaemic. 10 replicates of each level have been processed on 2 independent analytical runs.

The evaluation of interactions between different plasma matrix contaminants in the different lots and the instrumental response was conducted by evaluating the variability in the response factor (RF) calculated as follows: $RF_i = \frac{A_i}{C_i}$, where A_i was the single analyte area and C_i was the related concentration. The deviations for QCs in each plasma condition for each analyte were evaluated with percent difference of RF ($\Delta RF\%$) as follows: $\Delta RF\% = \frac{RF_i - \overline{RF}}{\overline{RF}} \bullet 100$, where the mean RF was calculated by relation $\overline{RF} = \frac{\sum_{i=1}^{n} RF_i}{n}$. In the last equation, the n = 4 was the number of concentration levels of QCs for each analyte.

To assess statistical deviation between repeatability in normal plasma condition and in haemolytic/lipaemic plasma condition, Fisher

Table 7
SET A: Chromatographic parameters, according to van Deemter model.

Analyte	RT (min)	Wb (min)	N	H (mm)
AVIBACTAM	2.92	8.50	6797	0.0147
SULBACTAM	2.95	10.11	4913	0.0204
TAZOBACTAM	3.12	8.30	8139	0.0123
VABORBACTAM	4.31	9.82	11141	0.0090
CEFTOLOZANE	3.16	7.41	10503	0.0095
CEFTOBIPROLE	3.11	6.50	13186	0.0076
CEFTAZIDIME	3.56	5.81	21700	0.0046
CEFIDEROCOL	3.82	5.92	24146	0.0041
CEFTRIAXONE	3.90	8.80	11313	0.0088
CEFTAROLINE	4.20	6.81	21973	0.0046
PIPERACILLIN	6.58	7.13	49471	0.0020

Table 8
SET B: Chromatographic parameters, according to van Deemter model.

Analyte	RT (min)	Wb (min)	N	H (mm)
LINEZOLID	5.62	0.14	25783	0.0039
LEVOFLOXACIN	4.01	0.12	17866	0.0056
TEDIZOLID	6.62	0.14	38241	0.0026
MEROPENEM	3.51	0.03	268861	0.0003
MOXIFLOXACIN	5.46	0.15	22173	0.0045
DAPTOMYCIN	8.12	0.12	74967	0.0013
DALBAVANCIN	6.85	0.15	32637	0.0030
ERTAPENEM	4.81	0.13	21813	0.0045

Table 9 (I)
SET A calibration curve parameters.

SET A	Avibactam	Sulbactam	Tazobactam	Vaborbactam	Piperacillin	Ceftaroline	Ceftriaxone	Cefiderocol	Ceftolozane	Ceftobiprole	Ceftazidime
Linearity range (μg/mL)	2.500–80.000	1.563–50.000	1.563–50.000	1.563–50.000	4.688–150.000	1.875–60.000	3.750–120.000	3.750–120.000	3.125–100.000	1.875–60.000	3.750–120.000
Correlation factor (R^2)	0.995	0.996	0.995	0.999	0.999	0.997	0.999	0.997	0.999	0.998	0.999
Slope (m)	6,30	3,46	16,37	$7{,}60 \times 10^{-4}$	$1{,}20 \times 10^{-4}$	$3{,}40 \times 10^{-4}$	$2{,}60 \times 10^{-4}$	$6{,}07 \times 10^{-5}$	$1{,}60 \times 10^{-4}$	$3{,}76 \times 10^{-1}$	$1{,}70 \times 10^{-4}$
Intercept (q)	-3819,160	2494,14	1699,57	60,62	-0,02	-0,02	-0,05	-0,10	-0,07	202,32	-0,09
$\Delta\overline{RF}$%	8430	8,13	13,83	5,70	1,41	3,76	3,89	9,30	3,60	4,81	3,66
limit of detection μg/mL (LOD)	0.750	0.450	0.450	0.450	1.350	0.550	1.100	1.100	0.950	0.550	1.100
limit of quantification μg/mL (LOQ)	2.380	1.490	1.490	1.490	4.460	1.780	3.570	3.570	2.980	1.780	3.570

Table 9 (II)
SET B calibration curve parameters.

SET B	Linezolid	Tedizolid	Levofloxacin	Moxifloxacin	Daptomycin	Dalbavancin	Meropenem	Ertapenem
Linearity range (μg/mL)	0.625–200.000	0.625–20.000	0.313–10.000	0.313–10.000	3.750–120.000	6.250–200.000	2.500–80.000	2.500–80.000
Correlation factor (R^2)	0.999	0.997	0.999	0.999	0.999	0.999	0.998	0.999
Slope (m)	$1{,}12 \times 10^{-6}$	$9{,}50 \times 10^{-6}$	$5{,}24 \times 10^{-6}$	$2{,}76 \times 10^{-6}$	$2{,}96 \times 10^{-7}$	$8{,}06 \times 10^{-8}$	$5{,}28 \times 10^{-6}$	23,74
Intercept (q)	0313	-0114	-0069	0373	0893	-0105	0,0002	3301,790
$\Delta\overline{RF}$%	3,95	6,73	7,48	8,35	7,49	8,18	8,77	1,61
limit of detection μg/mL (LOD)	0.185	0.089	0.098	0.098	1.100	1.990	0.750	0.750
limit of quantification μg/mL (LOQ)	0.612	0.276	0.306	0.306	3.675	6.124	2.45	2.45

Table 10 (I)
Considered parameters for method analytical validation referred to SET A.

SET A (I)	Avibactam				Sulbactam				Tazobactam				Vaborbactam				Piperacillin			
	QC1	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*
mean value of calculated concentration μg/mL (Xm)	4,97	10,12	24,58	48,52	3,77	7,81	13,69	30,70	3,71	6,64	23,28	22,50	3,69	6,30	25,73	34,15	9,46	16,37	62,30	87,82
standard deviation on repeatability (Sr)	0,36	0,64	1,58	2,84	0,21	0,35	0,55	0,38	0,21	0,37	0,67	1,01	0,26	0,45	1,12	2,52	0,31	1,51	4,16	7,13
confidence interval (CI)	0,28	0,56	0,99	2,18	0,16	0,27	0,42	0,29	0,16	0,29	0,51	0,77	0,20	0,35	0,86	1,94	0,24	1,17	3,20	5,48
repeatability coefficient (r)	1,18	1,86	2,88	9,26	0,70	1,15	1,79	1,24	0,67	1,21	2,18	3,28	0,84	1,47	3,66	8,22	1,01	4,94	13,58	23,24
relative standard deviation on repeatability (RSDr)	7,30	8,54	12,15	5,85	5,67	8,60	4,01	1,23	5,57	5,59	2,88	4,67	7,00	7,13	4,14	7,37	3,27	9,27	6,69	8,11
relative standard deviation on reproducibility (RSDR)	12,56	9,57	5,80	8,91	13,10	13,00	10,80	9,55	13,13	12,03	9,96	10,07	13,14	12,12	9,73	9,49	11,40	10,50	8,59	8,16
inaccuracy on repeatability (Δ%)	9,79	7,56	2,75	12,02	-5,06	8,87	4,12	14,01	-3,95	7,90	4,57	-12,37	-3,57	6,67	3,07	-8,71	9,59	11,87	7,35	13,52
inaccuracy on reproducibility (Δ%)	-10,62	9,01	3,71	13,84	6,09	2,34	1,15	9,10	4,07	2,45	1,45	0,53	3,44	-1,79	0,86	2,14	4,24	5,15	5,82	8,13
recovery (R%)	96,00				98,00				95,00				96,00				96,00			
uncertainty of measure (U(x))	25	24	17	18	26	26	22	19	26	24	20	20	26	24	19	18	22	21	17	16

Table 11
(I) Considered parameters for method analytical validation referred to SET B.

SET B (I)	Linezolid				Tedizolid				Levofloxacin				Moxifloxacin			
	QC1	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*
mean value of calculated concentration in μg/mL (Xm)	1,65	2,35	9,37	14,87	0,72	1,89	3,90	6,54	0,79	1,98	3,24	7,43	0,75	1,99	4,55	8,16
standard deviation on repeatability (Sr)	0,06	0,09	0,32	0,65	0,04	0,04	0,16	0,33	0,03	0,02	0,09	0,66	0,06	0,13	0,68	0,43
confidence interval (CI)	0,05	0,07	0,25	0,50	0,03	0,03	0,12	0,26	0,03	0,02	0,07	0,51	0,04	0,10	0,52	0,33
repeatability coefficient (r)	0,21	0,30	1,04	2,11	0,12	0,12	0,53	1,09	0,11	0,07	0,30	2,14	0,19	0,42	2,21	1,41
relative standard deviation on repeatability (RSDr)	3,80	3,87	3,40	4,30	4,93	1,80	4,14	5,09	4,20	1,10	2,85	8,85	7,66	6,50	14,00	5,27
relative standard deviation on reproducibility (RSDR)	14,83	14,06	11,42	10,65	14,90	14,41	13,04	12,06	14,82	14,42	13,40	11,83	14,70	14,41	12,74	11,66
Repeatability inaccuracy (Δ%)	3,87	2,21	-3,38	3,40	2,83	0,88	4,22	1,12	4,44	3,65	2,72	-1,16	-2,56	9,80	6,99	1,11
Reproducibility inaccuracy (Δ%)	-12,55	5,59	-4,47	6,46	-8,39	-2,22	-1,47	5,02	-8,03	-3,80	-5,80	3,34	13,05	12,13	-7,39	-11,23
limit of detection (LOD)	0185				0089				0098				0098			
limit of quantification (LOQ)	0612				0276				0306				0306			
recovery (R%)	98,00				95,00				97,00				97,50			
uncertainty of measure (U(x))	29	28	22	21	30	28	26	24	30	28	26	22	30	28	25	23

Table 10 (II)
Considered parameters for method analytical validation referred to SET A.

SET A (II)	Ceftaroline				Ceftriaxone				Cefiderocol				Ceftolozane				Ceftobiprole				Ceftazidime			
	QC1	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*
mean value of calculated concentration in μg/mL (Xm)	3,90	7,78	31,54	42,92	8,24	15,50	63,39	78,98	8,74	16,23	63,99	87,30	6,83	12,99	50,62	71,65	4,70	6,32	28,09	48,34	7,75	14,78	57,73	85,50
standard deviation on repeatability (Sr)	0,27	0,74	1,63	3,15	0,81	1,62	3,62	4,51	0,50	1,71	4,78	6,64	0,46	1,09	3,84	6,56	0,29	0,44	2,67	1,73	0,56	1,16	2,71	6,34
confidence interval (CI)	0,20	0,57	1,26	2,42	0,62	1,25	2,78	3,47	0,38	1,31	3,65	5,11	0,35	0,84	2,95	5,04	0,22	0,34	2,05	1,33	0,43	0,89	2,08	4,87
repeatability coefficient (r)	0,87	2,41	5,32	10,28	2,64	5,29	11,81	14,71	1,61	5,56	15,48	21,67	1,49	3,55	12,51	21,39	0,95	1,44	8,70	5,64	1,83	3,78	8,82	20,66
relative standard deviation on repeatability (RSDr)	6,82	9,51	5,18	7,34	9,84	10,47	5,71	5,72	5,68	10,50	7,41	7,16	6,69	8,37	7,58	9,15	6,09	6,99	9,50	3,58	7,27	7,83	4,68	7,41
relative standard deviation on reproducibility (RSDR)	13,03	11,75	9,52	9,08	11,60	10,60	8,57	8,30	11,50	10,50	8,56	8,20	12,00	10,90	8,86	8,50	9,00	12,10	9,68	9,00	12,00	10,70	8,69	8,00
inaccuracy on repeatability (Δ%)	-8,92	11,50	-9,64	-0,24	3,78	10,64	8,42	7,86	-2,10	13,32	9,70	-1,97	4,27	10,20	8,51	-0,36	12,28	12,84	6,57	-12,07	9,53	6,80	7,72	0,18
inaccuracy on reproducibility (Δ%)	2,81	4,76	7,46	7,35	4,99	6,21	6,65	8,49	-6,09	5,61	7,97	8,01	2,81	5,91	3,01	9,21	10,57	3,80	8,85	6,96	4,26	4,42	4,99	7,46
recovery (R%)	97,00				98,50				96,00				95,00				94,50				97,00			
uncertainty of measure (U(x))	26	23	19	18	23	21	17	17	23	21	17	16	24	22	18	17	18	24	19	18 12		21	17	8

Table 11
(II) Considered parameters for method analytical validation referred to SET B.

SET B (II)	Daptomycin				Dalbavancin				Meropenem				Ertapenem			
	QC1	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*	*QC1*	*QC2*	*QC3*	*QC4*
mean value of calculated concentration in μg/mL (Xm)	8,35	21,23	49,13	67,51	20,05	41,52	84,38	150,10	5,13	10,35	37,04	53,61	5,22	9,58	39,17	50,04
standard deviation on repeatability (Sr)	0,82	1,60	5,65	3,28	0,82	2,38	2,82	5,10	0,18	0,73	0,95	4,71	0,24	0,35	0,51	0,84
confidence interval (CI)	0,65	1,23	4,35	2,52	0,63	1,83	2,17	3,92	0,14	0,56	0,73	3,61	0,19	0,27	0,39	0,65
repeatability coefficient (r)	2,77	5,22	12,43	10,70	2,67	7,75	9,21	16,64	0,59	2,36	3,11	15,35	0,79	1,14	1,65	2,74
relative standard deviation on repeatability (RSDr)	10,19	6,87	8,18	4,86	4,08	5,72	3,35	3,41	3,49	7,01	2,57	8,77	4,63	3,65	1,29	1,67
relative standard deviation on reproducibility (RSDR)	11,62	9,96	8,46	8,48	10,18	9,13	8,21	7,52	12,50	11,25	9,29	8,78	12,47	11,39	9,21	8,87
Repeatability inaccuracy (Δ%)	5,31	4,80	6,40	4,71	14,39	3,39	4,56	2,61	12,61	10,31	14,11	8,81	11,20	1,98	3,04	14,17
Reproducibility inaccuracy (Δ%)	3,05	2,90	6,91	1,79	5,16	2,62	3,12	4,46	8,76	13,82	12,13	5,03	3,77	1,61	2,09	1,33
limit of detection (LOD)	1100				1990				0750				0750			
limit of quantification (LOQ)	3675				6124				2450				2450			
recovery (R%)	96,00				96,00				96,50				96,50			
uncertainty of measure (U(x))	22	19	17	16	20	18	16	15	24	22	18	16	24	22	18	17

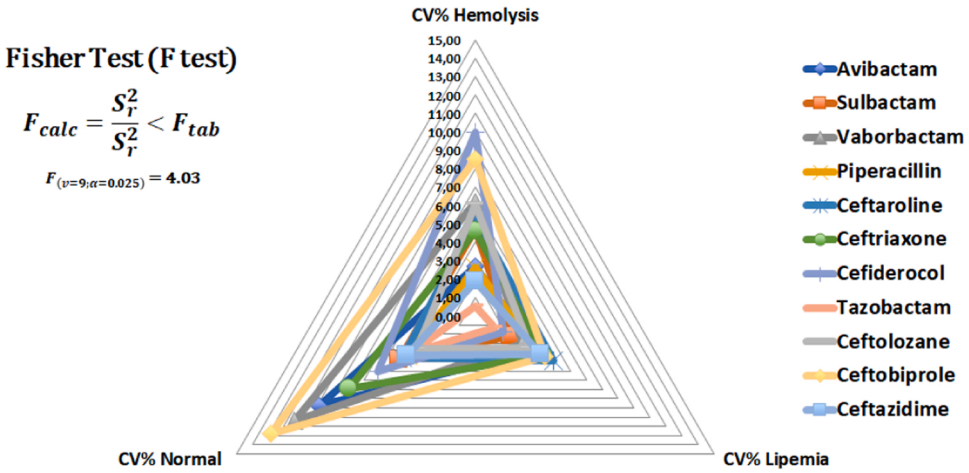


Fig. 3. SET A – ΔCV% of QCs (75% ULOQ for exemplification) in normal, haemolytic and lipaemic conditions.

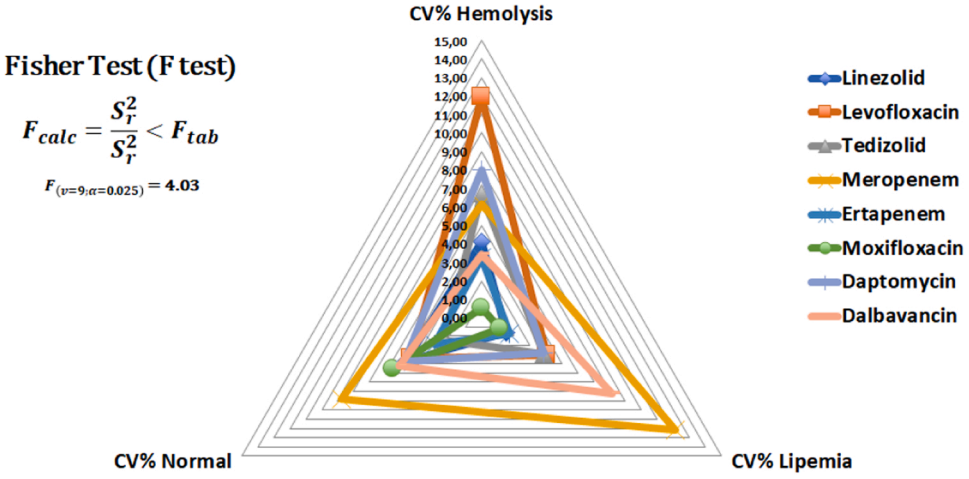
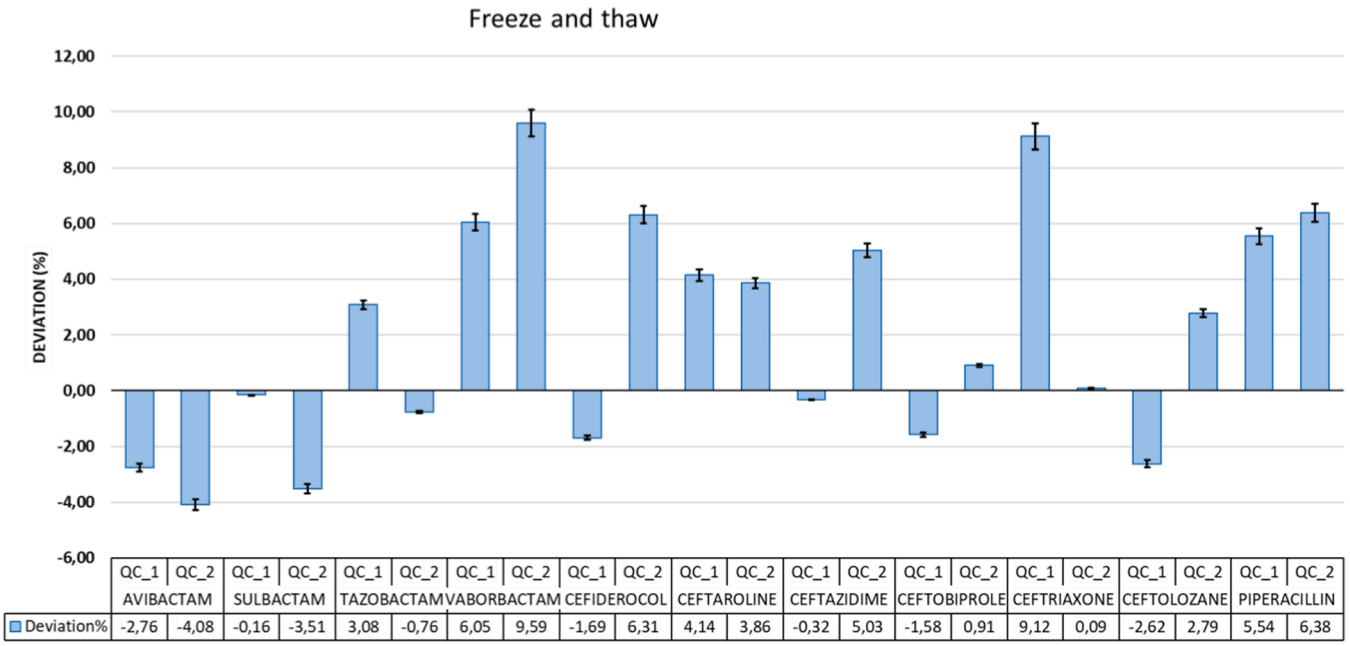


Fig. 4. SET B – ΔCV% of QCs (75% ULOQ for exemplification) in normal, haemolytic and lipaemic conditions.



	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2	QC_1	QC_2
	AVIBACTAM		SULBACTAM		TAZOBACTAM		VABORBACTAM		CEFIDEROCOL		CEFTAROLINE		CEFTAZIDIME		CEFTOBIPROLE		CEFTRIAXONE		CEFTOLOZANE		PIPERACILLIN			
Deviation%	-2,76	-4,08	-0,16	-3,51	3,08	-0,76	6,05	9,59	-1,69	6,31	4,14	3,86	-0,32	5,03	-1,58	0,91	9,12	0,09	-2,62	2,79	5,54	6,38		

Fig. 5. CV% expressed as "Deviation%" after 3 freeze and thaw cycles of QC1 and QC2 for each analyte of SET A.

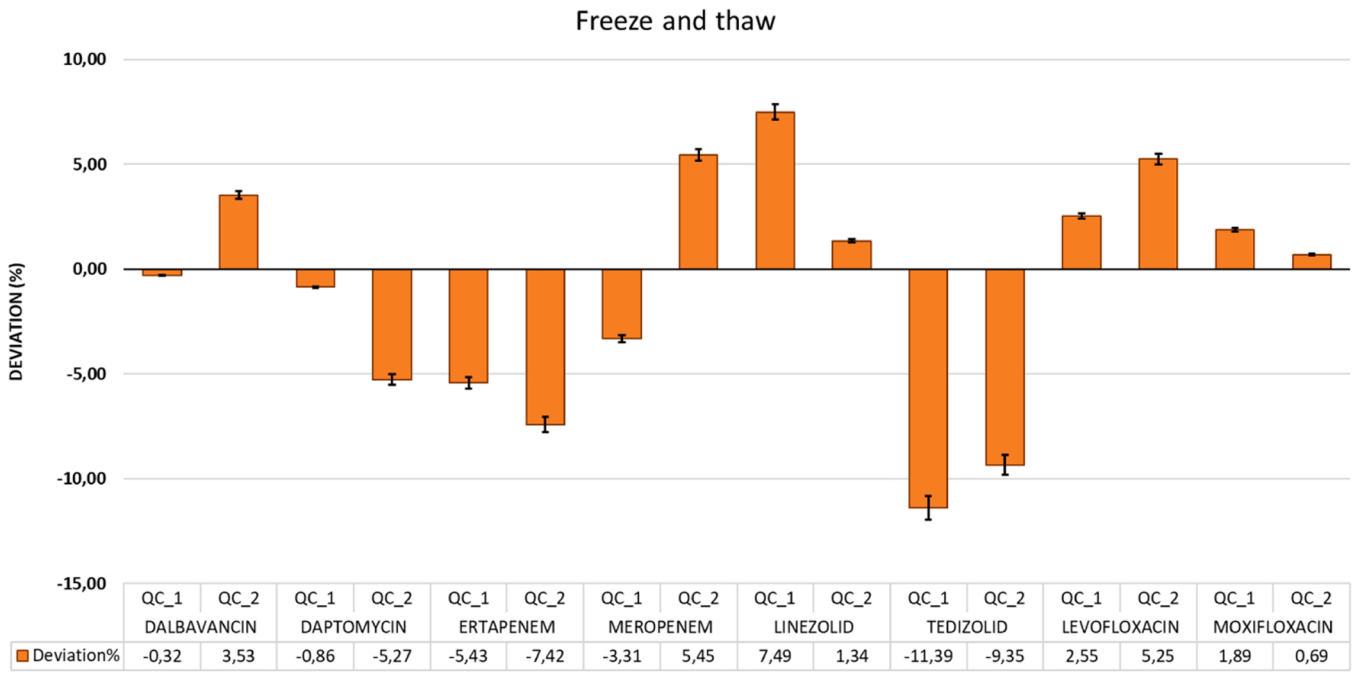


Fig. 6. CV% expressed as "Deviation%" after 3 freeze and thaw cycles of QC1 and QC2 for each analyte of SET B.

test at 97.5 level of confidence was performed, according to the following equation: $F = \frac{s_{r1}^2}{s_{r2}^2} < F_{tab}$; where $F_{(\nu=9;\alpha=0.025)} = 4,03$ and s_{r1} and s_{r2} represent, respectively, the standard deviation on repeatability under normal and haemolytic/lipaemic conditions.

In precision evaluation, CV% for each analyte in each plasma condition has been evaluated and acceptance criteria was ΔCV% between ± 15%, as recommended by ICH guidelines.

Furthermore, the matrix effect was evaluated on three replicates of QC_1 and QC_2 in six different plasma lots. The analytes response was compared to theorical concentration, with percent deviation in acceptability range of ± 15% for each concentration level. The same acceptability values were considered for CV% in precision evaluation of response data.

2.6.6. *Shelf life*

Stability study was conducted on samples at two different concentration levels, to evaluate the feasibility of samples and standards collection.

Samples were stored at −20 °C and −80 °C and the selected timings for the stability study included 10, 30 and 90 days.

Stability was calculated as the percent difference between analyte concentrations found in samples freshly extracted and samples stored at −20 °C and −80 °C.

In addition, analytes stability was assessed after three freeze and thaw cycles on both levels of QCs in five replicates. Analytes stability to freeze and thaw cycling (−80 °C) was calculated as deviation percentage (Δ%) in concentration for each analyte through following equation: $\Delta\% = \frac{[\overline{X}]_{t0} - [\overline{X}]_{t1}}{[\overline{X}]_{t0}} \bullet 100$; where $[\overline{X}]_{t0}$ represents mean concentration value for each analyte in 5 replicates of each fresh (t_0) QCs, while $[\overline{X}]_{t1}$ stands for mean concentration value for each analyte in 5 replicates of each QCs after every freeze and thaw cycle (t_1). Acceptance criteria was Δ% between ±15%..

Analytes showed stability up to 15 days and 3 months when stored at − 20 °C and − 80 °C, respectively.

2.6.7. *Clinical applications*

Described method has been applied on the real-life context processing samples from patients treated with antibiotics at the "Amedeo di Savoia" hospital (Turin, Italy). Inclusion criteria were the assumption of considered antibiotic drugs.

The study has been conducted in compliance with the declaration of Helsinki and local review board regulations; all patients gave written informed consent, according to the local ethics committee standards ("Appropriatezza farmacologica della terapia anti-infettiva", approved by Ethical Committee "A.O.U CITTA' DELLA SALUTE E DELLA SCIENZA DI TORINO – A.O. ORDINE MAURIZIANO DI TORINO – A.S.L CITTA' DI TORINO", n°456/2022).

For SET B, method has been applied on 6 samples of DBV; 4 samples of DPT; 4 samples of LEV; 5 samples of LZD; 3 samples of TDZ; 3 samples of MOX and 1 sample of MEM.

Concerning SET A, method application has been tested on 4 samples of CEFTRL; 6 samples of CFZD; 6 samples of CFTRX; 4 samples of CFDCOL; 5 samples of CEFTLZ; 5 samples of TAZ; 2 samples of CFBPL; 3 samples of PIP; 2 samples of VAB; and 2 samples of SUL.

Samples of DBV have been collected at three timings; T0, T1 and T2, corresponding to "before infusion", one-hour post-infusion and two-hours post-infusion; samples for DPT, CEFTRL, CFZD, CFTRX and CFDCOL were collected at 2 timings: PRE and POST, considered, in order, as before drug infusion and within one-hour post-infusion.

3. Results

Our results were in accordance with ICH recommendations and EMA guidelines, showing good linearity within range of measure, optimal accuracy as well as satisfactory results in terms of intra-day and inter-day precision. Method achieved expected criteria in robustness, sensitivity and matrix effect suppression.

3.1. *Chromatographic separation*

The whole chromatographic run was completed in 10.0 min.

Retention times of the selected analytes were reported in Tab. 3. The separation efficiency was assessed monitoring peak W_b, number and height of theorical plates and derives were prevented.

The peak of each analyte had compliant shape and symmetry factor was included between 0.90 and 1.10.

Fig. 1 and Fig. 2 show a chromatogram recorded from the middle

Table 12A
SET A antibiotic concentrations in human plasma samples.

SET A: analytes concentration (µg/mL) in real patient samples																				
Sample_ID	**CEFTRL**	**PZ_ID**	CFZD	PZ_ID	CFTRX	PZ_ID	CFDCOL	PZ_ID	CEFTLZ	PZ_ID	TAZ	PZ_ID	CFBPL	PZ_ID	PIP	PZ_ID	SUL	PZ_ID	VAB
PZ_36_PRE	0,76	PZ_38_PRE	2,4	PZ_41_PRE	14,43	PZ_50_PRE	12,05	PZ_52	60,18	PZ_52	8,59	PZ_57	1,52	PZ_59	34,84	PZ_62	3,18	PZ_64	137,80
PZ_36_POST	10,61	PZ_38_POST	26,31	PZ_41_POST	212,01	PZ_50_POST	247,00	PZ_53	53,23	PZ_53	6,30	PZ_58	2,40	PZ_60	217,00	PZ_63	30,90	P_65	138,90
PZ_37_PRE	0,28	PZ_39_PRE	10,51	PZ_42_PRE	19,39	PZ_51_PRE	13,5	PZ_54	61,38	PZ_54	7,63			PZ_61	110,00				
PZ_37_POST	17,81	PZ_39_POST	85,53	PZ_42_POST	264,91	PZ_51_POST	71,4	PZ_55	55,10	PZ_55	7,82								
		PZ_40_PRE	27,97	PZ_43_PRE	15,56			PZ_56	76,20	PZ_56	7,71								
		PZ_40_POST	116,2	PZ_43_POST	245,25														

point of the calibration curve for SET A and SET B respectively and the chromatographic parameters are reported in Table 7 and Table 8.

3.2. Calibration curve

The calibration curve was freshly prepared and extracted in three replicates on three different analytical sessions. The linearity results were consistent with acceptability criteria, as reported in Table 9 (I) and Table 9 (II) for SET A and SET B respectively, along with the calculated LOD and LOQ for each analyte of both sets.

3.3. Repeatability and reproducibility

The results of repeatability and reproducibility are compliant to acceptance range of accuracy and precision. The overall measurement parameters are reported in Table 10 (I-II) and Table 11 (I-II) for SET A and SET B, respectively.

3.4. Accuracy, precision, recovery and measurement uncertainty

The results of precision were reported in Table 10 (I-II) (I-II) and Table 11 (I-II) for SET A and SET B respectively. The percent deviation from nominal value and CV% were both in accordance with acceptability criteria in precision and accuracy evaluation.

The recovery for each compound was included between 92.5% and 99% (absolute value), while the Bias% was calculated for PIP, TAZ, SUL, CFZD, LZD and MEM (on available EQA) and ranged from 13,0 to 4,0% (absolute value). The uncertainty of measure was comprised between 8% and 26% for SET A and between 15% and 30% for SET B, calculated with coverage factor k = 2.0 (Table 10 (I-II) and Table 11 (I-II) for SET A and SET B, respectively).

3.5. Method robustness and matrix effect

ΔRF% for all analytes in each plasma conditions remained within ±15%; Considering precision evaluation, CV% for each analyte of both SETs was retained within acceptance criteria, in each plasma condition: results are reported in Fig. 3 and Fig. 4, for SET A and SET B respectively and referred to 75% of ULOQ concentration QC. In detail, for SET A, analytes showed a greater ΔCV%, in repeatability when considering normal plasma condition, with CFBPL showing a CV% of 13%; on the other hand, analytes of SETs B with greater ΔCV% were LEV under haemolytic plasma conditions (12%) and MEM and DBV under lipaemic plasma condition, 12% and 8,5% respectively.

No statistical deviation was observed between repeatability in normal plasma condition and in haemolytic/lipaemic conditions, with $F_{calc.} < 4.03$.

CV% for QC1 and QC2 in 6 different plasma batches was within 15% for all analytes, as requested by guidelines.

3.6. Shelf life

All analytes for both SETs were stable after 3 freeze and thaw cycles. CV% of QC1 and QC2 are reported in Fig. 5 and Fig. 6 for SET A and SET B respectively. Considering SET A, CV% ranges from − 2,76 to 9,12% for QC1 and from 0,09 to 9,59% for QC2; while for SET B, CV% ranges from − 11.39–7,49 and from − 9,35 to 5,45, for QC1 and QC2 respectively.

Analytes in plasma showed stability up to 15 days and 3 months when stored respectively at − 20 °C and − 80 °C.

3.7. Clinical applications

The method has been tested on real plasma samples from patients treated with antibiotics and was capable of correctly quantify target antibiotics within range of measure, data are summarized in Table 12A, Table 12B.

Table 12B
SET B antibiotic concentrations in human plasma samples.

SET B: analytes concentration (μg/mL) in real patient samples													
Sample_ID	DPT	Sample_ID	DBV	Sample_ID	LEV	Sample_ID	LZD	Sample_ID	MOX	Sample_ID	MEM	Sample_ID	TDZ
PZ_1	63,67	PZ_10_T0	3,65	PZ_20	8,93	PZ_24	0,20	PZ_29	1,17	PZ_32	85,28	PZ_33	3,88
PZ_2	26,10	PZ_10_T1	346,41	PZ_21	1,53	PZ_25	4,02	PZ_30	4,37			PZ_34	3,83
PZ_3_PRE	9,90	PZ_10_T2	310,05	PZ_22	1,28	PZ_26	16,31	PZ_31	0,98			PZ_35	2,04
PZ_3_POST	118,05	PZ_11	21,28	PZ_23	6,57	PZ_27	14,739						
		PZ_12	29,06			PZ_28	2,48						
		PZ_13	4,68										

4. Discussion

This work described a novel UHPLC MS/MS method intended to be implemented in a LC-MS/MS Kit. The analytical method has been fully validated according to EMA and ICH Harmonised Guideline for bioanalytical method validation recommendations [11] in the context of LOD, LOQ, specificity and selectivity, linearity range, accuracy, intra-day and inter-day precision, robustness, matrix-effect and stability under freezing/thawing condition. This method successfully group 19 antibiotics, and extractive procedure has been optimized to be fast and consistent: to maximize recovery of DBV and DPT, compounds with a high bound to plasma proteins, a freezing precipitation step has been added: the extractive solvent modifies plasma dielectric constant, inducing protein precipitation. The phospholipidic portion, which acted as important interference in LC-MS revelation of more lipophilic compounds, was further removed by keeping samples at − 20 °C and adding an ultracentrifugation step at 10000 rpm, In robustness evaluation, mean CV% for all compounds in QCs remained within 15%, highlighting method capability to suppress matrix effect in different plasma conditions: haemolytic, lipaemic and physiological. In detail, measurements were in accordance with compound chemical profiles, with more lipophilic compounds, like DBV, showing greater CV% in lipaemic condition and more hydrophilic ones, like LEV, displaying great variation in haemolytic condition. Freeze and thaw cycles appeared not to have effect on analytes stability: method ionization conditions assured good response also for more sensitive antibiotics, like cephalosporins and carbapenems.

Considering previously published papers, this method comprehends the largest number of analytes and a wider range of β-lactams [19,20].

The described method allows for quantification of new generation cephalosporins, like CFTRL, innovative lipoglycopeptide, like DBV and β-lactamase inhibitors like AVI, TAZ and VAB that have been re-evaluated in their combination-use with cephalosporins, considering the increment of the last years in multi drug resistance phenomena [21].

Another important aspect is the use of 13 isotopic-labelled IS to correct for undesired effects: other published method generally reports the use of deuterated analogues and competitors on the market prevalently consider the use of deuterated to limit the cost of production [22]. For these reasons our IS working solution has been optimized at a concentration to allow minimum IS powders use.

Since the presented method is meant to be used in a TDM routinary context, the possible matrix effect of haemolytic and lipaemic samples has been investigated, as well as calibrators and QCs stability to freeze and thaw cycles. When considering other published works, these aspects are generally poorly explored during method validation.

Limitation of this assay is the fact that it allows for quantification of total antibiotic concentrations; nonetheless for those antibiotics with low protein binding, the free fraction can be estimated from total concentrations [23].

When tested on clinical samples, the validated method was able to correctly quantify target antibiotics within range of measurement. "PRE" samples corresponded to the timing before a second drug administration and, for this reason, the antibiotic is detectable, and the response is not n.d. (not detectable) as expected. The presented method could be useful to reach the PK/PD target for considered antibiotics: describing, for example, the area under the concentration/time curve (AUC) for concentration/time dependant antibiotics, like DBV and correctly quantifying plasma concentrations of time dependant antibiotics, like LZD. Finally, the presented method/KIT has been used in research clinical settings [24–27] and in ongoing routinary TDM daily activity.

5. Conclusions

This work describes the analytical validation process of a novel multi-residual UHPLC MS/MS method for 19 antibiotics determination in plasma. Method validation has been carried out according to EMA and ICH Harmonised Guidelines for bioanalytical method validation.

Simple extractive protocol and fast run time made this method suitable for clinical chemistry lab. Method testing on several samples in a long-term clinical study is planned, in order be fully implemented in a validated LC-MS/MS analytical Kit useful for routinely TDM application. To conclude, this method could serve as an instrument to guide therapeutic adjustment, especially for those antibiotics where TDM is recommended [9]; given its fast-processing protocol and its analytical consistency, method suitability to automatic preparatory could be considered.

No patents.

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Conflict of interest statement

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marco Simiele reports a relationship with CoQua Lab srl that includes: equity or stocks. Alessandra Manca reports a relationship with CoQua Lab srl that includes:

equity or stocks. Antonio D'Avolio reports a relationship with CoQua Lab srl that includes: equity or stocks.

Data availability statement

Data are available on request by the corresponding author.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114790.

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