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Liquid chromatography-tandem mass spectrometry for the quantification of moxifloxacin, ciprofloxacin, daptomycin, caspofungin, and isavuconazole in human plasma

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ABSTRACT

A simple and precise ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method was developed for the simultaneous analysis of five anti-infective agents used to treat severe infections [three antibiotics (daptomycin, moxifloxacin, ciprofloxacin) and two antifungals (isavuconazole, caspofungin)] in human plasma. Sample preparation was based on protein precipitation with ice cold methanol. All five agents were analyzed with the corresponding isotopically labeled internal standards. All analytes were detected in multiple reactions monitoring (MRM) using API 4000 triple-quadrupole mass spectrometer with electrospray (ESI) source operating in positive mode. The calibration curves were linear over the selected ranges ($r > 0.99$). The method is precise and accurate with a total run time of 5.5 min. Accuracy of all target analytes ranged between 95.9–116.6%, measured with an imprecision of less than 10.8%. The lower limit of quantification was 1.25 mg/L for caspofungin, 0.3125 mg/L for isavuconazole, 3.125 mg/L for daptomycin, 0.075 mg/L for ciprofloxacin, and 0.1875 mg/L for moxifloxacin. The successful application of the method in patient samples proved its suitability for the medical surveillance of antimicrobial therapy in intensive care units as well as to other pharmacokinetic studies.

1. Introduction

Inadequate treatment of severe bacterial or fungal infections increases the mortality rate of intensive care units (ICU)' patients [1]. Challenges in eradicating infection in ICU patients are due to the lack of guidelines for the antimicrobial therapy intended for the critically ill. These patients show, depending on disease severity, different pathophysiological changes which impact the anticipated levels of the treatment and, therefore, the clinical outcome. Under the current circumstances and when standard dosing is applied, it is not possible to foresee in which cases an over or under exposure occur [2]. In the latter case resistance development and selection of drug resistant microbes become an additional burden [3]. Even though in the fight of bacterial infections in ICU' patients ß-lactams represent the first line of treatment, other classes of antibiotics that have considerable safe profile and are active against resistant bacteria are also often required [4–7]. For instance, ciprofloxacin (CPX) is a 2nd generation broad spectrum fluoroquinolone used to treat serious infections, especially Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Enterobacteriaceae* strains resistant to gentamicin [8,9], as well as against resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* strains (MRSA) [9,10]. Furthermore, moxifloxacin (MOX), a 4th generation broad spectrum fluoroquinolone with limited side effects, is used against problematic respiratory tract infections caused by *Streptococcus pneumonia* and *Haemophilus influenzae,* even those showing resistance to penicillin antibiotics [11,12]. In contrast to the aforementioned broad spectrum fluoroquinolones, daptomycin (DAP) is a cyclic lipopeptide antibiotic [13] active only against Gram-positive bacteria including methicillin-susceptible *S. aureus,* MRSA, as well as to other strains such as Streptococci and Enterococci resistant to vancomycin [14–16]. In addition to the challenges in treating severe bacterial infections, treatments of invasive fungal infections (IFI) represent also a significant problem for ICU patients. This is supported by the fact that delayed and inade-

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quately treated IFI, such as those caused by *Candida* and *Aspergillus* spp., are associated with high mortality rates [17,18] especially in immunocompromised patients. Different classes of antifungal agents such as echinocandins and triazoles are used to combat IFI [19,20]. Although triazoles revolutionized the prevention and treatment of IFI, most of the available ones suffer from high inter- and intra-individual pharmacokinetic variability and are associated with major drug-drug interactions [21]. Isavuonazole (ISC) is a relativly new triazole supposed to overcome problems encountered with existing ones [22]. ISC is active against a variety of fungi including *Aspergillosis* spp., *Candida* spp., as well as to other rare fungi [23,24]. Yet, as a more recent drug, studies regarding its pharmacokinetic profile in different types of patients are scarce. Caspofungin (CAS), an echinocandin, is the first cyclic lipopeptide used for the treatment of invasive Aspergillosis, invasive Candidiasis, and other suspected IFI in febrile and neutropenic patients [25]. CAS represents an alternative choice for patients when azoles are contradicted or not well-tolerated [19].

Ample evidence shows that the large variability reported in the concentrations of anti-infective agents in critically ill patients is not restricted to specific class of agents whereas in addition to the variations observed with ß-lactams, variations in the levels of other agents such as CAS [7,26], DAP [27,28], MOX [29,30] and CPX [31,32] are also reported. Taken together, the current knowledge emphasises the importance of therapeutic drug monitoring (TDM) to increase the clinical outcome of the antimicrobial therapy in ICU patients [33].

Several analytical methods such as liquid chromatography (LC)-coupled to UV (LC-UV) or mass spectrometry (LC–MS/MS) are reported for the analysis of CPX, MOX, DAP, ISC, and CAS (Fig. 1) alone or in combination with other compounds (Supplemental data, Table 1); yet, the established methods suffer in some cases from the limited covered range [34–36] and/or from a long run time [37,38], which make them less suitable for routine application. Furthermore, none of the methods allows their simultaneous analysis. This is probably due to the enormous differences in their structures, sizes, physico-chemical properties, as well as in their expected plasma levels in patients. While it is not possible to predict whether or not TDM will be required for ISC, it is essential to monitor the concentrations of CPX, MOX, DAP, and CAS that are used against resistant pathogens to limit the occurrence of resistance due to improper dosing. Moreover, ISC analysis will help in TDM and also in other pharmacokinetic studies to better delineate its level in patients with heterogeneous health conditions. As LC–MS/MS has emerged as the current platform of choice for TDM [34,35,37–41]; therefore, the aim of this work is to establish a selective LC–MS/MS method for the simultaneous analysis of CPX, MOX, DAP, ISC, and CAS at concentrations that fit within the reported ranges in patients and fast enough to be suitable for routine analysis and pharmacokinetic studies.

2. Materials and methods

2.1. Standards and reagents

Ciprofloxacin and moxifloxacin hydrochloride were received from Sigma-Aldrich (Munich, Germany). Caspofungin acetate, caspofungin acetate-$^2H_4$ (CAS-$^2H_4$), daptomycin, daptomycin-$^2H_5$ trifluoroacetic acid salt (DAP-$^2H_5$), ciprofloxacin-$^2H_8$ (CPX-$^2H_8$), and the cis enantiomer of moxifloxacin-$^2H_4$ hydrochloride (MOX-$^2H_4$) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Isavuconazole (BAL4815) and isavuconazole-$^2H_4$ (ISC-$^2H_4$, BAL4815-003) were kindly provided by Basilea Pharmaceutica Ltd. (Basel, Switzerland). Sterile water was bought from B. Braun Melsungen AG (Melsungen, Germany). All reagents used were of the highest available analytical grades. Drug free human plasma was from the Blood Donors' Center in Regensburg (Haema, Regensburg, Germany).

2.2. Instrumentation

Analysis was performed using an Agilent 1200 series liquid chromatography system consisting of G1312B binary pump, CTC-PAL Autosampler (CTC Analytics AG, Zwingen, Switzerland) and G1316 B column oven module, linked to an API 4000 triple-quadrupole mass spectrometer featured with electrospray (ESI) source (Applied Biosystems/Sciex, Germany). A reversed phase Kinetex F5 column with TMS endcapping (50 mm × 2.1 mm, 2.6 μm) (Phenomenex, Germany) and an UltraLine UHPLC In-Line Filter (In-Line Assembly with Filter) (Restek, USA) were used for chromatographic separation. The temperature of the column was set at 55 °C while that of the auto-sampler was kept at 8 °C. Separation of the different tested compounds was achieved over 5.5 min using two mobile phases:

A (water-isopropanol-formic acid (90:10:0.1, v/v/v)- ammonium acetate 2 mM) and B (methanol-isopropanol-formic acid (90:10:0.1, v/v/v)- ammonium acetate 2 mM). A binary pump delivered the mobile phases at a flow rate of 0.6 mL/min using the following gradient: From the start to 0.10 min the elution consisted of 0% B which was followed by a linear increase of B to 100%. During the last 0.9 min the column was re-equilibrated to the initial condition. The injection volume was 10 μL. The MS/MS instrument was set with capillary voltage (5.5 kV) and desolvation gas (nitrogen) heated at 400 °C. The dwell time for all the transitions was 10 ms.

2.3. Preparation of stock solutions, standards, and quality control samples

Individual stock solutions prepared at concentrations ranging from 2000 to 30000 mg/L, depending on the analyte, were stored at −80 °C. A first set of standard calibrators (SD) and quality control (QC) were prepared in water. These samples were further diluted in pooled human plasma to generate the respective working solution of SDs and QCs. Individual stock solutions of the isotopically labeled internal standards were prepared in methanol at a concentration of 1000 mg/L. For every analysis internal standard working solution (ISWS) was freshly prepared in ice-cold methanol.

The QC samples were tested at four different concentrations selected to cover the tested range of the analytes: Quality control high [QCH, 80% of upper limit of quantification], QC medium [QCM, 50% of selected range], QC low [QCL, 2–4 times the lower limit of quantification (LLOQ)] and QC at LLOQ (QCLLOQ).The individual range of the different analytes covered in the current method are: Caspofungin (1.25 mg/L–40 mg/L), isavuconazole (0.3125 mg/L–10 mg/L), ciprofloxacin (0.075 mg/L–12 mg/L), daptomycin (3.125 mg/L–100 mg/L), and moxifloxacin (0.1875 mg/L–12 mg/L).

2.4. Sample preparation

Protein precipitation with methanol containing the corresponding isotopically labeled internal standards (ISWS) was used for sample cleanup. 60 μL of spiked plasma was extracted with 200 μL ISWS. After 3 min of vortex the samples were centrifuged for 5 min (13000*g*, 4 °C). Subsequently, 100 μL of the clear supernatant was transferred to an autosampler vial containing 300 μL mobile phase A. 10 μL were injected into the system.

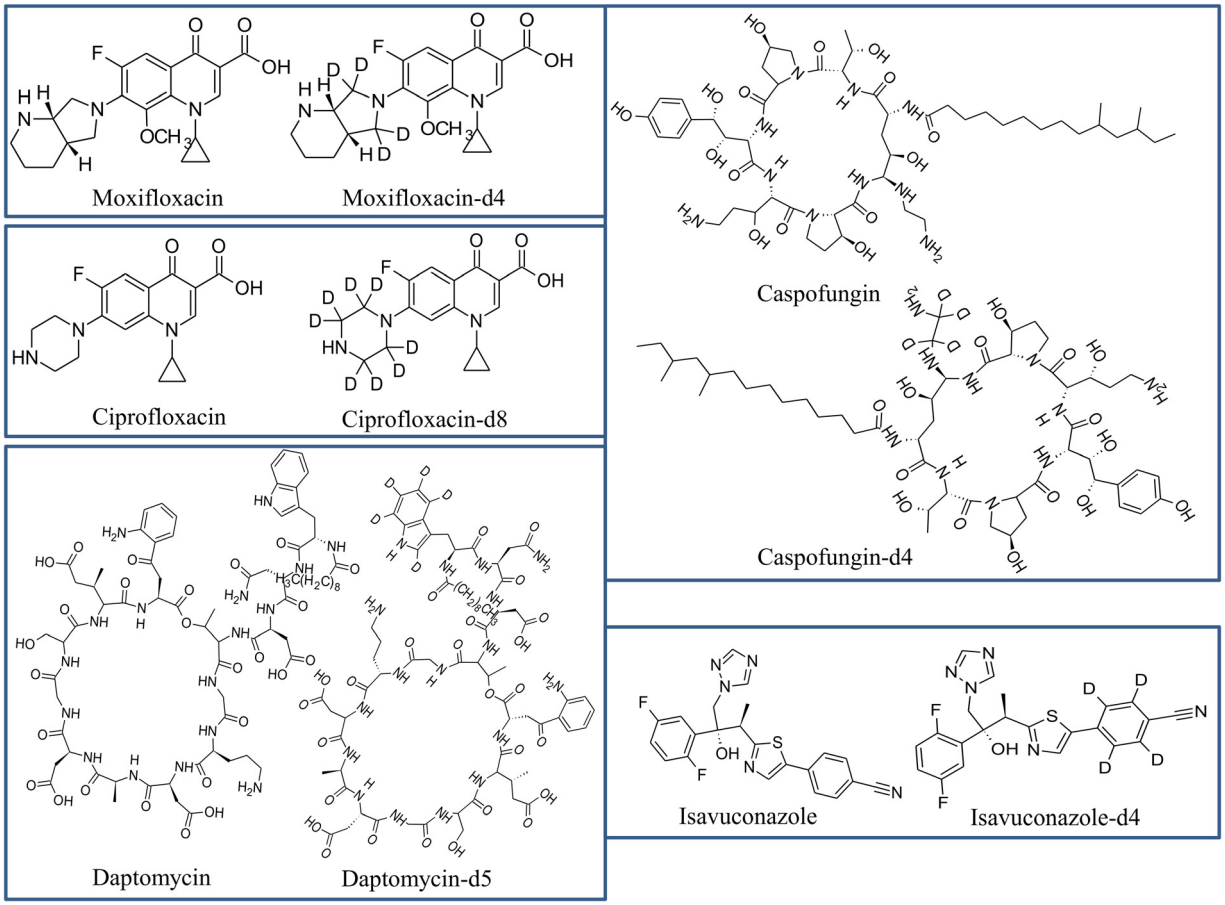

Fig. 1. Structures of the analytes under study and their corresponding internal standard.

2.5. Method validation

For method validation selectivity, lower limit of quantification, linearity, accuracy, precision, matrix effect, as well as to the stability of the analytes and internal standards over the entire period of storage and processing conditions were evaluated as recommended by the European-Medicines-Agency guidelines (EMA) for bioanalytical method validation [42]. The entire process of validation was performed by the use of pooled human plasma, except for process efficiency and matrix effect for which plasma from 8 different sources was utilized.

2.5.1. Selectivity

A selective method should differentiate the analytes and the IS apart from all the other compounds present in the matrix (plasma). In order to evaluate if there are interferences, like similar peaks with same transition at the same retention time, between the analytes or the IS and excipients of the plasma, blank plasma samples from different sources were tested. The EMA guidelines recommend using at least 6 different sources of matrix. A selective method should not have interference of more than 20% of the lower limit of quantification of the analyte and more of 5% of the IS [42].

2.5.2. Lower limit of quantification

Referring to the EMA guidelines the signal of the analyte at the lower limit of quantification (LLOQ) should be at least 5 times higher than that of a blank sample. LLOQ should be measured with a variation not exceeding 20% of the nominal expected value [42]. For every tested compound the LLOQ was selected as the lower concentration covered by the selected range.

2.5.3. Calibration curve/Linearity

Every run included a set of seven standard calibrators with known concentration, a blank sample (plasma processed with ISWS), and a double blank (plasma extracted with methanol without analytes/IS). Every analyte was analyzed according to its respective calibration standard. Linearity was accepted whenever the coefficient of correlation (r) exceeds 0.99. The back calculated concentrations of at least 75% of the calibrators should be within a 15% margin, yet a 20% margin for the LLOQ of the nominal expected value is accepted [42].

2.5.4. Accuracy and precision

Accuracy of the method is obtained by comparing the concentration of QC samples determined against a SD curve with the expected nominal values. QC samples are prepared from different stock solutions than the SD. According to the EMA the accuracy has to be determined in one run using at least five different replicates on the same day (intra-day accuracy) as well as on different days (inter-day accuracy).

The precision of the analytical method also termed as “coefficient of variation” tells the confirmation of the repeated measurements. Similar to the accuracy, the inter- and intra-day precision was also examined. The inter- and intra-day accuracy and precision were measured at 4 QC levels that cover the entire calibration range (QCH, QCM, QCL, and QCLLOQ). Each run consisted of a new set of SD and QC samples (6 samples per level) freshly prepared for every run. According to EMA the values of inaccuracy and imprecision should not exceed a 15% margin, except for the LLOQ at which deviation of 20% is acceptable [42].

2.5.5. Matrix effect and process efficiency

Components of the matrix can inadvertently influence the measurements of the method by suppressing or enhancing the signal of the tested analytes (known as the matrix effect (MF)). Plasma from at least 5 different donors should be tested [42]. The process efficiency (PE) tells how robust/reliable the processing of the samples is. MF and PE were calculated by the following equations: MF(%) = B/A*100 and PE(%) = C/A*100. Set A followed regular sample preparation where spiked plasma samples were extracted with ISWS. For Set B the plasma samples were spiked after extraction. In case of Set C the analytes were prepared in vehicle. Every set was tested at two concentrations (QCH and QCL). MF and PE were calculated for all analytes and isotopically labeled IS using 8 different sources of plasma. Normalized MF and PE were obtained by dividing the valued reported for the analytes by those of the IS.

2.5.6. Stability

The stability of the analytes was tested by comparing accuracy and precision of QC samples (QCH and QCL) kept under different storage conditions to freshly prepared calibrators. The stability of analytes in plasma was tested immediately after sample preparation and after storing them at the conditions to be evaluated: after keeping the samples at room temperature (Benchtop stability), after storage in −80 °C, and after three cycles of freeze and thaw. In this later condition, samples stored for a minimum of 12 h in −80 °C, were kept at room temperature for at least 30 min followed by freezing in −80 °C for a minimum of 12 h. Post-operative stability was also evaluated by rerunning the samples stored for 16 h at 8 °C (Autosampler stability). Deviation of <15% of the nominal value is considered to confirm the stability of the analytes [42].

2.5.7. Dilution integrity

The dilution integrity was examined to ascertain that an unknown sample with concentration exceeding the upper limit of a compound´s calibration range, could be diluted with drug free plasma without influencing the accuracy and precision of the measurement [42]. To achieve this aim SD and QCH samples were prepared at 2 fold higher concentration followed by dilution (1:1) in blank plasma before extraction. The inaccuracy and imprecision of the diluted samples should not deviate by more than 15% [42].

3. Results and discussion

3.1. Method development (MS/MS conditions, chromatographic separation, and sample preparation)

3.1.1. MS/MS conditions

The current method involves the analysis of two lipopeptides [caspofungin (CAS) and daptomycin (DAP)], two fluoroquinolones [moxifloxacin (MOX) and ciprofloxacin (CPX)], and one triazole (isavuconazole (ISC)) (Fig. 1). All of the analytes were detected in positive ion mode. Yet, while MOX, CPX, and ISC were detected as single charged molecular ions $[M+H]^{1+}$, CAS and DAP, due to their lipopetide moiety, were detected with double charged molecular ions $[M+2H]^{2+}$. The analytes were detected in multiple reactions monitoring (MRM). Two transitions were monitored for each analyte. One served as a quantifier and one as a qualifier. Due to their large size a third transition has been monitored as an additional qualifier for caspofungin and daptomycin. Only one MRM transition was used for the internal standard (Table 1). The declustering potential, collision energy, and exit potential were optimized for every analyte in MRM analysis. Among the MS/MS settings that needed optimization was the sheath gas temperature. The intensity of the different analytes was monitored with different temperatures (350–450 °C). A temperature of 400 °C seemed ideal as a decrease in the intensity of several analytes was observed when higher and lower temperatures were used.

Table 1
MRM transitions.

	MRM-Transitions (*m*/*z*)	
	Quantifier	Qualifier
Name	Q1 → Q3	Q1 → Q3
MOX	402.2 → 358.1	402.2 → 364.2
MOX-$^2H_4$	406.3 → 362.2	
CAS	547.5 → 131.0	547.5 → 538.4
		547.5 → 137.2
CAS-$^2H_4$	549.5 → 540.3	
CPX	332.1 → 231.1	332.1 → 288.1
CPX-$^2H_8$	340.3 → 296.1	
DAP	811.3 → 341.4	811.3 → 159.1
		811.3 → 313.2
DAP-$^2H_5$	814.2 → 162.9	
ISC	438.2 → 224.0	438.2 → 369.1
ISC-$^2H_4$	442.2 → 224.0	

Q1: precursor ion. Q3: production.

3.1.2. Chromatographic separation

Kinetex F5 which allows the separation of different compounds with different mechanisms of interaction proved to be suitable for the separation of the five analytes under study. Yet, the large variation in the properties of the analyzed compounds impacted their retention and the quality of peak shape under the different tested chromatographic conditions. Therefore, it was necessary to carefully optimize the best mobile phases that will result in reasonable separation along with improved peak shape to ascertain high reliability in the analysis. Several mobile phases were tested. A combination of water and methanol at different ratio were tested and proved insufficient to ensure good elution and avoid carry over of the more lipophilic compounds. This problem was overcome by adding isopropanol at a final level of 10% (V/V) to the mobile phases and by heating the column to 55 °C.

3.1.3. Sample preparation

An additional challenge was the selection of the volume of plasma and the dilution step convenient for the simultaneous analysis of the different analytes at their expected concentrations in the plasma of patients. Different plasma volumes (10–60 μL), different extraction, and different dilution factors following extraction were evaluated. Less than 60 μL plasma was not enough for the detection of ISC and DAP at the lower range and decreasing the dilution resulted in carry over. After several attempts of combining different volumes of plasma for extraction along with different dilutions, the best compromise between peak shape, sensitivity, and carry over was attained by extracting 60 μL plasma with 200 μL ISWS followed by dilution of 100 μL of the supernatant in 300 μL mobile phase A.

3.2. Method validation

3.2.1. Matrix effect and process efficiency

Matrix effect (MF) and process efficiency (PE) of the different analytes were determined at two concentrations of QC (QCL and QCH) in 8 different plasma samples. As shown in Table 2 significant ion suppression exceeding 15% was observed for MOX (29.6%), DAP (20.3%), and ISC (19.7%). At QCH ion suppression was only observed for MOX (27.5%) and DAP (16.9%). The use of IS successfully compensated ion suppression whereas the compensated values of all analytes ranged from 90.3-102.3% at QCL and 93.2-101.3% at QCH and, consequently, fulfill the criteria specified by EMA (85–115%) [42]. Similarly, the compensated PE of all analytes falls in the range specified by the EMA [42].

Table 2
Matrix effect (MF) and Process efficiency (PE).

		Low Concentration (QCL)		High Concentration (QCH)	
	n = 8	MF (%) + CV	PE (%) + CV	MF (%) + CV	PE (%) + CV
CPX	Absolute	94.3 + 6.4	97.4 + 19.8	103.4 + 7.7	98.5 + 2.9
	Compensated	100.1 + 2.3	100.7 + 4.2	95.9 + 8.1	92.0 + 3.9
MOX	Absolute	70.4 + 9.0	83.4 + 7.3	72.5 + 10.6	75.8 + 5.8
	Compensated	100.8 + 6.5	106.8 + 4.3	93.2 + 9.8	91.9 + 6.0
CAS	Absolute	89.8 + 10.4	59.1 + 7.3	86.0 + 8.9	63.6 + 5.4
	Compensated	99.2 + 8.6	113.8 + 5.6	95.0 + 9.3	104.6 + 2.9
DAP	Absolute	79.7 + 13.5	106.6 + 6.5	83.1 + 12.0	83.8 + 7.3
	Compensated	90.0 + 10.5	116.3 + 7.0	96.7 + 13.2	101.4 + 5.9
ISC	Absolute	80.3 + 17.7	79.8 + 9.2	92.4 + 10.9	112.1 + 4.9
	Compensated	102.3 + 8.1	109.2 + 7.6	101.3 + 9.2	114.3 + 3.1

CV: Coefficient of variation. n: number of replicates.

3.2.2. Linearity and lower limit of quantification

Table 3 presents a summary of inaccuracy and imprecision of five selected points of the calibration curve covering the specific range of each analyte under study. The linearity of all calibrators, run on 5 different days, over the specified range was confirmed whereas the coefficient of correlation (*r*) of all analytes was above 0.993. All calibrators were measured with an inaccuracy and imprecision of less than 11.1% and 9.6%, respectively. The lowest calibration point was accepted as the lowest limit of quantification. A representative chromatogram of the different analytes at their lower limit of quantification is depicted in Supplemental data (Supplemental data, Fig. 1). Concentrations of the four QCs (QCH, QCM, QCL, and QCLLOQ) are provided in Table 3.

3.2.3. Selectivity and carry-over

3.2.3.1. Selectivity. None of the tested blank plasma obtained from eight different sources showed any detectable interference from endogenous compounds with the same transitions and retention times of the studied analytes and their respective IS.

3.2.3.2. Carry over. The evaluation of carry-over was performed by injecting blank plasma after the highest calibrator. No carry over was observed for any of the IS used (<1%). The carry over observed with the different analytes is less than 20% of the LLOQ [CAS (18.21%), MOX (17.29%), CPX (18.46%), ISC (19.14%), and DAP (17.95%)]. Therefore, all analytes and IS pass the acceptance criteria specified by the EMA [42].

3.2.4. Accuracy and precision

Intra and inter-day assays accuracy and precision were evaluated using 6 freshly prepared replicates at each tested QC level (QCLLOQ, QCL, QCM, and QCH). At QCH, QCM and QCL the intra-day accuracy ranged from 95.0% to 115.1% (Table 4). At the QCLLOQ MOX, CAS, and CPX were measured with inaccuracy below 15%, while for DAP and ISC the inaccuracy was 16.6% and 20.7%, respectively. The intra-day imprecision was less than 10.7% for all compounds at all tested concentrations. For all compounds combined inter-day imprecision was less than 15% at QCH, QCM, and QCL, and less than 16.6% at the QCLLOQ.

3.2.5. Stability

Stability of analytes in plasma was examined under different storage conditions using two concentrations of QC (QCL and QCH). QC samples stored at −80 °C were stable up to 16 days and after 3 cycles of freeze and thaw. All samples were measured with inaccuracy and imprecision that did not exceed 15% (Table 5). Benchtop stability test proved that all five analytes are stable in plasma for 1.5 h at room temperature. All analytes were accurately measured (99.7%–116.8%) with an imprecision that didn't exceed 10%. All analytes showed a degradation of less than 13% after storage for 16 h in the autosampler (Table 5). QC stock solutions prepared in water are also stable when stored in −80 °C for up to 2 months period.

3.2.6. Dilution integrity

Dilution integrity was also evaluated to check whether dilution of samples prepared at concentrations exceeding the upper limit of quantification does not affect the accuracy and precision of the analysis. The accuracy and imprecision of the diluted samples were: CPX (104.2; 2.5), MOX (101.5; 2.7), CAS (102.9; 3.1), DAP (105.8; 4.7), and ISC (114.7; 1.5). Collectively, this data indicate that sample dilution does not affect the accuracy and precision of the analysis.

3.2.7. Comparison to previously published methods

Within a short runtime of 5.5 min the developed UPLC–MS/MS method allows the simultaneous analysis of five analytes (CAS, ISC, CPX, DAP, and MOX) used against severe infections. The ranges covered by the current method (Table 3) makes it suitable for the measurements of reported maximum and minimum concentrations (Cmax, Cmin) of all analytes in the plasma of patients (Supplemental Data, Table 1). In comparison to other methods, the current one has an extended upper limit for CAS, MOX, and CPX and a comparable upper limit for ISC and DAP. Even though lower quantification limits are attained by other methods [34–37,39–41]; yet, the long runtime along with the achieved modest upper quantification limit can be a drawback for their application for routine analysis. For instance, some of the methods are not suitable for the measurements of Cmax expected in critically ill patients (Supplemental Data, Table 1). Cmax is essential for the determination of Cmax/minimum inhibitory concentration (MIC) and area under the curve over 24 h ($AUC_{0\text{-}24h}$)/MIC, important determinants to avoid resistance and define the strength of bacterial killing, respectively, as in the case of fluoroquinolones per se.

3.3. Clinical application

The Clinical Ethics Consultation Service of the University Hospital Regensburg approved the measurement of DAP concentrations in plasma from a critically ill patient admitted to ICU. The patient suffered from bloodstream infection and endocarditis due to *Enterococcus faecialis*, which can be life-threatening due to the high levels of antibiotic resistance to many antibiotics including ampicillin, cephalosporines, meropenem, cotrimoxazol, and macrolide. Therapy with DAP (5 mg/kg body weight) was initiated and later increased to 10 mg/kg body weight as the infection persisted. The method was applied for the determination of DAP concentrations in plasma to ascertain that the delay in the eradication of infection was not due to inadequate dosing. Two different plasma samples were obtained from different time interval and analyzed by the current established method. The determined concentrations (40 and 105 mg/L) proved that increasing the dosing regimen of DAP was

Table 3
Calibration standards.

		Calibrators					*r*	CV (%)	Quality Controls (mg/L)			
		LLOQ				ULOQ						
MOX	NC (mg/L)	0.1875	0.375	3	6	12	0.997	0.24	0.192	0.38	3.84	9.60
	Accuracy (%)	103.1	92.8	104.4	100.4	98.6						
	Imprecision (%)	3.3	7.0	1.6	6.8	3.5						
CPX	NC (mg/L)	0.0750	0.375	3	6	12	0.997	0.23	0.077	0.192	3.84	9.60
	Accuracy (%)	98.8	98.6	96.5	98.1	104.8						
	Imprecision (%)	3.1	8.3	6.5	3.3	6.5						
DAP	NC (mg/L)	3.1250	6.25	25	50	100	0.995	0.24	3.20	8.00	32.0	80.0
	Accuracy (%)	103.9	89.8	102.4	100.0	102.3						
	Imprecision (%)	1.9	3.2	4.1	9.4	9.6						
ISC	NC (mg/L)	0.3125	0.625	2.5	5	10	0.993	0.25	0.32	0.80	3.20	8.00
	Accuracy (%)	102.8	91.3	87.9	102.3	110.4						
	Imprecision (%)	2.1	0.2	3.5	5.8	6.5						
CAS	NC (mg/L)	1.2500	2.5	10	20	40	0.999	0.09	1.28	3.20	12.80	32.0
	Accuracy (%)	100.8	96.5	102.8	100.3	98.5						
	Imprecision (%)	1.7	4.7	2.4	4.9	5.2						

CV: coefficient of variation. *r*: coefficient of correlation.

Table 4
Summary of the intra- and inter- day accuracy and precision.

		Intra-day		Inter-day	
	NC (mg/L)	Accuracy (%)	Imprecision (CV%)	Accuracy (%)	Imprecision (CV%)
MOX	0.192	106.5	3.7	99.8	6.2
	0.384	107.6	5.8	97.5	10.3
	3.84	97.5	4.6	97.9	10.8
	9.6	112.3	4.5	100.1	14.0
CAS	1.28	109.4	8.0	110.3	15.8
	3.20	102.2	2.5	103.3	9.3
	12.8	100.5	3.8	101.5	8.5
	32.0	95.9	6.2	96.5	4.7
CPX	0.077	114.6	3.9	102.2	9.6
	0.192	115.1	3.5	98.9	10.1
	3.84	102.8	3.8	96.7	6.6
	9.6	103.6	3.3	96.6	7.1
DAP	3.20	116.6	4.6	118.8	16.5
	8.00	114.3	9.9	105.4	12.0
	32.0	106.1	4.4	102.4	13.3
	80.0	110.5	10.7	101.6	10.9
ISC	0.32	120.7	5.1	115.8	10.7
	0.8	95.0	4.1	94.0	11.5
	3.2	100.7	4.3	101.2	15.9
	8.0	114.5	5.2	105.8	9.5

NC: nominal concentration. CV: coefficient of variation.

Table 5
Stability under different storage conditions.

		Freeze and Thaw		Bench Top 1.5 h		Auto-sampler (16 h)	
	NC (mg/L)	Accuracy (%)	Imprecision (CV%)	Accuracy (%)	Imprecision (CV%)	Accuracy (%)	Imprecision (CV%)
MOX	0.384–9.6	101.7–97.1	4.1–6.7	103.8–90.3	4.5–7.2	108.1–104.3	5.6–4.2
CAS	3.20–32.0	98.4–92.4	6.3–3.8	116.4–100.8	8.4–1.6	88.2–87.9	2.6–2.6
CPX	0.192–9.6	104.6–94.7	1.6–6.7	99.7–96.3	3.9–7.1	103.6–100.0	3.7–4.5
DAP	8.0–80.0	91.1–100.2	7.9–8.9	116.8–106.0	5.4–9.7	96.1–94.6	4.9–1.0
ISC	0.8–8.0	86.3–100.6	11.6–14.7	108.0–112.2	7.1–5.3	94.0–90.1	2.4–3.6

All stability tests were performed on 6 replicates. NC: nominal concentration. CV: coefficient of variation.

appropriate and the patient's health condition improved slowly over the following days, such that the patient could then be released from the hospital. Collectively, the first experience with the method confirms its suitability for clinical application.

3.4. Conclusion

Antimicrobial therapy is an essential need for the treatment of critically ill patients. However, the pathophysiological changes observed in these multimorbid patients underscore the need for therapeutic drug monitoring to provide an optimized clinical outcome. The newly developed and validated method allows accurate and fast quantification of five different agents used to treat complicated bacterial and fungal infections in human plasma. The method is simple and covers the reported therapeutic ranges (maximum and trough concentrations) expected in patients. None of the currently published methods combine the simultaneous analysis of CPX, MOX, DAP, ISC, and CAS, probably because of the chemical and physical differences between the compounds as well as their expected concentrations in plasma. With respect to other methods this is the only method in which all five analytes are analyzed with respect to their respective isotopically labeled internal standard.

The method was successfully applied for the determination of DAP in the plasma of a critically ill patient in intensive care unit. Collectively, as with other methods established in our laboratory [43,44], the new method which allows the fast and simultaneous detection of anti-infective drugs in small volumes of patient' samples is also suitable for routine analysis of antimicrobial therapy surveillance in intensive care units.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.05.015.

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