Development and validation of a reversed-phase high-performance liquid chromatography assay for polymyxin B in human plasma

Guoying Cao1,2, Feda’ Emad Atta Ali1, Francis Chiu3, Alexandre P. Zavascki4,5, Roger L. Nation1† and Jian Li1*†

1Facility for Anti-infective Drug Development and Innovation, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia; 2Department of Pharmacy, Beijing Hospital, Beijing 100730, P. R. China; 3Centre for Drug Candidate Optimisation, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia; 4Infectious Diseases Service, Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul, Brazil; 5Division of Infectious Diseases, Hospital de Clínicas de Porto Alegre, 2350 Ramiro Barcelos Street 90035-903, Porto Alegre, Brazil

Received 6 May 2008; returned 18 June 2008; revised 14 July 2008; accepted 1 August 2008

Objectives: The purpose of this study was to develop a specific, sensitive, accurate and reproducible high-performance liquid chromatographic (HPLC) method to measure polymyxin B in human plasma.

Methods: Derivatization of polymyxin B with fluorescent 9-fluorenylmethyl chloroformate (FMOC-Cl) was performed in the same solid-phase extraction C18 cartridge used for the sample pre-treatment. Reversed-phase HPLC was employed with fluorometric detection. The summed peak areas of polymyxin B1 and B2 derivatives were used for quantification. Stability of polymyxin B FMOC derivatives was examined at room temperature for 6 days. Specificity was investigated against seven potentially co-administered antibiotics. Accuracy and reproducibility of the HPLC assay were determined by inter- and intra-day validation.

Results: The derivatives of polymyxin B2 and B1 were well resolved and had retention times of 4.75 and 5.55 min, respectively. Good linearity ($r^2 > 0.99$) was obtained between 0.125 and 4.00 mg/L polymyxin B in human plasma with good accuracy and reproducibility at the limit of quantification (0.125 mg/L). Intra- and inter-day validation demonstrated good accuracy and reproducibility for quality control samples with nominal concentrations of 0.30 and 3.00 mg/L. FMOC derivatives of polymyxin B were stable for at least 3 days at room temperature. None of the possibly co-administered antibiotics tested interfered with the chromatographic analysis of the polymyxin B FMOC derivatives.

Conclusions: A rapid, specific, sensitive, accurate and reproducible HPLC method has been developed and validated to measure polymyxin B in human plasma. The method is suitable for clinical pharmacokinetic studies.

Keywords: derivatization, HPLC, polymyxin B sulphate, 9-fluorenylmethyl chloroformate

Introduction

Since the 1990s, emergence of multidrug-resistant Gram-negative bacteria, such as Pseudomonas aeruginosa, Acinetobacter baumannii and Klebsiella pneumoniae has been presenting a global medical challenge.1–3 In many cases, few antibiotic options are available, except polymyxins. Although the pharmacology of polymyxins is very limited and most data were obtained more than three decades ago, they have been increasingly used as the last resort for multidrug-resistant Gram-negative pathogens that are resistant to all other currently available antibiotics.4–8

There are two polymyxins used clinically, polymyxin B and colistin (polymyxin E), both of which have similar antibacterial spectra and rapid bactericidal activity.9–11 There is
cross-resistance between polymyxin B and colistin. Unlike colistin, which is administered parenterally in the form of its inactive prodrug colistin methanesulphonate (sodium salt), polymyxin B is administered as its sulphate salt, i.e. polymyxin B sulphate. Polymyxin B is a cyclic heptapeptide with a tripeptide side chain acylated with a fatty acid (Figure 1). Like many antibiotics, polymyxin B is a multi-component antibiotic with polymyxin B1 and B2 being the two major components; they differ from each other only in the fatty acid moiety (Figure 1).

Several methods have been developed for the measurement of polymyxin B, mainly for formulation analysis, including microbiological assays, capillary zone electrophoresis with ultraviolet (UV) detection, thin-layer chromatography, and no native fluorescence. This study describes an HPLC method that involves the formation of a fluorescent derivative allowing quantification of clinically relevant concentrations of polymyxin B in human plasma.

**Materials and methods**

**Chemicals and reagents**

Polymyxin B sulphate was purchased from Fluka (≥6000 USP units per mg, Castle Hill, NSW, Australia). The derivatizing reagent, 9-fluorenylmethyl chloroformate (FMOC-Cl), was ordered from Sigma-Aldrich (NSW, Australia), ciprofloxacin from Santa Cruz Biotechnology (VIC, Australia), azithromycin from Pfizer (NSW, Australia), ceftazidime from GlaxoSmithKline (VIC, Australia), meropenem from AstraZeneca (NSW, Australia), aztreonam from Bristol-Myers Squibb (VIC, Australia), piperacillin from David Bull Laboratory (VIC, Australia) and tobramycin from David Bull Laboratory. Tetrahydrofuran (Science Supply, VIC, Australia), acetonitrile, acetone and methanol (Biolab, VIC, Australia) were of HPLC grade. All other reagents were of analytical grade. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). All solutions were stored at 4°C.

**Plasma sample pre-treatment, derivatization in solid-phase extraction (SPE) cartridges and HPLC**

Prior to use, the SPE cartridges (C18, 100 mg, Sep-Pak®, Waters, Milford, MA, USA) were washed with 1 mL of acetone and conditioned with 1 mL of methanol followed by 1 mL of carbonate buffer (1%, w/w, pH 10). All manipulations involving the SPE cartridges were performed using a vacuum manifold (Supelco Visiprep 24TM, Bellefonte, PA, USA). An aliquot (100 µL) of acetonitrile was added to 100 µL of human plasma in a 1.5 mL centrifuge tube. After vortex-mixing for 30 s and centrifugation (10 000 g for 10 min), all of the supernatant was transferred to a conditioned SPE cartridge. After the cartridge was washed with 1 mL of acetone buffer, 110 µL of FMOC-Cl solution (containing 30 µL of 100 mM FMOC-Cl in acetonitrile and 80 µL of methanol) was added. Following 10 min of reaction, the polymyxin B derivatives were eluted with 900 µL of acetonitrile. The eluate was mixed with 600 µL of boric acid (0.20 M) and 500 µL of acetonitrile. After vortex-mixing, 30 µL was injected onto the HPLC column.

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10AS pump, an SIL-10ADvp auto injector (at room temperature) and an RF-10AXL fluorescence detector connected to a data processing system (Class-VP 6.13). A 50 × 4.6 mm (ID) Onyx Monolithic C18 column coupled with a 4 × 3.0 mm C18 guard column (at 25°C) was employed (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile/tetrahydrofuran/water (50:25:25, v/v) and the flow rate was 1.0 mL/min. Detection by monitoring for fluorescence was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm.

**Validation of the assay**

**Confirmation of identity of the polymyxin B derivatives.** An aliquot (10 µL) of polymyxin B sulphate in water (1 mg/mL) was chromatographed on a reversed-phase Phenosphere-NEXT C18 column (5 µm, 250 × 4.6 mm; Phenomenex) coupled with a C18 guard column (5 µm, 4.0 × 3.0 mm, Phenomenex). The mobile phase, a mixture of acetonitrile/35 mM triethylamine (pH 2.5) (21.79; v/v), was pumped at 1.0 mL/min for a run time of 15 min. UV absorbance of the eluent was monitored at 210 nm. The eluate fractions corresponding to the two dominant peaks were collected and analysed by electrospray mass spectrometry (LCMS-2010EV, Shimadzu). For MS conditions, the tuning voltages were fixed for the interface, CDL and Q-array. The detector voltage was 1.5 kV. The temperatures were maintained at 200°C for both the CDL and heat block. Nitrogen gas flow rates of 1.5 and 10 L/min were employed for nebulizer gas and drying gas, respectively. Positive ion electrospray mass spectral analysis was conducted by scanning between 200 and 1300 m/z, and the data were analysed using LCMS Solutions (version 4.2, Shimadzu). To identify the peaks from chromatographic analysis of the FMOC derivatized eluate collected in the HPLC/UV assay, corresponding to each of the two main components of polymyxin B (polymyxin B1 and B2), was reacted with FMOC-Cl as described above. An aliquot (5 µL) of the derivative solution was injected onto the analytical column used for the analysis of plasma samples (the Onyx Monolithic C18 column) and the

**Figure 1.** Chemical structure of polymyxin B. Fatty acid: 6-methylocyclohexanoic acid for polymyxin B1 and 6-methylheptanoic acid for B2. Thr, threonine; Leu, leucine; Dab, α,γ-diaminobutyric acid; Phe, phenylalanine; α and γ indicate the respective amino group involved in the peptide linkage.
respective derivative was detected using fluorescence, as described above.

Specificity, recovery and stability of the FMOC derivatives. The specificity of the method was examined with blank human plasma (Australian Red Cross, Melbourne, Australia) spiked with ciprofloxacin, azithromycin, cefazidime, meropenem, aztreonam, piperacillin or tobramycin individually at a concentration of 4.00 mg/L. These antibiotics may possibly be co-administered with polymyxin B clinically. Recovery was calculated by comparing the peak areas of plasma samples with those of water samples spiked with 0.50, 2.00 and 4.00 mg/L polymyxin B; water samples and plasma samples were derivatized inside conditioned SPE cartridges under the same conditions (see above). Stability of the derivatives was examined at room temperature by comparison of the peak areas of polymyxin B1 and B2 and the sum of the two at 4.00 mg/L on days 1, 2, 3 and 6 with those at time zero.

Linearity, accuracy and reproducibility. One working standard of polymyxin B sulphate in water (0.10 mg/mL) was used to prepare calibration standards in blank human plasma (Australian Red Cross) at concentrations of 0.125, 0.25, 0.50, 1.00, 2.00 and 4.00 mg/L. Calibration curves were obtained by linear least-squares regression analysis of the summed peak areas of polymyxin B1 and B2 versus drug concentrations (x) using 1/x weighting. Quality control (QC) samples at 0.30 and 3.00 mg/L were prepared independently with separate stock solutions. Accuracy and reproducibility were assessed by the inter-day assay (with three consecutive analyses of QC samples of 0.30 and 3.00 mg/L polymyxin B in human plasma on three separate occasions; the QC samples were prepared independently) and by the intra-day assay (with six consecutive analyses of the QC samples). The limit of quantification was determined by measuring the concentration of polymyxin B in six human plasma samples prepared independently at 0.125 mg/L.

Results

Figure 2 shows the typical chromatograms of blank human plasma and a plasma sample collected from a critically ill patient 3 h after intravenous administration of polymyxin B (sulphate, 1.50 mg/kg). The derivatives of polymyxin B2 and B1 were well resolved and had retention times of 4.75 and 5.55 min, respectively. There was no chromatographic interference to the derivatives of polymyxin B from endogenous compounds or reaction by-products.

Figure 2. Typical chromatograms of blank human plasma (a) and a plasma sample (1.62 mg/L) from a critically ill patient at 3 h after intravenous administration of 1.50 mg/kg polymyxin B sulphate (b).
The two major derivative peaks in the analysis of plasma samples on the Onyx Monolithic C18 column were identified indirectly. With UV detection at 210 nm, two main components of underivatized polymyxin B were well resolved on the PhenoSphere-NEXT C18 column with retention times of 6.3 and 11.7 min for polymyxin B2 and B1, respectively. The mass spectra of the individual components obtained from the HPLC fractions collected contained ions with \( m/z \) 602.4 \([\text{M}+2\text{H}]^2+\) and 401.9 \([\text{M}+3\text{H}]^3+\) (Figure 3a), and 595.4 \([\text{M}+2\text{H}]^2+\) and 397.2 \([\text{M}+3\text{H}]^3+\) (Figure 3b). These were consistent with the protonated molecular ions of polymyxin B1 (theoretical molecular weight 1202) and polymyxin B2 (theoretical molecular weight 1188), respectively. Hence, in the component analysis of underivatized polymyxin B, the first peak corresponded to polymyxin B2 and the second to polymyxin B1. Correspondingly, in the HPLC chromatograms of polymyxin B FMOC derivatives, the first peak corresponds to the derivative of polymyxin B2 and the second to that of polymyxin B1 (Figure 2).

None of ciprofloxacin, azithromycin, ceftazidime, meropenem, aztreonam, piperacillin or tobramycin interfered with the chromatographic analysis of the FMOC derivatives of polymyxin B1 and B2. Mean recoveries of 103%, 93.2% and 105% \((n = 3)\) were achieved when comparing the peak areas of plasma samples with those of water samples spiked with 0.50, 2.00 and 4.00 mg/L polymyxin B, respectively. The derivatives of polymyxin B1 and B2 were stable for at least 3 days (>95% HPLC peak area in comparison with time zero) at room temperature.

For calibration curves \((0.125–4.00 \text{ mg/L})\) prepared on different days \((n = 6)\), the relative standard deviation (RSD) of slopes was 10.9% and the mean \((\pm \text{SD})\) coefficient of determination was 0.9957 ± 0.004. The limit of quantification was 0.125 mg/L, at which concentration the mean measured value was 0.13 mg/L and the RSD was 8.23% \((n = 6)\). Inter-day and intra-day reproducibility and accuracy are shown in Table 1. This method has been intensively employed in pharmacokinetic studies of polymyxin B in patients. A plasma concentration versus time profile of polymyxin B obtained with this HPLC method, from a patient who was administered 1.25 mg/kg intravenously, is shown in Figure 4.

**Discussion**

In the last few decades, emergence of nosocomial Gram-negative bacteria with acquired resistance to almost all available antimicrobial agents\(^1\) has severely threatened therapeutic

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Mass spectra of polymyxin B1 (a) and polymyxin B2 (b) obtained by electrospray ionization MS. Arrows show the protonated molecular ion \([\text{M}+2\text{H}]^2+\) of polymyxin B1 and B2.
Polymyxins (polymyxin B and colistin) have become the last resort for treatment of infections caused by multidrug-resistant Gram-negative pathogens. Although intravenous administration of polymyxin B (sulphate) has substantially increased recently, knowledge of its pharmacology is extremely limited. Recent emergence of resistance to polymyxins, including hetero-resistance, highlights the urgency of systematically investigating their pharmacology to avoid suboptimal clinical use. Most recent clinical experience with polymyxins focuses on colistin. There are no solid pharmacokinetic data available in the literature for polymyxin B supporting the current intravenous dosage regimens in humans. Even in the Product Information of Polymyxin B Sulphate for Injection, no plasma concentration or half-life values are provided. Lack of a reliable assay for polymyxin B in biological fluids is likely to be one of the major contributors to this dearth of knowledge.

To date, only one LC/MS method has been reported for the measurement of polymyxin B1 in biological fluids; the B2 component was not quantified, and as a result, the method would underestimate the concentration of polymyxin B. Unfortunately, LC/MS may not be commonly available in laboratories for the measurement of drug concentrations in human fluids. Microbiological assays lack specificity, particularly when samples contain other co-administered antibiotics that are active against the test strain. In the present study, an accurate and reproducible HPLC assay was developed for polymyxin B in human plasma, based on our previously reported assay for colistin, but with major improvements (e.g. simpler sample pre-treatment and a substantially shorter HPLC run time). Since there is only one amino acid difference between polymyxin B and colistin, it is believed that the conditions for colistin derivatization with FMOC-Cl are similar for polymyxin B.

A very simple sample pre-treatment involved precipitation of proteins in plasma by addition of an equal volume of acetonitrile. Washing the SPE cartridges with 1 mL of acetone prior to their use and 1 mL of carbonate buffer after loading the supernatant from plasma sample pretreatment resulted in cleaner chromatograms. The advantages of FMOC-CI over other derivatizing reagents are rapid reaction with both primary and secondary amino groups and stable fluorescent derivatives. In this study, FMOC polymyxin B derivatives were very stable (95% HPLC peak area in comparison with time zero) in the employed matrix at room temperature for up to 3 days; even after 6 days, the peak area was >93%.

Remarkably, HPLC baseline resolution was achieved for the polymyxin B1 and B2 derivatives within a 7 min total run time (Figure 2), even though there is only one carbon difference in their fatty acid chains (Figure 1). The 7 min run time, which is much shorter than that for colistin (35 min), enhanced the efficiency of this polymyxin B assay. The calibration range of 0.125–4.00 mg/L was chosen by taking account of clinically relevant plasma concentrations of polymyxin B and analytical considerations. The highest concentration (4.00 mg/L) was employed to avoid fluorescence saturation at higher concentrations and also to maintain an acceptable lower limit of quantification (0.125 mg/L) for pharmacokinetic studies in humans (Figure 4). Concentrations above 4.00 mg/L can be easily measured by appropriate dilution(s) of derivative samples. This method has been employed to investigate the pharmacokinetics of polymyxin B in critically ill patients, in that clinical study, meropenem, ciprofloxacin, levofloxacin, vancomycin, aztreonam, ceftazidime and/or trimethoprim/sulfamethoxazole was co-administered with polymyxin B, and no interference in quantification of polymyxin B was observed. Thus, the method maintains its utility even for plasma samples collected from patients receiving antibiotics in combination with polymyxin B.

**Conclusions**

In summary, a rapid, specific, sensitive, accurate and reproducible HPLC method has been developed and validated for the measurement of polymyxin B in human plasma. The method is suitable for clinical pharmacokinetic studies.

**Acknowledgements**

We are grateful for the excellent technical support of Ms Roxanne Owen and Ms Anima Poudyal at the Facility for

Funding

This study was supported by internal research group funding. J. L. is an Australian National Health and Medical Research Council R. Douglas Wright Research Fellow. A. P. Z. has received financial support from The National Council for Scientific and Technological Development (CNPq), Ministry of Science and Technology, Brazil (152041/2006-0).

Transparency declarations

A. P. Z. has received speaking honoraria from Forest Laboratories, UK. Other authors: none to declare.

References