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LC–MS/MS method development and validation for the determination of polymyxins and vancomycin in rat plasma

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ABSTRACT

Simple, sensitive and robust liquid chromatography–tandem mass spectrometer (LC–MS/MS) methods were developed and validated for the determination of lipopeptide polymyxins and glycopeptide vancomycin in rat plasma. The effect of trichloroacetic acid (TCA) concentration on sample recoveries (peak area of sample recovered from plasma/peak area of sample from neat solvent solutions) was studied and an optimized concentration of 30% TCA were determined that gives the best sample recovery for the peptides from rat plasma. The effect of the TCA concentration on the chromatographic behavior of peptides was studied on a Phenomenex Jupiter C18 5 μ 300 Å 50 mm × 2 mm column using a mobile phase with a pH of 2.8. Other than protein precipitation, TCA also acted as ion pairing reagent and was only present in the samples but not in the mobile phases. The data demonstrated that by increasing the TCA concentration, the analyte retention and sensitivity were improved. The absence of TCA in mobile phase helped to reduce the ion source contamination and to achieve good reproducibility. The plasma method was linearly calibrated from 5 to 5000 ng/mL for polymyxins with precisions to be of 2.3–10.8%, and accuracies to be 91.7–107.4% for polymyxin B1, B2, E1, E2, respectively. For vancomycin the calibration is from 1 to 5000 ng/mL with precisions to be of 7.8–10.3 and accuracies to be 96.2–102.0%. The LLOQs corresponding with a coefficient of variation less than 20% were 7.5, 18.1, 7.3, 5.0 and 1.0 ng/mL for polymyxin B1, B2, E1, E2 and vancomycin, respectively.

1. Introduction

In the past 30 years, the emergence of multi-drug resistance (MDR) bacteria has created a situation in which there are few or no treatment options for infections by certain microorganisms. For example, the emerging MDR Gram-negative bacteria, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, are resistant to all β-lactams, fluoroquinolones, and aminoglycosides [1,2]. Additionally, methicillin resistant *Staphylococcus aureus* (MRSA) has evolved into a significant pathogen among hospitalized patients around the world [3]. Lipopeptide PMXs and glycopeptide VCM interact noncovalently to their target ligands, usually cell-wall or cell-membrane structures. As the noncovalent interactions are nonspecific than covalent interactions, it is more difficult for bacteria to develop resistance to these agents [4]. This mechanist opportunity is used in developing antibacterial peptide drugs against MDR bacteria [5]. This has led to the resurgence in the use of PMX antibiotics which are active against a wide spectrum of Gram-negative bacteria despite their known nephrotoxicity [6,7]. Moreover, there exist renewed interests in the exploration of VCM and other glycopeptides modifications that are active against Gram-positive bacteria [8].

The two clinically used PMXs, PMB and PME, are cyclic lipodecapeptides. In these peptides, the amino acid units 1–3 are linear and 4–10 form a 23-membered ring. Each molecule carries 5 free amino groups and, accordingly, 5 positive charges are present under physiological conditions [9]. The main difference between PMB and PME is in the amino acid components. PMB is comprised mainly of PMB1 and PMB2 [10], and PME (also known as colistin), is comprised mainly of PME1 (colistin A) and PME2 (colistin B) [11,12]. The cationic molecules of PMX compete and displace Ca^{2+} and Mg^{2+} ions, and the hydrophobic segments of PMX microscopically form complexes with bacterial lipopolysaccharide, which causes local disturbance of the cell membrane, and increases cell permeability, cell lysis and death [13–16]. They display sub-micromolar minimum inhibitory concentration (MIC) values against a variety of Gram-negative bacteria [2,6,8,9,17,18].

Currently there is a lack of reliable information concerning the pharmacokinetic data for PMXs in humans [2,10]. PMXs are

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highly soluble in water and poorly soluble in organic solvents [19]. The unique molecular properties of PMXs present chromatographic challenges with a variety of conventional reverse phase LC columns. Since all the main components of the PMXs possess five free amino groups which tend to adsorb onto silica surface [20], severe peak tailing are observed for untreated PMX samples with LC. Therefore, either derivatization [21] or further purification are required for optimal bioanalysis. Bioanalytical methods such as capillary zone electrophoresis (CZE) [20,22,23], high-performance liquid chromatography (HPLC) with fluorescence detector, UV spectrophotometric detector or scanning fluorescence detector [21,24,25], and LC–MS/MS [26,27] have been used for quantitative analysis. Since CZE and LC with UV and fluorescence detection lack of structure-specific selection [24], and fluorescence detection requires compound derivatization for a sensitive and specific method [21,24,25,28], LC–MS/MS is the choice for pharmaceutical industry because of its high sensitivity and structural specificity. The reappraisal of PMXs as the only available active antibiotics for some bacteria species as well as the combined-drug synergy study of PMXs with other antibacterial compounds [2] demand a simple and accurate analytical method with adequate dynamic range and sensitivity for the determination of PMXs in biological samples.

Recently, LC–MS/MS methods have been developed for quantification of PME (colistin) in milk and animal tissues [27,29]. The methods required the use of strong and highly concentrated acids for sample recovery followed by laborious sample clean-up, preconcentration, and long separation time. LC–MS/MS methods have also been reported for the analysis of PME in human plasma and urine [26,30]. These methods are unsatisfactory since they require a long and expensive procedure of SPE, consumption and injection of a large volume of samples (100–200 μL), and long separation time with poor chromatography. The reported sensitivity for PME in any matrices ranges from 30 to 300 ng/mL (g) per 10 μL injection.

Another class of antibiotic peptide drugs is glycopeptide antibiotics. This class is composed of glycosylated cyclic or polycyclic nonribosomal peptides, neutral sugars and an amino sugar. The peptides consist of cross-linked unusual aromatic amino acids and convention amino acids such as aspartic acid [31]. Significant glycopeptide antibiotics include VCM, dalbavancin, teicoplanin, telavancin, bleomycin, ramoplanin, and decaplanin [32,33]. They are soluble in aqueous solvent but not in nonpolar organic solvents. This class of drugs inhibits the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. They bind to the amino acids within the cell wall, preventing the addition of new units to the peptidoglycan.

VCM is a benchmark compound for various preclinical pharmacology models treating endocarditis [34,35]. However, the unique molecular properties of VCM presented similar bioanalytical challenges as PMXs. Current LC–MS methods include using strong cation exchange SPE for sample preparation from serum followed by LC-full scan Fourier transform MS [36], online sample extraction followed by column switching technique [37], and an offline sample extraction technique using TFA and methanol [38]. The LLOQ obtained ranged from 1 to 10 ng/mL. The above methods require complicated extraction procedure, large injection volume and long separation time. Moreover, the previous studies did not apply sample recovery optimization.

The two classes of antibiotics, lipopeptide PMXs and glycopeptide VCM, actually have similarities. They are both peptide drugs of similar molecular weight range; the sizes of the peptide parts are dominant in either the lipopeptide molecules or in the glycopeptide molecule. The purpose of the study is to develop and validate a general bioanalytical method based on the same principle for the above antibacterial peptide compounds.

2. Experimental

2.1. Chemicals and reagents

All solvents used were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (88%) was supplied by J. T. Baker (Phillipsberg, NJ, USA). TCA (99+%) was purchased from Acros (Morris Plains, NJ, USA). Control Rat Plasma in EDTA K2 (Individual MALE 031-APEK2-MI) was purchased from Bichemed (Wichester, MA, USA). PMB (polymyxin B sulfate), PME (colistin methanesulfonate), VCM and dalbavancin were obtained from Pfizer Global Research & Development (Groton, CT, USA). [Glu[1]]-Fibrinopeptide B human (≥97%) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Equipment

A standard multitube vortex-mixer from VWR Scientific Products (West Chester, PA, USA) was used for vortex-mixing, and an Eppendorf centrifuge model 5810R from Brinkmann Instruments Inc. (Westbury, NY, USA) was used for centrifugation. An Applied Biosystems/MDS Sciex (Concord, ON, Canada) model API 4000 triple quadrupole mass spectrometer equipped with a Shimadzu LC-10AD Prominence solvent delivery system, degasser and SCL-10 Avp system controller (Columnbia, MD, USA) was used for LC–MS/MS analysis. A Leap Technologies CTC PAL autosampler with Shimadzu 10AD pump was used. A Harvard Apparatus (South Natick, MA, USA) syringe pump with a 500 μL syringe from Hamilton Co. (Reno, NE, USA) was employed for compound infusion.

2.3. Sample preparation using TCA or acetonitrile (ACN) induced plasma protein precipitation

For TCA induced plasma precipitation, TCA was diluted in water to obtain 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30% and 35% concentration (w/v). To 50 μL plasma samples, 30 μL TCA at various concentrations were added; white protein precipitation was observed; then 170 μL water was added. Samples were centrifuged at 4000 rpm for 5 min, and 50 μL of the supernatants were aliquoted into a 1.2 mL polypropylene 96-well plates for sample analysis. For ACN induced plasma precipitation, to 50 μL plasma samples, 25–200 μL ACN (at various ACN/water ratios) were added to 50 μL of plasma samples, and protein precipitation was observed. 50 μL of the supernatants were aliquoted and reconstituted in 10% ACN for sample analysis. Analyte recovery was calculated by peak areas count ratios of samples recovered from plasma and samples from water solutions at correspondent TCA concentrations. Samples at each concentration level were analyzed in triplicate over three independent batch runs.

2.4. Preparation of calibration standards

Stock solutions of PMB (containing PMB1 and PMB2), PME (containing PME1 and PME2), Fibrinopeptide B, VCM and dalbavancin were prepared as 1 mg/mL concentration in water with their purity factors considered. Fibrinopeptide B and dalbavancin were further diluted to 500 ng/mL for use as internal standards for PMXs and VCM, respectively. Stock solutions were serially diluted with rat plasma or water. Analytical standards used to construct calibration curves were prepared separately for each type of extraction method. The stock solutions of the compounds were prepared in water and the stock standard solutions were carried out by serial dilutions of the stock solutions to desired concentrations. Plasma and neat solvent working standards were prepared by spiking known quantities of the stock standard solutions to the blank rat plasma and water, respectively. The final concentrations for PMXs

Table 1
Compound structures and MRM parameters for MS analysis.

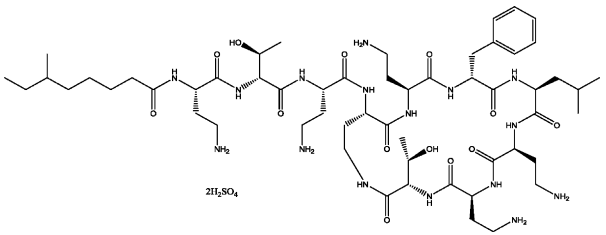
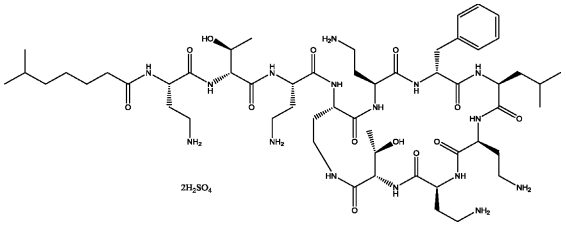
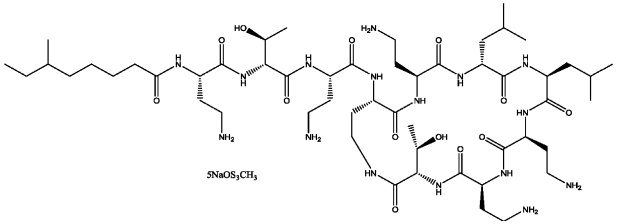
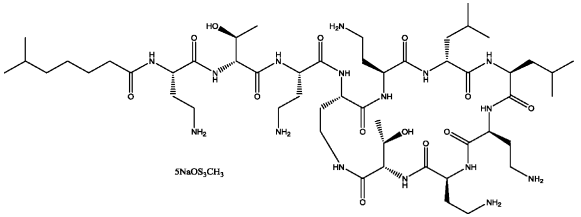
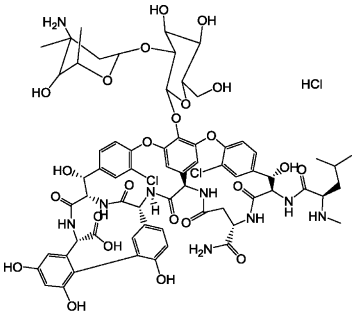
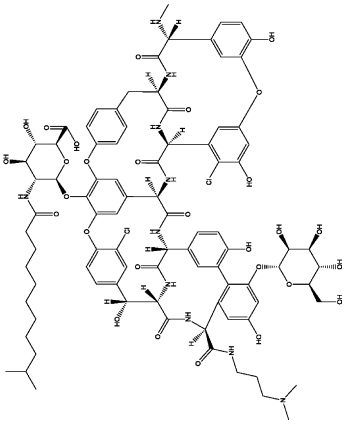
Commercial name	Chemical structure	FW	FW of salt	Salt purity		Q1	Q3	DP	CE	CXP	EP
				Factor	%						
Polymyxin B1		1203.5	1399.6	1.16	95	602.5	241.2	70	34	10	10
Polymyxin B2		1189.5	1385.6	1.16	95	595.6	227.5	70	38	10	10
Polymyxin E1		1169.5	1759.9	1.50	95	578.5	227.2	70	36	10	10
Polymyxin E2		1155.4	1745.9	1.51	95	585.6	241.3	70	34	10	10
Vancomycin		1449.3	1485.7	1.03	90	725.5	144.0	70	46	10	10

Table 1 (*Continued*)

Commercial name	Chemical structure	FW	FW of salt	Salt purity		Q1	Q3	DP	CE	CXP	EP
				Factor	%						
Dalbavancin		1816.7	1816.7	1.00	100	909.5	340.6	110	40	10	10
[Glu1]-Fibrinopeptide B human	Glu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg	1570.6	1570.6	1.00	90	786.3	187.3	70	52	10	10

Q1: molecular ion; Q3: daughter ion; DP: declustering potential (V); CE: collision energy (V); EP: entrance potential; (V) CXP: collision cell exit potential (V).

working standards are: 5, 10, 25, 50, 100, 250, 500, 1000, 2500, 5000 ng/mL; for VCM: 1, 2, 5, 10, 25, 50,100, 250, 500, 1000, 2500, 5000 ng/mL. Accuracy (% RE) and precision (% CV) of the assay were assessed by analyzing quality control samples of 19.5, 156, 1250 ng/mL for PMB1 and PME1 and 39.1, 313, 2500 ng/mL concentration for PMB2, PME2 and VCM. Quality control samples were prepared identically to the analytical standards.

2.5. LC–MS/MS analysis

A Phenomenex Jupiter C18 5 μ 300 Å 50 mm × 2 mm column (Torrance, CA, USA) was used for the analysis. Gradient chromatography was performed with 0.1% formic acid in 100% water (A) and 0.1% formic acid in 100% ACN (B) at a flow rate of 0.25 mL/min. The gradient used was 0–0.5 min, 5% B; 1.5 min, 70% B; 2.5 min, 90% B; 3.0–3.5 min, 5% B; 3.6 min, stop. The injection volume was 10 μL. To test the impact of the silica pore size of the column with VCM, isocratic elution was applied at 6% B with 0.1% acetic acid. The column was equilibrated for 1 min before each run.

Positive ion electrospray tandem mass spectra were recorded using an AB Sciex API 4000 triple quadrupole mass spectrometer with multiple reaction monitoring (MRM) detection mode controlled by Analyst (version 1.41) operating software. The ionspray voltage was set to 5000 V, and the probe temperature was set at 500 °C. Nitrogen was used as the collision gas. And the nebulizer (GS1), curtain, and turbo gas (GS2) were set to 40, 10, and 50 psi, respectively. MRM parameters of test compounds were set as described in Table 1. Dwell times were set to 200 ms for each transition.

3. Result and discussion

3.1. TCA induced plasma protein precipitation

Plasma protein precipitation with organic solvents is commonly used for analyte recovery. However, because of the very low solubility of the peptide compounds in organic solvents, their analyte recoveries were less than 20% at various ACN/water volume ratios (0.5:1, 1:1, 2:1, 4:1). In order to obtain better recoveries for high throughput liquid–liquid extraction methods, chlorine-containing acid induced protein precipitation had been used. In our research, we choose TCA over HCl or $HClO_4$ since it was studied that protein precipitation is not dictated by pH but is strongly dependent on the trichloro group [39]. No precipitation was observed at 0.1% and 1% TCA concentration, but the protein was partly changed to a molten globule state. Protein precipitation initiated at 5% TCA, and reached a maximum at about 30% TCA. Fig. 1 shows analyte recovery for PMB, PME, Fibrinopeptide B and VCM when various TCA concentrations were used for plasma protein precipitation. The data shows that analyte recoveries increased as TCA concentration increased for all the peptides. Since VCM is less polar than PMX, its recovery plateaued about 15–35% TCA. For PMX the analyte recoveries surpassed 100% when TCA concentration was higher than 20%, which indicated that adding TCA not only helped protein precipitation but also helped to increase the mass spectrometric response of PMX. It was determined 30% TCA was the concentration to optimize analyte recoveries.

3.2. Chromatographic conditions optimization

One important aspect of liquid chromatography separation involves matching the pore size of the packed silica with the size of the analyte molecules. Several columns with different pore sizes had been tested with VCM as shown in Table 2. It was found that amongst important column retention parameters such as carbon load, surface area, coverage, and pore size, increasing pore size can

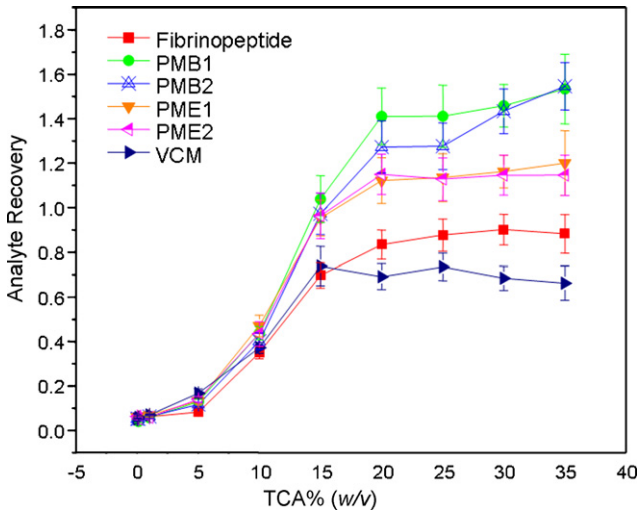


Fig. 1. Analyte recovery versus TCA concentration for peptide molecules.

improve peptide retention while holding other parameters constant. The molecular weights (MW) of the antibacterial peptide compounds are greater than 1000 Da and the Phenomenex Jupiter C18 5 μ 300 Å 50 mm × 2 mm column was selected for LC–MS/MS analysis. PMX molecules were also tested, and the Phonomenex column was found to offer the best performance.

For PMX, the chromatography was optimized by employing a gradient elution that started at a very low ACN percentage (5%) where it was held for 0.5 min to allow the analyte to achieve good retention. After 1.5 min, ACN percentage was increased to 90%, as high organic content helped the analyte achieve better ionization efficiency. Fig. 2 shows the effects of TCA concentration on retention of PMB1, PME1 and VCM (the data for PMB2 and PME2 were very similar to PMB1 and PME1 and are not shown) in both neat (water) solution [Fig. 2(a)] and in rat plasma [Fig. 2(b)]. In both matrices, increasing the TCA percentage (only added in the samples) from 5% to 20% quickly increased the retention time of the PMXs and VCM until the retention time remained steady after 20% TCA. The increase of the retention time is derived from the ion-pairing effect of TCA. When TCA was added to the samples during sample preparation, TCA formed ion pairs with the polar molecules, increased their hydrophobicity, changed their charge status, and changed the interaction between the analyte and the column surface. When the percentage of TCA was greater than 20%, a single, sharper peak was observed for both PMB1 and PMB2 compared to not adding TCA, indicating the ion-pairing effect had reached its maximum. It was also seen that the buffering capacity of the plasma supernatant had reduced the retention differences between PMX and VCM.

To determine the ratios of PMB1 to PMB2 and PME1 to PME2, it was assumed that analyte pairs have the same response factor for MRM detection. This is reasonable since the molecules differ by a single CH_2 group [26,27,40]. As purified PMB1, PMB2, PME1 and PME2 are unavailable and their compositions differ between manufacturers and batches, the percentage of the components were determined by their peak area ratios with respect to the total peak

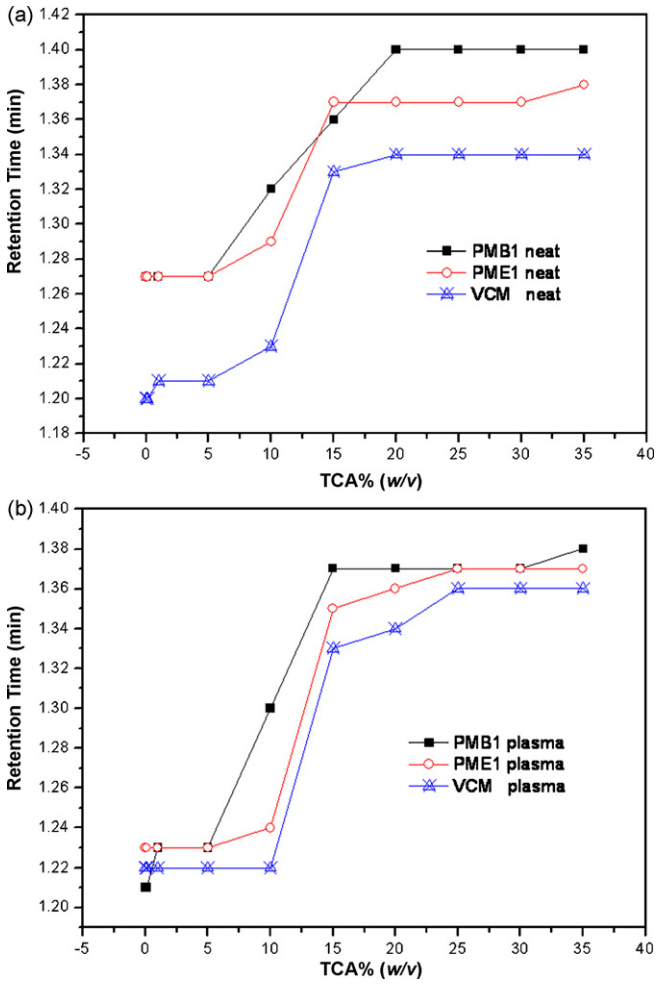


Fig. 2. The effect of TCA concentration on retention of PMB1, PME1 and VCM tested with gradient elution in (a) neat solution and (b) rat plasma.

area. The percentage of PMB1 and PMB2 was found to be 78.0 ± 0.8% and 17.0 ± 0.8%. The percentage of PME1 and PME2 was found to be 71.0 ± 1.1% and 24.0 ± 1.1%.

3.3. Quantitation

The calibration curves for PMXs were generated from MRM analysis of five replicate rat plasma samples at the calibration standard concentration level covering the range of 5–5000 ng/mL, with Fibrinopeptide B as the internal standard at 500 ng/mL and an injection volume of 10 μL. VCM was prepared in the same manner as PMXs except the concentration range was 1–5000 ng/mL, with dalbavancin as the internal standard. Good responses over the concentration ranges were obtained. Calibration curve regression was weighted as $1/x$ and analyzed using linear fit of quantities versus peak area ratios. Precision and accuracy data are shown in Table 3. The standard calibration curves were linear over the concentration range with a correlation coefficient

Table 2
Column parameters versus retention times for VCM.

Column	Retention time (min)	Surface area (m^2/g)	Carbon load (%)	Bonded phase coverage (μmol/m)	Bonded phase coverage (μmol/g)	Pore size (A)
Phenomenex Jupiter 5uC18 50 mm × 2 mm	3.0	170	13.3	5.50	935	300
Varian Intersil 5μ ODS 3 100 mm × 2 mm	2.6	320	15.0	3.23	1034	100
Phenomenex Lunar C(18)2 5u C18 50 mm × 2 mm	1.6	400	13.5	5.50	2200	100
Varian MetaSil AQ3uC18 50 mm × 2 mm	1.1	220	12.0	2.52	554	80

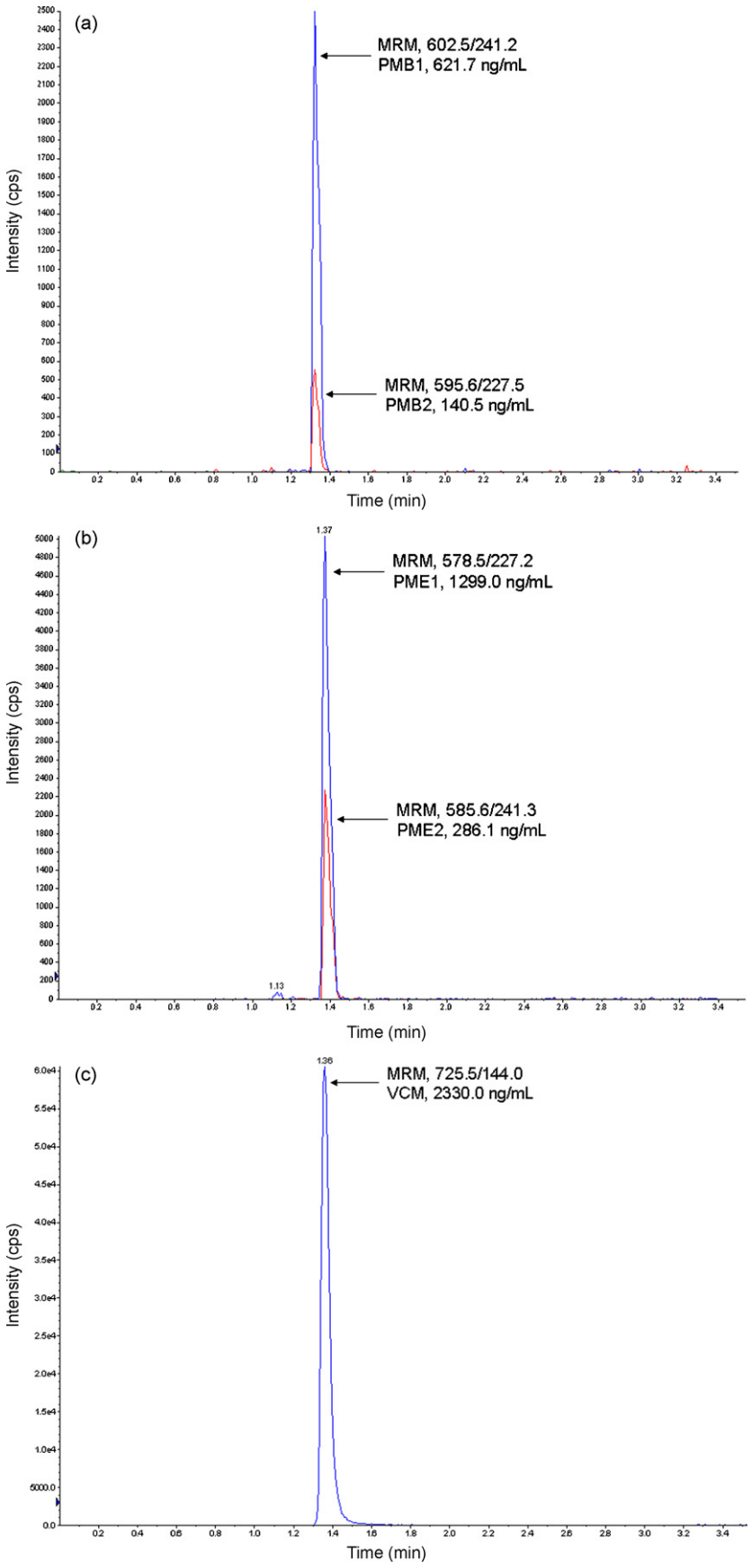


Fig. 3. The chromatograms of peptide drugs rat plasma sample collected at 4 h (a) PMB1 and PMB2, after IV dose of PMB at 2 mg/kg; (b) PME1 and PME2, after IV dose of PME at 2 mg/kg; (c) VCM, after SC dose of VCM at 200 mg/kg.

better than 0.9989. The method validation yielded precision results of: 4.3–7.4%, 2.3–9.2%, 5.1–10.8%, 3.8–9.4%, and 7.8–10.3% and accuracy results of: 91.7–104.2%, 91.7–105.1%, 94.9–104.8%, 94.3–107.4%, and 96.2–102.0% for PMB1, PMB2, PME1, PME2, and VCM, respectively. The LLOQs corresponding to a coefficient of variation less than 20% were 7.5, 18.1, 7.3, 5.0 and 1.0 ng/mL for PMB1, PMB2, PME1, PME2 and VCM, respectively. Since TCA was only added to the samples but not to the mobile phases, the ion suppression which would cause the non-linear standard curves was not observed. The data demonstrated that good accuracy and precision of this assay was developed for rat plasma samples. This is a significant improvement over assays reported in literatures in terms

Table 3
Precision and accuracy data for PMXs and VCM.

Nomonal conc. (ng/mL)	Inter or intraday	Measured conc. (ng/mL)	%RSD	%RE	Nomonal conc. (ng/mL)	Inter or intraday	Measured conc. (ng/mL)	%RSD	%RE
Polymyxcin Bl					*Polymyxcin B2*				
7.5 (LLOQ)	Intraday	7.47			18.1 (LLOQ)	Intraday	21.5		
	Intraday	7.62				Intraday	18.5		
	Intraday	7.23				Intraday	17.1		
	Interday	8.05				Interday	19.8		
	Interday	8.44	6.4	103.5		Interday	18.2	9.2	105.1
19.5 (LQC)	Intraday	17.0			39.1 (LQC)	Intraday	38.2		
	Intraday	17.5				Intraday	39.8		
	Intraday	19.0				Intraday	35.2		
	Interday	18.2				Interday	34.7		
	Interday	20.7	7.4	94.8		Interday	41.5	7.5	96.9
156 (MQC)	Intraday	135			313 (MQC)	Intraday	295.9		
	Intraday	143				Intraday	279.6		
	Intraday	150				Intraday	293.6		
	Interday	137				Interday	281.9		
	Interday	150	4.5	91.7		Interday	284.3	2.3	91.7
1250 (HQC)	Intraday	1300			2500 (HQC)	Intraday	2469.8		
	Intraday	1250				Intraday	2376.6		
	Intraday	1370				Intraday	2399.9		
	Interday	1250				Interday	2423.2		
	Interday	1340	4.3	104.2		Interday	2353.3	3.6	96.2
Polymyxcin E1					*Polymyxcin E2*				
7.3 (LLOQ)	Intraday	6.91			5.0 (LLOQ)	Intraday	5.79		
	Intraday	7.55				Intraday	5.72		
	Intraday	6.87				Intraday	4.67		
	Interday	8.43				Interday	5.11		
	Interday	8.50	10.8	104.8		Interday	5.56	9.4	107.4
19.5 (LQC)	Intraday	19.1			39.1 (LQC)	Intraday	44.4		
	Intraday	20.0				Intraday	43.1		
	Intraday	17.9				Intraday	36.8		
	Interday	20.3				Interday	42.3		
	Interday	18.6	5.1	98.4		Interday	40.0	7.7	105.7
156 (MQC)	Intraday	159			313 (MQC)	Intraday	294		
	Intraday	151				Intraday	290		
	Intraday	149				Intraday	281		
	Interday	135				Interday	313		
	Interday	146	5.6	94.9		Interday	298	3.8	94.3
1250 (HQC)	Intraday	1280			2500 (HQC)	Intraday	2450		
	Intraday	1240				Intraday	2520		
	Intraday	1230				Intraday	2460		
	Interday	1230				Interday	2560		
	Interday	1390	5.4	101.9		Interday	2430	4.3	99.4
Vancomycin									
1.0 (LLOQ)	Intraday	0.909							
	Intraday	1.02							
	Intraday	1.04							
	Interday	0.900							
	Interday	1.08	8.1	99.0					
39.1 (LQC)	Intraday	35.0							
	Intraday	42.7							
	Intraday	36.1							
	Interday	35.5							
	Interday	38.9	8.2	96.2					
313 (MQC)	Intraday	270							
	Intraday	356							
	Intraday	323							
	Interday	329							
	Interday	302	10.3	101.0					
2500 (HQC)	Intraday	2530							
	Intraday	2600							
	Intraday	2590							
	Interday	2640							
	Interday	2390	7.8	102.0					

LLOQ: lower limit of quantitation; LQC: low quality control; MQC: medium quality control; HQC: high quality control; %RSD relative standard deviation; %RE: percent relative error.

of sensitivity, simplicity and understanding of the chromatography challenges for both PMXs [26,27,29,30] and VCM [36–38]. For PMXs the reported methods require a long and expensive procedure of SPE, consumption and injection of a large volume of samples (100–200 μL), and long separation time (7–16 min) with poor chromatography (peak tailing and peak fronting) and low sensitivity (for PME in any matrices ranges from 30–300 ng/mL (g) per 10 μL injection). Similarly for VCM our method avoided complicated extraction procedure, large injection volume, or long separation time, yet it achieved better or similar sensitivity than the literature reports (1–10 ng/mL).

3.4. Real sample analysis

PMB and PME were administrated individually though intravenous (IV) route into the tail vein of Sprague–Dawley (SD) rats to obtain their pharmacokinetic profiles. The dosed amounts were 0.2, 0.4 and 2 mg/kg of compound in sterile saline. VCM were administrated individually via subcutaneous (SC) route into bacterially infected Sprague–Dawley (SD) rats in order to obtain the pharmacokinetic and pharmacodynamic (PK–PD) profiles. The dose amounts were 20, 60, and 200 mg/kg in sterile saline. Whole blood samples were collected at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h and processed by centrifugation to obtain plasma. Fig. 3(a) shows the chromatograms for PMB1 and PMB2, Fig. 3(b) for PME1 and PME2, and Fig. 3(c) for VCM plasma sample collected at the 4 h time point when the dose amounts for PMB and PME were 2 mg/kg and for VCM was 200 mg/kg.

4. Conclusions

In the present work, we have studied the effect of the concentration of TCA on plasma protein precipitation and sample recovery efficiency for antibacterial peptide compounds. It was found that the TCA sample precipitation method gave better sample recovery than the ACN sample precipitation method when the concentration of TCA reached 25–30% for these polar peptide molecules. It can be concluded that the TCA sample precipitation method is a general sample preparation method for hydrophilic peptide compound with MW less than 2000 Da. Moreover, when TCA is used, it has the effect of increasing the retention of the peptide molecules as well as sharpening the elution peaks. LC–MS/MS methods have been developed and validated for the analysis of PMB and PMB with Fibrinopeptide B as the internal standard and VCM with dalbavancin as the internal standard. The method used TCA protein precipitation, a reversed phase C-18 column with pore size of 300 Å, and a very high aqueous content buffer to afford acceptable retention. Satisfactory LLOQs of 7.5, 18.1, 7.3, 5.0 and 1.0 ng/mL for PMB1, PMB2, PME1, PME2 and VCM, respectively, were obtained using an injection volume of 10 μL. Compared with existing methods, the method detailed in this paper avoided using ion pairing reagents in the mobile phase, derivatization, SPE, organic solvent extraction and long separation time, yet it yielded similar or better sensitivity for the compounds studied.

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