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Simultaneous quantification of antimicrobial agents for multidrug-resistant bacterial infections in human plasma by ultra-high-pressure liquid chromatography–tandem mass spectrometry

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ABSTRACT

Antibiotic-resistant bacterial infection is one of the most serious clinical problems worldwide. Vancomycin, teicoplanin, daptomycin, and colistin are glycopeptide and lipopeptide antibiotics that are frequently used to treat multidrug-resistant bacterial infections. Therapeutic drug monitoring is recommended to ensure both safety and efficacy and to improve clinical outcomes. This study developed a fast, simple, and sensitive ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the simultaneous determination of the concentrations of these four drugs in human plasma. The sample preparation process includes a simple protein denaturation step using acetonitrile, followed by an 11-fold dilution with 0.1% formic acid. Eight target peaks for the four drugs can be analyzed within 3 min using a Kinetex™ 2.6 μm C18 column. The mass spectrometry parameters were optimized, and two transitions for each target peak were used for multiple reaction monitoring, which provided high sensitivity and specificity. The UHPLC–MS/MS method was validated over clinical concentration ranges. The intra-day and inter-day precisions for the ratio of the peak area of each analyte to the peak area of the internal standard were all below 12.7 and 14.7% relative standard deviations, respectively. The accuracy at low, medium, and high concentrations of the eight target peaks was between 89.3 and 110.7%. The standard curves for the analytes were linear and had coefficients of determination higher than 0.997. The limits of detection were all below 70 ng mL^{-1}. The use of this method to analyze patient plasma samples confirmed that it is effective for the therapeutic drug monitoring of these four drugs and can be used to improve the therapeutic efficacy and safety of treatment with antibiotics.

1. Introduction

Drug-resistant bacterial infection is one of the most serious clinical problems worldwide. Several multidrug-resistant (MDR) microorganisms, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp.*, have recently been referred to by the term "ESKAPE" because of their escape from antibiotics [1]. Of these MDR microorganisms, the prevalent Gram-positive bacterium methicillin-resistant *S. aureus* (MRSA) is associated with complicated skin-structure infections, hospital-acquired infections, and ventilator-associated pneumonia. In 2005, an estimate of 18,650 in-hospital deaths were due to invasive MRSA infections in the United States [2]. The emergence of MRSA is one of the most important aspects of nosocomial infections worldwide in the last two decades. The prevalence of hospital-acquired MRSA (HA-MRSA) is greater than 50% in North America, South America, Asia and Malta [3]. In additional to Gram-positive MDR bacterial infections, outbreaks of Gram-negative MDR bacterial infections including *A. baumannii* (MDRAB) and MDR *P. aeruginosa* (MDRPA) have been reported in Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan, and Korea in recent years [4,5]. It has been reported that MDRAB and MDRPA infections significantly increase treatment complexity and the duration of hospital stays for patients. Studies have demonstrated that ensuring the effectiveness of antibiotic treatment is of prime importance for the control of MDR infections. Glycopeptide antibiotics such as vancomycin and

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teicoplanin (six sub-components), lipopeptide antibiotic daptomycin, and oxazolidinone antibiotic linzolid are now widely used to treat MRSA. In contrast to the many antibiotics available to treat Gram-positive bacterial infections, there are very few effective antibiotics for treating Gram-negative MDR strains. Colistin, an old lipopeptide antibiotic with known nephrotoxicity and neurotoxicity, was withdrawn in the 1970s but is now being used to treat Gram-negative MDR strains. [6].

Various factors such as drug absorption, liver and kidney deficiency, obesity, and critical illness affect pharmacokinetic (PK) performance and result in different drug concentrations in patients' plasma. Among the antimicrobial agents for multidrug-resistant bacterial infections, vancomycin, teicoplanin, daptomycin and colistin are renally excreted, and therapeutic drug monitoring (TDM) is recommended for patients being treated with the aforementioned four drugs to ensure pharmaceutical efficacy and prevent toxicity, especially for patients with renal and critical illnesses. In contrast to these four antibiotics, no dose adjustment is recommended for patients with renal insufficiency when receiving linezolid due to MDR bacterial infections [7]. It has been reported that the trough concentrations of vancomycin and teicoplanin should be greater than 10–20 mg L^{-1} to ensure their efficacy [8,9]. A daptomycin concentration above 0.5 μg mL^{-1} should be maintained to inhibit 90% of several staphylococcal strains (MIC_{90}) [10], and effective peak plasma concentration of daptomycin was suggested to be higher than 60 times of the MIC [11]. Steady state plasma concentration of colistin was suggested to be between 1 and 5 μg mL^{-1} [12]. Because of the limited pharmacokinetic/pharmacodynamic data available for colistin and daptomycin, both of the therapeutic index and treatment regimen are still being seriously discussed. Severe adverse effects, including nephrotoxicity, ototoxicity, and hypotension, have been reported for vancomycin. Studies indicate that higher incidences of adverse effects occur when the plasma vancomycin concentration is above 80–100 mg L^{-1} [13,14]. Teicoplanin is less toxic than vancomycin. However, patients have reported experiencing adverse reactions such as ototoxicity and nephrotoxicity when using teicoplanin [15]. Both myopathy and eosinophilic pneumonia have been observed in

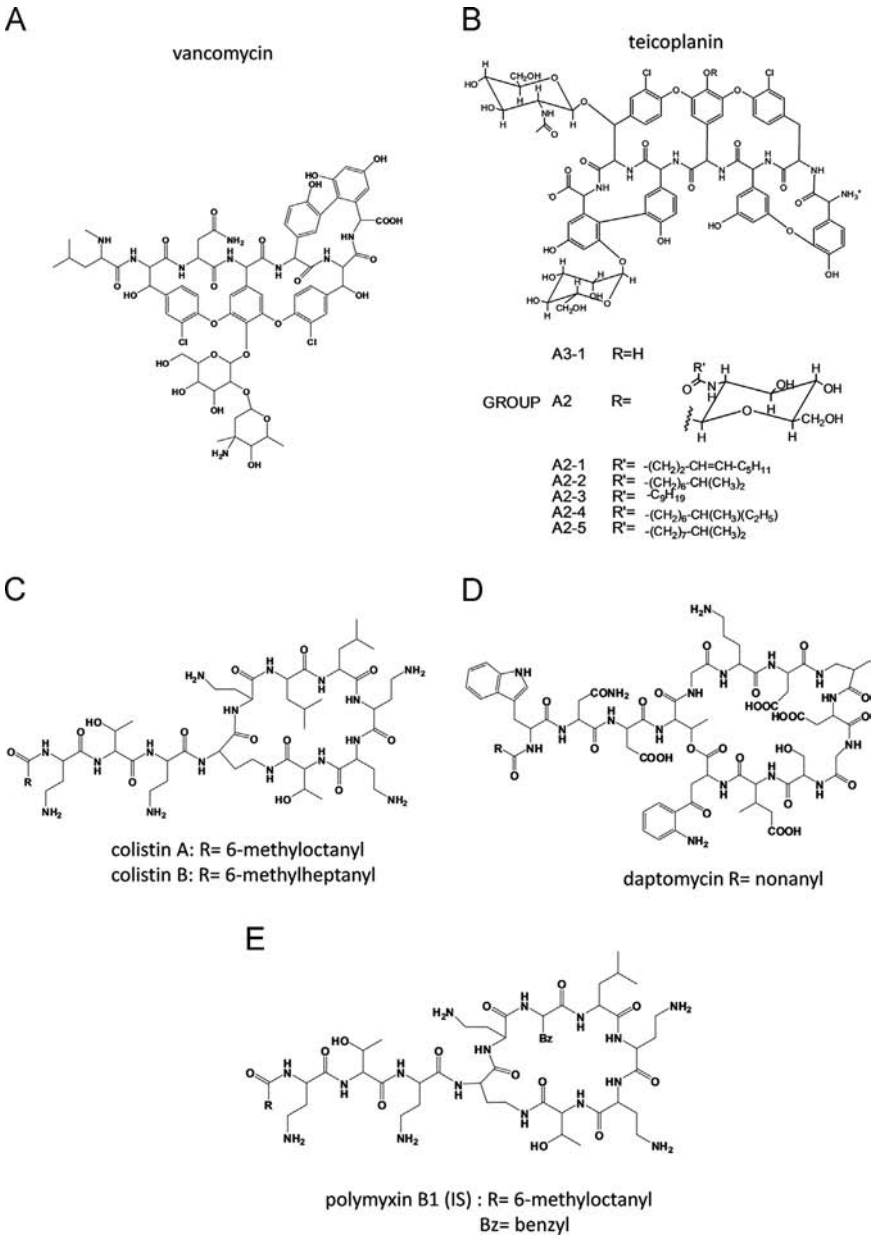

Fig. 1. Structures of (A) vancomycin, (B) the six sub-components of teicoplanin, (C) colistin A and colistin B, (D) daptomycin, and (E) IS: polymyxin B1.

patients treated with daptomycin [16]. Nephrotoxicity and neurotoxicity have been reported in patients treated with colistin. Both of these adverse effects seem to be dose dependent and reversible [17]. Given the efficacy and safety issues with the four antibiotics, having a sensitive and selective analytical method for determining the concentrations of these drugs in biological samples is important.

Different analytical methods have been developed for individually quantifying vancomycin, teicoplanin, daptomycin and colistin in biological samples. Florescence polarization immunoassays (FPIAs) have been developed and are widely used to determine vancomycin and teicoplanin concentrations [18–23]. These methods are convenient and require no separation or washing steps. However, the results from FPIA methods are easily affected by light scattering, endogenous fluorophores, and cross reactivity in a complex sample matrix [24]. High-performance liquid chromatography methods using an ultraviolet detector (HPLC–UV) have been developed to quantify vancomycin [25,26], teicoplanin [27,28], and daptomycin [29,30] in serum and plasma. The analysis time required for these methods to provide sufficient separation is relatively long, and sample pretreatment steps, such as a liquid–liquid extraction or solid-phase extraction, are usually necessary to decrease endogenous interference. Several studies have used an HPLC system coupled with a fluorescence detector to improve the analytical sensitivity and selectivity for determining the vancomycin [31], teicoplanin [32] and colistin [33,34] concentrations. Although these methods possess superior sensitivity, laborious derivatization steps were required. In addition, the lipophilicity usually increases for the derivatized products, thus increasing the retention times and the total analysis time. In recent years, liquid chromatography coupled with mass spectrometry (LC–MS) has gained popularity as a highly sensitive and selective compound analysis technique. Different studies have described LC–MS/MS methods to individually quantify the concentrations of vancomycin, colistin, and daptomycin in plasma or urine [35–43]. Teicoplanin is widely used to clinically treat MRSA. However, only one recent study, by Fung et al., used LC–MS/MS to analyze teicoplanin, which is possibly due to the size and complexity of the subcomponents of this drug. In that study, only two of the six major components of teicoplanin were chosen as targets to quantify its concentration [44].

Due to the emerging issues with MDR bacterial infections worldwide, a universal analytical method with simple preparation procedures for frequently used drugs can greatly benefit concentration adjustment in clinical practice. However, current available analytical methods are only capable of quantifying individual antibiotics for MDR infections. Therapeutic drug monitoring has been demonstrated to improve safety and efficacy for vancomycin, teicoplanin, daptomycin, and colistin treatments in MDR infections, and our goal was to develop a simple and efficient LC–MS/MS method for these four antibiotics (Fig. 1). We explored the feasibility of a protein precipitation method followed by sample dilution to simplify the sample preparation method. We reduced the total run time for LC analysis to 10 min to achieve efficient analysis. Finally, the developed method was applied to clinical samples. To our knowledge, this is the first LC–MS/MS method that can simultaneously determine the concentrations of vancomycin, teicoplanin, daptomycin, and colistin in human plasma.

2. Material and methods

2.1. Standards and reagents

Vancomycin, daptomycin, colistin, colistin sodium methanesulfonate (CMS), and polymyxin B standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). The colistin standard contains two major components, colistin A and colistin B, with composition percentages of 26% and 54%, respectively. A teicoplanin standard (purity > 97%) containing six major components was purchased from Dongkook Pharm. (Seoul, Korea). The composition percentages of the six components were as follows: teicoplanin A3-1, 5.4%; A2-1, 2.8%; A2-2, 58.4%; A2-3, 5.4%; A2-4, 18.2%; and A2-5, 9.8%. Formic acid (purity > 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC–MS-grade water and acetonitrile (ACN) were purchased from Scharlau (Sentmenat, Spain) and J.T. Baker (Phillipsburg, NJ, USA), respectively. All reagents and solvents were of either analytical or LC–MS grade.

2.2. Liquid chromatography

All of the LC analyses were performed using an Agilent 1290 UHPLC system equipped with a quaternary solvent pump, an autosampler, a sample reservoir, and a column oven (Agilent Technologies, Waldbronn, Germany). A Kinetex C18 2.1 × 50 mm (2.6 μm) column (Phenomenex, Torrance, USA) was used to separate the compounds. The mobile phase was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B) with a flow rate of 0.3 mL/min. The linear gradient went from 5% solvent B to 100% at 2.5 min. Isocratic elution with 100% solvent B was maintained for 3.5 min, and then, the column was re-equilibrated with 5% solvent B for 4 min. The sample reservoir and column oven were maintained at 4 °C and 40 °C, respectively. The injection volume was 10 μL.

2.3. Mass spectrometry

The mass spectrometric analysis was performed using an Agilent 6460 triple quadrupole system (Agilent Technologies, Waldbronn, Germany). The positive electrospray ionization mode was used with the following parameters: a dry gas temperature of 300 °C, a dry gas flow rate of 5 L/min, a nebulizer pressure of 35 psi, a sheath gas temperature of 325 °C, a sheath gas flow rate of 11 L/min, a nozzle voltage of 500 V, and a capillary voltage of 3500 V. The data gathered for the transition pairs, the fragmentor voltage (FV), and the collision energy (CE) of the eight target peaks (one for vancomycin, two for colistin, four for teicoplanin, and one for daptomycin) are listed in Table 1.

2.4. Sample pretreatment

A 100 μL aliquot of human plasma was transferred to a clean glass tube, and 35 μL of an internal standard (polymyxin B, 10 μg mL^{-1}) was added. After the mixture was vortexed, 150 μL of acetonitrile was added to precipitate the proteins. The sample was then centrifuged at 3000 RCF for 5 min, and 20 μL of the supernatant was transferred into a glass vial containing 200 μL of 0.1% formic acid in water. The sample was directly introduced into the LC–MS/MS system after mixing.

2.5. Validation

2.5.1. Selectivity

Six blank plasma samples were collected from three healthy females and three healthy males. An aliquot of each blank plasma sample was treated according to the sample preparation method described in Section 2.4. The six blank plasma samples were used to determine the level of endogenous interference at the retention times of the target antibiotics under the UHPLC–MS/MS conditions that were developed.

Table 1
Retention times (Rt) and MRM parameters of the eight target peaks and the internal standard.

Compound name	Formula	Rt (min)	FV (V)	CE (V)	Precursor ion (*m*/*z*)	Product ion[a]	
						Q1 (*m*/*z*)	Q2 (*m*/*z*)
Vancomycin	$C_{66}H_{75}Cl_2N_9O_{24}$	1.62	135	10	724.7	144.0	99.9
Colistin B	$C_{52}H_{98}N_{16}O_{13}$	1.72	135	10	385.9	101.0	227.0
Colistin A	$C_{53}H_{100}N_{16}O_{13}$	1.74	135	10	390.6	101.1	241.0
Teicoplanin A3-1	$C_{72}H_{68}Cl_2N_8O_{28}$	1.73	150	15	782.4	203.7	125.8
Teicoplanin A2-1	$C_{88}H_{95}Cl_2N_9O_{33}$	1.93	150	15	939.8	314.3	296.2
Teicoplanin A2-2 & A2-3	$C_{88}H_{97}Cl_2N_9O_{33}$	1.96	150	15	940.6	316.2	298.2
Teicoplanin A2-4 & A2-5	$C_{89}H_{99}Cl_2N_9O_{33}$	1.99	150	15	947.9	330.2	204.1
Daptomycin	$C_{72}H_{101}N_{17}O_{26}$	2.25	150	25	810.9	313.1	158.9
Polymyxin B1 (IS)	$C_{55}H_{96}N_{16}O_{13}$	1.77	135	10	401.9	101.0	241.1

[a] Two transitions were used for MRM detection. The first one was used for quantification, and the second one was used for confirmation.

Table 2
Inter-day and intra-day precisions for the eight target peaks at low, medium, and high concentrations.

Compound name	Spike concentration (μg mL^{-1})			Intra-day precision (% RSD, $n=9$)				Inter-day precision[b] (% RSD, $n=3$)			
				Rt[a]	Peak area ratio			Rt	Peak area ratio		
	Low	Medium	High		Low	Medium	High		Low	Medium	High
Vancomycin	0.50	10.0	100.0	0.13	5.05	1.90	1.45	0.76	6.69	2.72	8.67
Colistin B	0.27	5.4	54.0	0.08	5.80	2.52	1.72	0.84	5.22	2.12	2.15
Colistin A	0.13	2.6	26.0	0.11	4.36	2.49	1.72	0.27	4.03	3.25	2.17
Teicoplanin A3-1	0.27	2.7	10.8	0.14	3.89	4.68	5.80	0.33	14.73	9.35	12.33
Teicoplanin A2-1	0.14	1.4	5.6	0.12	2.49	6.65	3.73	0.36	3.29	8.08	10.59
Teicoplanin A2-2 & A2-3	0.32	6.4	63.8	0.20	12.36	3.02	2.57	0.26	12.56	4.26	4.03
Teicoplanin A2-4 & A2-5	0.14	2.8	28.0	0.20	12.74	4.56	1.84	0.32	11.22	6.65	2.80
Daptomycin	0.50	10.0	100.0	0.04	5.62	3.02	2.32	0.49	7.98	4.42	3.31

[a] Rt, retention time.
[b] Inter-day precision was estimated using measurements taken on three different occasions.

2.5.2. Precision and accuracy

The intra-day precision ($n=9$) and inter-day precision (three different occasions) of both the retention times and the peak area ratios are reported as relative standard deviations (RSDs). The precisions of the peak area ratios were determined at low, medium, and high antibiotic concentrations. The accuracy was also investigated at low, medium, and high concentrations and is reported as the percent recovery (measured concentration relative to the target concentration). The investigated concentrations are indicated in Table 2.

2.5.3. Calibration curve, lower limit of quantification (LLOQ), and limit of detection (LOD)

A calibration curve was generated for each analyte using six concentration levels. Teicoplanin A2-2/A2-3 and teicoplanin A2-4/A2-5 are pairs of isomers with the same fragmentation patterns and retention times. Therefore, one calibration curve was generated for the total concentration of A2-2 and A2-3 and another was generated for the total concentration of A2-4 and A2-5. Eight calibration curves were constructed using quadratic least-squares regression with 1/concentration as the weighting factor, and the linear ranges of the teicoplanin and colistin sub-components were calculated using their composition percentages. The LLOQ of each target peak was defined as the lowest concentration on the calibration curve with a precision of $<15\%$ RSD and an accuracy between 85 and 115%. The limit of detection (LOD) was defined as the point at which the signal-to-noise ratio equaled 3.

2.5.4. Recovery of the sample preparation method and the matrix effect

The recoveries was investigated for the eight target peaks at a total concentration of 6 μg mL^{-1} for the four antibiotics. Blank plasma samples from three different healthy volunteers were used. The recoveries were calculated by dividing the peak area for the pre-spiked sample by the peak area for the post-spiked sample and multiplying by 100%. The matrix effects were investigated by post-column infusion method. Analytes were continuously infused to the UHPLC elute at a flow rate of 10 μL min^{-1}. Infused analyte concentration: vancomycin, 1 μg mL^{-1}; colistin B, 5.4 μg mL^{-1}; colistin A, 2.6 μg mL^{-1}; teicoplanin A3-1, 5.4 μg mL^{-1}; teicoplanin 2-1, 2.8 μg mL^{-1}; teicoplanin A2-2 & A2-3, 6.4 μg mL^{-1}; teicoplanin A2-4 & A2-5, 2.8 μg mL^{-1}; daptomycin, 1 μg mL^{-1}; IS (polymyxin B1), 1 μg mL^{-1}. Samples of drug free plasma were prepared according to the sample pretreatment method and introduced to LC–MS/MS analysis. MRM transitions for the eight target peaks were used to monitor the ion suppression or ion enhancement of the analytes.

2.5.5. Stability

All of the samples used for the stability investigations were blank human plasma samples spiked with low and high concentrations of antibiotics ($n=3$). Short-term stability was evaluated by keeping the samples at room temperature for up to 24 h. Long-term stability was evaluated by keeping the samples at -80 °C for 30 days. The three freeze-thaw cycles used a temperature of -80 °C. The stability of the target compounds is presented as the recovery (%) relative to that for freshly prepared samples. The post-preparation stability was evaluated by comparing the results for samples analyzed immediately after preparation and those analyzed after storage in a sample reservoir at 4 °C for 24 h. To study the influence of the hydrolysis of CMS (a pro-drug of colistin) on the colistin quantification, plasma was spiked with 10 μg mL^{-1} CMS and analyzed using the developed method. The amounts of colistin A and B produced from the CMS were also investigated.

2.6. Application

The developed method was used to quantify antibiotics in patient plasma. Plasma samples containing antibiotics were collected from patients undergoing antibiotic treatment at the National Taiwan University Hospital. This study was approved by the ethics committee, and signed informed consent forms were received from all patients who participated in the study. The results for the quantification of the teicoplanin and colistin concentrations are reported as the summation of the sub-components.

3. Result and discussion

3.1. Optimization of analytical conditions

The structures of vancomycin, teicoplanin, daptomycin, and colistin are shown in Fig. 1. To develop a fast and sensitive analytical method for simultaneous determination of the four antibiotics, the parameters that affect the performance of ultra-high-pressure liquid chromatography coupled with tandem mass spectrometry were optimized. A linear gradient with a steep slope was used for analytes possessing wide lipophilicity ranges. Precursor-to-product ion transitions specific to eight target peaks were also investigated in the multiple reaction monitoring (MRM) analysis.

3.1.1. MS/MS optimization

Due to the polypeptide moiety of the analytes used in this study, molecular ions with multiple charges were generated by the ESI source. The addition of 0.1% formic acid to the mobile phase resulted in the formation of doubly and triply charged molecular ions. For vancomycin, the six components of teicoplanin, and daptomycin, the doubly charged molecular ion $[M+2H]^{2+}$ with the highest intensity was chosen as the precursor ion for the tandem MS study. The three dominant ions in the scan mode mass spectrum were $[M+2H]^{2+}$, $[M+H+Na]^{2+}$, and $[M+3H]^{3+}$ for colistin B, colistin A, and polymyxin B1 (IS). Although $[M+H+Na]^{2+}$ possessed the highest intensity, it was not stable enough in the MRM mode to serve as a precursor ion. Therefore, the $[M+3H]^{3+}$ peak, which had a higher intensity than the $[M+2H]^{2+}$ peak, was chosen as the precursor ion for these three compounds. The fragmentor voltage (FV) and collision energy (CE) for each analytical compound in the MRM analysis were optimized, and two dominant product ions were selected for each analyte. The precursor-to-product ion transition with the highest peak intensity was used for quantification, and the other was used for qualification. All of the information concerning the FV, the CE, and the precursor-to-product ion transitions are listed in Table 1. As indicated in Section 2.5.3, both teicoplanin A2-2/A2-3 (isomers 1) and teicoplanin A2-4/A2-5 (isomers 2) are isomer pairs. Each isomer pair had the same mass spectrometry performance and retention time ($Rt_{A2\text{-}2 \text{ and } A2\text{-}3}$ = 1.96 min, $Rt_{A2\text{-}2 \text{ and } A2\text{-}3}$ = 1.99 min); therefore, teicoplanin A2-2/A2-3 (isomers 1) and teicoplanin A2-4/A2-5 (isomers 2) were separately treated as two peaks.

Other optimized mass spectrometry parameters include the sheath gas temperature (275–350 °C), the sheath gas flow rate (9–12 L/min), the nebulizer voltage (20–45 V), the capillary voltage (2000–4500 V), the nozzle voltage (0–2000 V), the dry gas temperature (250–325 °C), and the dry gas flow rate (5–8 L/min). The temperature and flow rate for the dry gas had little influence on the peak intensity, whereas the sheath gas flow rate and the nozzle voltage significantly influenced the peak intensities. From our observations, the signal intensities of vancomycin, colistin B, and colistin A increased greatly as the sheath gas flow rate increased from 9 to 12 L/min. However, the signal intensity of daptomycin decreased as the sheath gas flow rate increased. With different nozzle voltages, colistin B and A showed higher signal intensities with nozzle voltage at 500 V, whereas vancomycin and daptomycin preferred nozzle voltage at 1000 V. Due to the diverse natural properties of the four drugs, the final MS conditions were a compromise among the optimal conditions of all of the analytes. The final MS parameters were as follows: a dry gas temperature of 300 °C, a dry gas flow rate of 5 L/min, a nebulizer pressure of 35 psi, a sheath gas heater temperature of 325 °C, a sheath gas flow rate of 11 L/min, a nozzle voltage of 500 V, and a capillary voltage of 3500 V.

3.1.2. UHPLC condition optimization

Our goal was to develop a high-throughput analysis method for clinical use; therefore, a short C18 column with a core–shell silica support was used to decrease the analysis time and provide good peak efficiencies. A linear gradient of 5% to 100% ACN over 2.5 min was used to elute the four drugs in 3 min (Fig. 2). Although all eight peaks for the four drugs (vancomycin, two peaks for colistin, four peaks for teicoplanin, and daptomycin) eluted close to one another, the tandem mass spectrometry using MRM transition pairs was able to provide sufficient selectivity. Matrix effect is one of the most undesirable phenomena that affects the quantification accuracy in LC–ESI–MS. To minimize matrix effect, one approach is to optimize the LC gradient to separate the analytes with endogenous interferences. The initial percentage of ACN was optimized to minimize the matrix effect for vancomycin. It was observed that using 10% ACN as the initial eluent decreased both the retention time and the peak intensity for vancomycin due to its co-elution with other hydrophilic endogenous components. This matrix effect was attenuated by decreasing the percentage of ACN to 5%. Due to the high lipophilicity of daptomycin, the final solvent strength was increased to 100% ACN to provide sufficient elution strength. After the elution of daptomycin, 100% ACN was maintained for 3.5 min to elute all lipophilic components of the plasma, and the LC flow was switched to waste mode at 4 min to prevent MS source contamination. With the organic solvent wash and flow switch steps, the performance of the analytical system was stable for up to 24 h.

3.2. Development of sample pretreatment procedures

To achieve efficient analysis for the four antibiotics, it is necessary to develop a simple, general, and timesaving sample pretreatment method. The sample pretreatment methods commonly used prior to the LC–MS/MS analysis of antibiotics in plasma samples include solid-phase extraction (SPE) and protein precipitation with liquid extraction. SPE involves conditioning, loading, washing, and elution steps [35,41]. Further evaporation of the eluent followed by reconstitution using the proper sample solution is often required to improve analytical performance [38]. Such laborious preparation procedures make this method less amenable for the routine analysis of clinical samples. Specific SPE method is also not suitable for multiple analytes with different physical and chemical properties, which may cause diversity in the extraction recoveries among the analytes. In addition, SPE cartridges are usually made from plastic, and adsorption of the lipopeptide antibiotics may occur during processing, which may result in decreased recovery rates [35,40]. Protein precipitation is a relatively simple method that uses an organic solvent or acid to denature proteins. In this study, we used protein precipitation followed by sample dilution as a simple, cost-effective, and timesaving sample pretreatment method. To reduce the matrix effects, improve the ionization efficiency, and maintain a sample concentration in the therapeutic range, both the composition of dilution

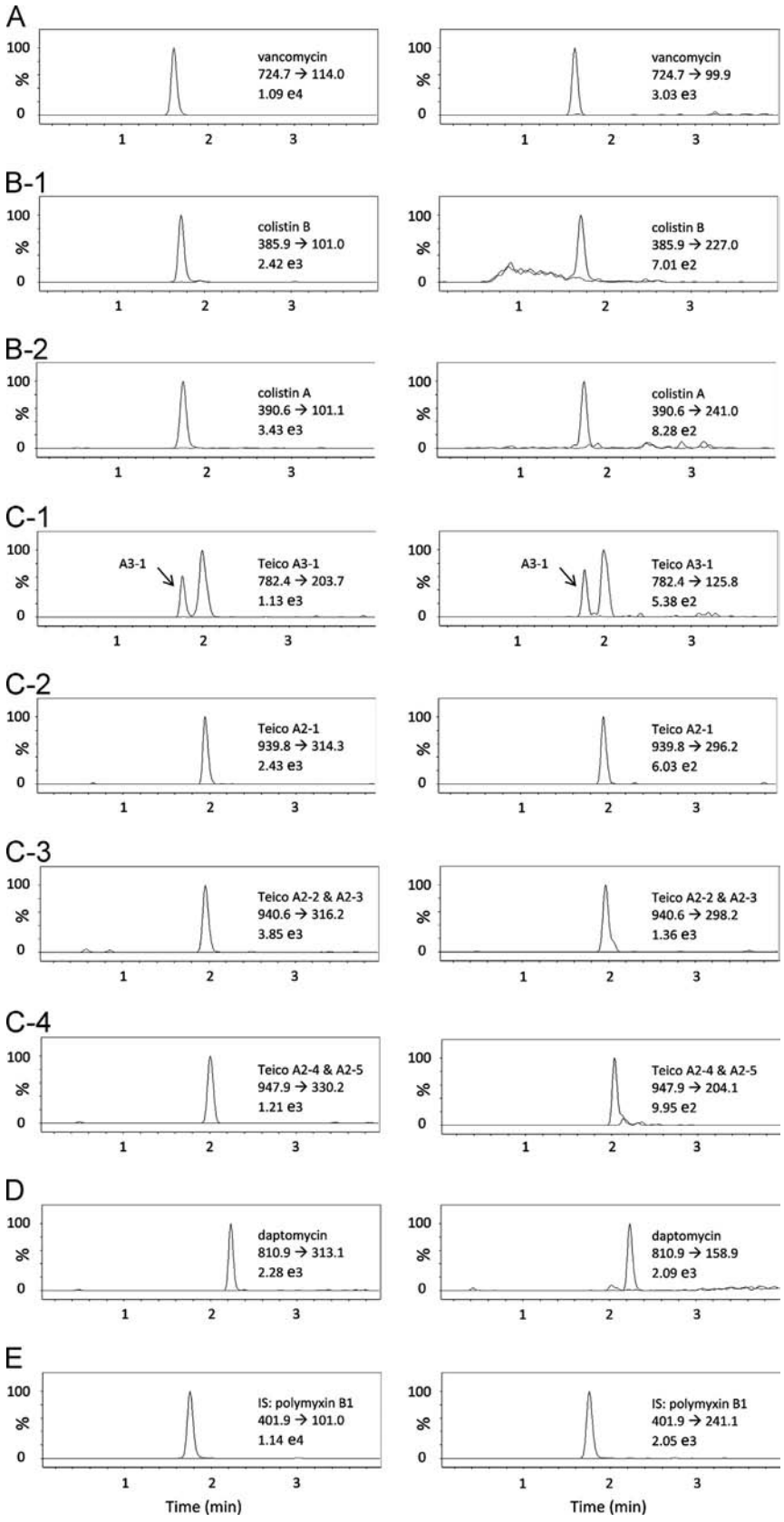

Fig. 2. Extracted ion chromatograms of the eight target peaks in plasma spiked at the LLOQ and overlapped with an EIC plasma blank. (A) vancomycin, (B1 and B2) colistin B and colistin A, (C1 to C4) teicoplanin sub-components, (D) daptomycin, and (E) IS: polymyxin B1. The EIC of mass transition used for quantification is depicted on the left, and the EIC of mass transition used for qualification is depicted on the right.

solution and the dilution factor were investigated for all four of the antibiotics.

After protein precipitation with ACN, the supernatant was diluted with deionized water containing 0.1% formic acid. We found that the presence of small amounts of acid is necessary to obtain reproducible peak areas, especially for colistin B and A. This might be due to the reason that their molecular ions are triply protonated, and the presence of formic acid in sample solution provides a source of proton donor for the drugs to form stable preformed-ions. The dilution factor for the supernatant was optimized to obtain a linear range suitable for therapeutic drug monitoring and to decrease the matrix effects. Dilution factors of 5, 11, and 16 were investigated based on both the lower and upper limits of quantification. With a 5-fold dilution, signal saturation occurred at high concentrations. On the other hand, it was found that the LLOQ was insufficient for therapeutic concentrations of the four antibiotics when using a 16-fold dilution. The most suitable dilution factor was thus found to be 11, which provided sufficient sensitivity and suitable linear range for clinical use. The sample preparation process can be completed in 10 min using a simple protein precipitation step and dilution. The recoveries for the four antibiotics were between 70.2 and 110.3%.

3.3. Method validation

3.3.1. Selectivity, precision, accuracy, linearity and sensitivity

The method validation was performed according to FDA guidelines [45]. Method validation for these four antibiotics with eight peaks was performed using plasma samples spiked with concentrations in the clinical ranges except in the selectivity test. Blank plasma samples were spiked with a suitable amount of antibiotics and analyzed using the developed LC–MS/MS method. In the selectivity test, there was no endogenous interference in the six blank plasma samples at the mass transitions of the antibiotics at the same retention times. The relative standard deviations (RSDs) of the intra-day ($n=9$) and inter-day precisions (three different occasions) for the retention times of the eight peaks were below 0.2% and 0.8%, respectively. The RSDs of intra-day ($n=9$) and inter-day precisions (three different occasions) of the peak area ratios for the eight peaks at low, medium, and high concentrations were found to be less than 12.7% and 14.7%, respectively. The eight calibration curves were fitted using quadratic least-square regression with 1/concentration as the weighting factor. All of the coefficients of determination (r^2) were above 0.997. The linearity ranges covered the therapeutic ranges for all four antibiotics and can be used for either pharmacokinetic studies or therapeutic drug monitoring. The LODs were all below 70 ng mL^{-1}. The accuracies of the eight peaks were expressed as percent recoveries. The recoveries for the eight peaks at low, medium, and high concentrations were between 89.3 and 110.7%. Previous study reported by Kuhn et al. indicated that the co-elution of drug metabolites with the target drugs might affect the quantification results due to in-source fragmentation [46]. From the literature survey, the four antibiotics do not show apparent metabolism and are excreted as unchanged form [47–52]. Therefore, the quantification results of target drugs will not be affected by the MS signals from metabolites due to in-source fragmentation. The results of the method validation are summarized in Tables 2 and 3.

3.3.2. Matrix effects

The results of matrix effects investigated by the post-column infusion method are shown in Fig. 3. Severe ion suppression was observed between 0.3 and 1 min. Signal enhancement for teicoplanin components and daptomycin was observed at 1.6 min. At the retention time of the eight target peaks and the internal standard, the matrix effect did not significantly decrease or increase the signal intensity. The negligible matrix effect revealed that our sample preparation process is suitable for drug analysis. We used 1.5-fold ACN for plasma protein denaturation and the resulting solution was further diluted for 11 folds, which provided total dilution factor of 16.5 for the sample matrix.

3.3.3. Stability

The short-term, long-term, freeze-thaw, and post-preparation stabilities of the four antibiotics were investigated. For the short-term stability, plasma samples spiked with low and high antibiotic concentrations were kept at room temperature for up to 24 h. Subsequent analysis using the developed method indicated that the recoveries of the eight target peaks were between 88.2 and 116.0% (Table 4). The long-term recoveries for the low and high concentrations after 30 days of storage at −80 °C were between 89.1 to 111.4%. In addition, the four antibiotics were stable after three freeze-thaw cycles at the concentrations tested.

The sample solutions contained 0.1% formic acid after sample preparation. To investigate the stability of the analyte in the sample solution, we analyzed the sample both immediately and 24 h after sample preparation (at 4 °C). The results of the post-preparation stability test indicate that the analytes were stable in 0.1% formic acid if the temperature of the sample reservoir was maintained at 4 °C.

Table 3
Linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy for the eight target peaks at low, medium, and high concentrations, and the therapeutic/general plasma concentration for the four drugs.

Compound name	*Linearity ($y=ax^2+bx+c$)*					LOD (ng mL^{-1})	LOQ (μg mL^{-1})	Accuracy (%)[a]			Therapeutic concentration[b] (μg mL^{-1})
	Range (μg mL^{-1})	*a*	*b*	Intercept	R^2			Low	Medium	High	
Vancomycin	0.50–100.0	−9.84E−05	0.20763	0.04095	0.997	1.1	0.50	110.7 ± 5.8	97.3 ± 2.7	93.8 ± 2.0	C_{min} > 10–20 [8,9]
Colistin B	0.27–54.0	−9.51E−04	0.42403	−0.0152	0.999	3.9	0.27	102.3 ± 8.6	105.0 ± 2.7	110.0 ± 1.7	Css: 1–5 [12]
Colistin A	0.13–26.0	−2.02E−03	0.53869	0.0456	0.999	0.9	0.13	110.5 ± 4.3	98.9 ± 1.8	105.3 ± 1.5	
Teico A3-1	0.27–10.8	3.02E−03	0.11134	−0.0093	0.997	55.2	0.27	95.3 ± 1.0	104.0 ± 1.7	95.3 ± 1.0	C_{min} > 10–20 [8,9]
Teico A2-1	0.14–5.6	6.95 E−03	0.29732	−0.0044	0.998	7.3	0.14	99.9 ± 5.2	91.2 ± 2.9	106.1 ± 4.5	
Teico A2-2 & A2-3	0.32–63.8	−5.03E−04	0.18921	−0.0166	0.999	17.3	0.32	105.8 ± 1.8	89.3 ± 1.3	90.7 ± 2.2	
Teico A2-4 & A2-5	0.14–28.0	1.47E−03	0.08328	−0.0017	0.999	10.5	0.14	92.3 ± 8.0	94.9 ± 1.8	95.3 ± 1.4	
Daptomycin	0.50–100.0	8.44E−04	0.50129	−0.1343	0.999	68.7	0.50	108.6 ± 3.0	91.0 ± 3.5	103.3 ± 2.6	C_{max} > 30 [10,11]

[a] The low, medium, and high concentrations for eight target peaks were the same as those listed in Table 2.

[b] C_{min}, trough concentration; C_{max}, peak concentration; C_{ss}, steady state concentration; The therapeutic concentraions for vancomycin and teicoplanin are well established. C_{ss} for colistin was suggested to be between 1 to 5 μg mL^{-1}, and C_{max} for daptomycin was indicated to be higher than 60 times of the MIC_{90} (minimum inhibitory concentration, 0.5 μg mL^{-1} for MRSA). Therapeutic concentration ranges for colistin and daptomycin are still under investigation.

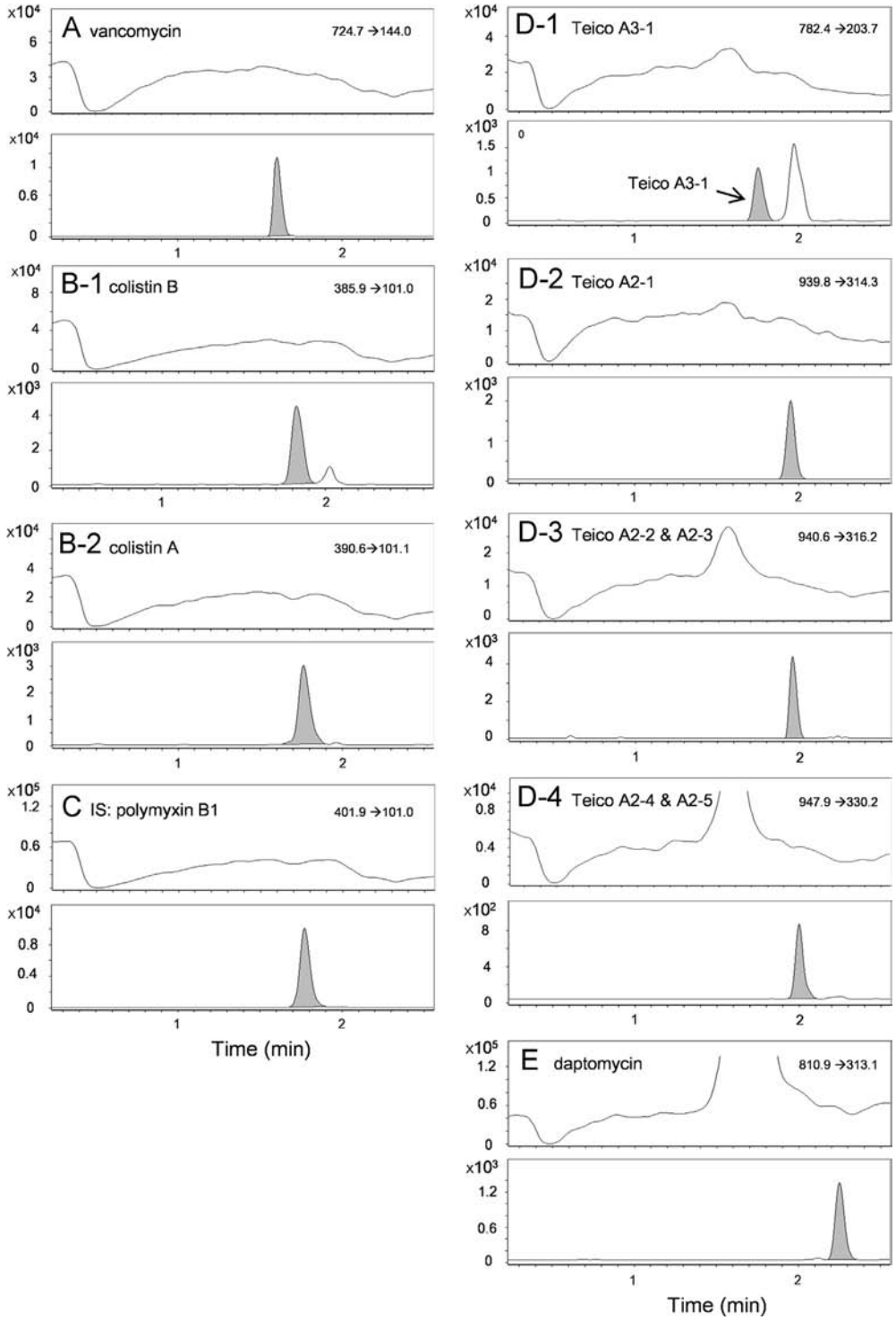

Fig. 3. Post-column infusion LC–MS/MS chromatograms of the target analytes. (A) vancomycin, (B1 and B2) colistin B and colistin A, (C) IS: polymyxin B1, (D1 to D4) teicoplanin sub-components, and (E) daptomycin. Chromatograms of target peaks at the concentration of LOQ are overlapped to indicate the position of peaks.

CMS is a pro-drug of colistin, and previous studies have indicated CMS is easily hydrolyzed to colistin in buffers, aqueous solutions, and biological samples [53]. It is possible that CMS in patient plasma samples will be hydrolyzed to colistin after sample preparation in 0.1% formic acid, thus affecting the quantitative results. To precisely quantify the colistin concentration, we determined the post-preparation effect of CMS hydrolysis by analyzing plasma samples from patients treated with CMS. The peak area ratios of colistin A and B to the IS remained the same for up to 8 h (the peak area ratio changed by less than 5%). In addition, we spiked a blank plasma sample with 10 $\mu g\ mL^{-1}$ CMS and analyzed the colistin A and B signals to monitor hydrolysis. The colistin A and B concentrations were only approximately 0.3 $\mu g\ mL^{-1}$ and 0.1 $\mu g\ mL^{-1}$, respectively, after 5 h of storage at 4 °C (the clinically observed concentration for colistin was higher than 1 $\mu g\ mL^{-1}$). These results demonstrate that our sample preparation process, sample solution and sample reservoir conditions will not result in the hydrolysis of CMS and thus provide accurate quantification results.

Table 4
Stability of eight target peaks.

Compound name	Conc.[a]	Short term[b] 24 h (%)	Long term[c] 30 days (%)	Freeze-thaw[d] (%)	Post-preparation[e] (%)
Vancomycin	Low	102.3 ± 3.7	99.2 ± 2.3	91.6 ± 2.5	93.9 ± 0.5
	High	93.3 ± 1.4	95.5 ± 4.1	107.6 ± 0.9	88.1 ± 1.0
Colistin B	Low	99.0 ± 4.4	103.0 ± 6.1	94.1 ± 2.5	103.5 ± 8.9
	High	102.8 ± 1.3	99.4 ± 2.2	103.9 ± 0.9	88.5 ± 1.1
Colistin A	Low	101.1 ± 4.8	103.4 ± 4.7	104.9 ± 10.9	101.3 ± 2.6
	High	102.2 ± 1.0	98.2 ± 0.4	102.7 ± 1.2	88.0 ± 0.7
Teico A3-1	Low	98.9 ± 11.6	97.5 ± 13.7	95.5 ± 6.1	99.1 ± 3.7
	High	116.0 ± 5.0	106.0 ± 9.2	98.2 ± 3.9	89.5 ± 1.0
Teico A2-1	Low	102.9 ± 5.8	95.1 ± 10.1	93.9 ± 8.9	110.8 ± 1.5
	High	104.1 ± 5.5	90.9 ± 2.8	100.0 ± 1.6	90.3 ± 1.8
Teico A2-2 & A2-3	Low	90.7 ± 1.5	107.0 ± 5.0	102.5 ± 8.4	101.6 ± 9.9
	High	88.6 ± 2.2	100.4 ± 0.5	109.4 ± 2.9	89.2 ± 0.2
Teico A2-4 & A2-5	Low	105.0 ± 8.5	102.9 ± 3.6	97.4 ± 12.7	96.8 ± 4.2
	High	88.2 ± 0.4	89.1 ± 0.9	108.8 ± 8.1	90.9 ± 1.2
Daptomycin	Low	96.1 ± 6.9	100.0 ± 10.2	106.9 ± 16.2	94.1 ± 4.6
	High	106.3 ± 4.7	111.4 ± 3.3	93.4 ± 12.6	92.6 ± 1.2

[a] The low and high concentrations were the same as indicated in Table 2.
[b] Short-term stability was assessed after storage at room temperature for 24 h.
[c] Samples were kept in a −80 °C freezer for 30 days for the long-term stability test.
[d] Samples were frozen at −80 °C and thawed at room temperature for three cycles.
[e] The samples were kept at 4 °C for the post-preparation stability test.

3.3.4. Comparison with previous LC–MS methods

The validation results indicate that the developed LC–MS/MS method can be used to simultaneously analyze all four antibiotics with good precision and accuracy in clinical therapeutic ranges. Cass et al. used an on-line sample extraction and HPLC–MS/MS method to determine the vancomycin concentration in serum for a pharmacokinetic study [36]. The linear range of Cass's method was from 0.001 to 10 μg mL^{-1}. Zhang et al. used LC coupled with full-scan mass spectrometry to determine vancomycin concentrations in a linear range from 0.05 to 10 μg mL^{-1} [54]. For daptomycin, Verdier et al. and Gika et al. developed LC–MS/MS methods with linear ranges from 0.5 to 120 μg mL^{-1} [37,40]. Bazoti et al. developed an LC–MS/MS method for daptomycin with a linear range from 0.01 to 10 μg mL^{-1} [42]. The linear ranges in several studies were approximately 0.01 to 7 μg mL^{-1} or 0.01 to 30 μg mL^{-1} for colistin quantification [35,38,41,43]. Compared to these methods, the sensitivity and the separation speed of our LC–MS/MS method were comparable or even better than these methods. In previous studies, only one drug can be analyzed using a particular method, and samples need to be analyzed using different methods if patients are receiving combination antibiotic therapy. Our developed LC–MS/MS method is the first analytical platform that can simultaneously determine the concentrations of vancomycin, teicoplanin, daptomycin, and colistin in human plasma, which can maximize the lab resource utilization.

3.4. Application

The developed method was applied to plasma samples from patients with MDR bacterial infections. Fig. 3 shows the extracted ion chromatograms for the four antibiotics from three patients. The drug concentrations were quantified, and the plasma concentrations of vancomycin and colistin (Fig. 4A) for patient No. 1 were 23.6 and 1.1 μg mL^{-1}, respectively. The teicoplanin concentration was 31.8 μg mL^{-1} in the plasma from patient No. 2 (Fig. 4B), and the daptomycin concentration was 6.8 μg mL^{-1} in the plasma from patient no. 3 (Fig. 4C). The quantification results for the first patient also indicate that this method is efficient for determining the concentrations of co-administered antibiotics in a single analysis, which is both cost effective and timesaving.

Recently, Fung et al. used LC–MS/MS to quantify teicoplanin A2-2 and A2-3 to determine the total teicoplanin concentration in patient plasma samples [44]. However, the analytical results for the plasma samples used in our study indicate that teicoplanin sub-components A2-4 and A2-5 contribute nearly half of the total concentration (A2-4/A2-5: 13.5 μg mL^{-1}; teicoplanin total concentration: 31.8 μg mL^{-1}). The previous study also indicated that teicoplanin A2-4 and A2-5 accumulated in human plasma after multiple treatment doses [55]. Therefore, the quantification of all six sub-components is necessary to accurately quantify the teicoplanin concentration in patient plasma.

4. Conclusions

Ensuring the effectiveness of antibiotic treatments is of prime importance in the control of drug-resistant bacterial infections. In this study, we developed a UHPLC–MS/MS method to simultaneously quantify the concentrations of four important antibiotics that are used to treat MDR bacterial infections: vancomycin, teicoplanin, daptomycin, and colistin. This method is both sensitive and selective, and it showed good validation performance, with wide linear ranges suitable for clinical use. The analysis time is only 10 min using the developed UHPLC–MS/MS method, and the retention times of the eight target peaks are less than 3 min. The sample preparation process involves a timesaving protein precipitation step followed by an 11-fold dilution and can be completed within 10 min.

Although previous LC–MS/MS study only measured teicoplanin A2-2 and A2-3 for the determination of the total teicoplanin concentration in patient plasma samples, we observed teicoplanin sub-components A2-4 and A2-5 contribute to nearly half of the total concentration. The developed method is the first LC–MS method that can determine all of the six major components of teicoplanin, which provides more accurate quantification results. The UHPLC–MS/MS method was applied to quantify four antibiotics in plasma samples from patients receiving single antibiotic treatment or subjecting to combination therapy. The results indicate that the developed method is both high throughput and can be used for efficient therapeutic drug monitoring.

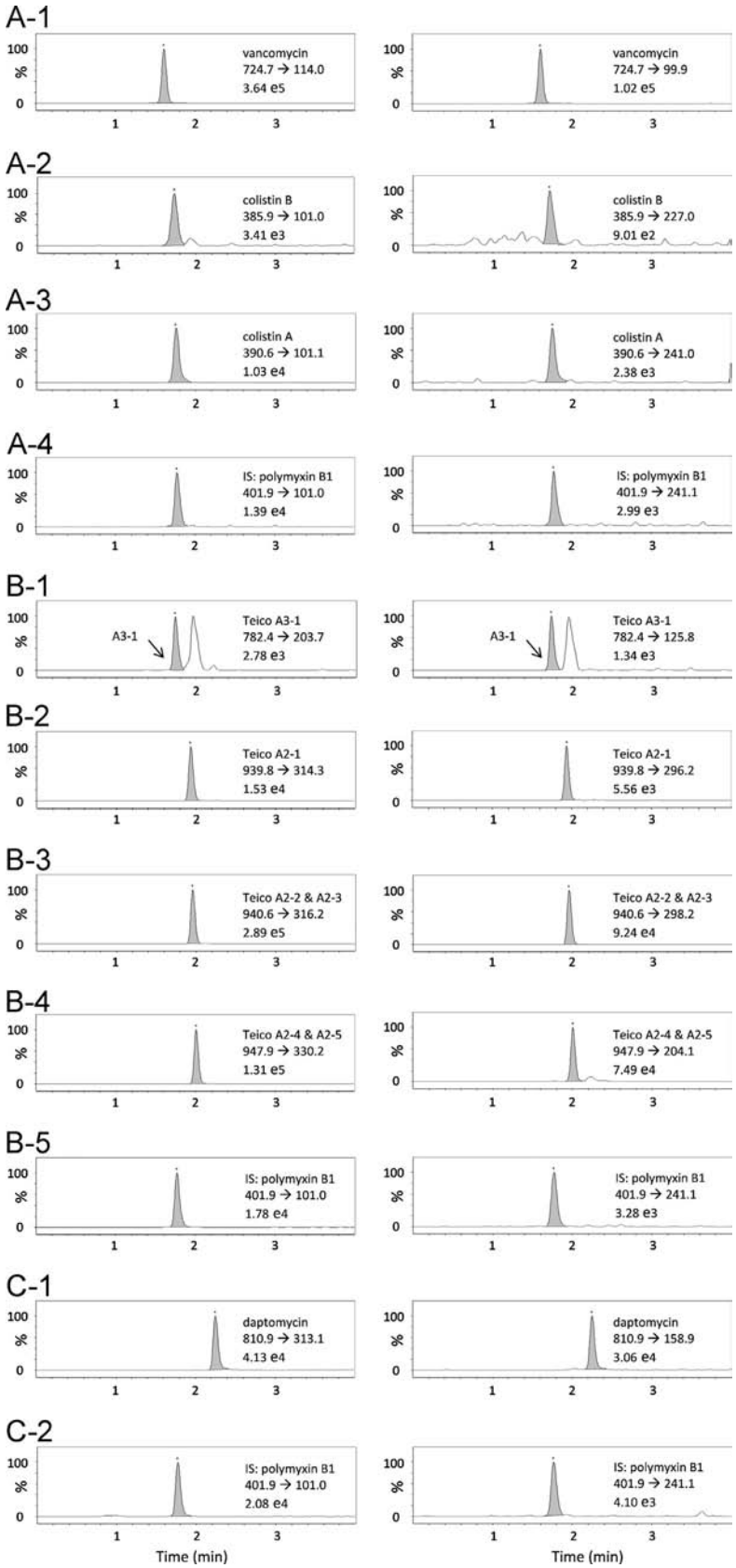

Fig. 4. Extracted ion chromatograms of the eight target peaks for the four antibiotics in patient plasma. The vancomycin and colistin peaks were from the plasma of the first patient. The teicoplanin and daptomycin peaks were from the plasma samples of the second and third patients, respectively. (A1) vancomycin, (A2 and A3) colistin B and colistin A, (B1 to B4) teicoplanin sub-components, (C1) daptomycin, and (A4, B5, C2) IS: polymyxin B1. The EIC of mass transition used for quantification is depicted on the left, and the EIC of mass transition used for qualification is depicted on the right.

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