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Development and validation of a liquid chromatography–mass spectrometry assay for polymyxin B in bacterial growth media

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ABSTRACT

There is increasing interest in the optimization of polymyxin B dosing regimens to treat infections caused by multidrug-resistant Gram-negative bacteria. We aimed to develop and validate a liquid chromatography–single quadrupole mass spectrometry (LC–MS) method to quantify polymyxin B in two growth media commonly used in *in vitro* pharmacodynamic studies, cation-adjusted Mueller-Hinton and tryptone soya broth. Samples were pre-treated with sodium hydroxide (1.0 M) and formic acid in acetonitrile (1:100, v/v) before analysis. The summed peak areas of polymyxin B1 and B2 relative to the summed peak areas of colistin A and B (internal standard) were used to quantify polymyxin B. Quality control samples were prepared and analyzed to assess the intra- and inter-day accuracy and precision. The robustness of the assay in the presence of bacteria and commonly co-administered antibiotics (rifampicin, doripenem, imipenem, cefepime and tigecycline) was also examined. Chromatographic separation was achieved with retention times of approximately 9.7 min for polymyxin B2 and 10.4 min for polymyxin B1. Calibration curves were linear between 0.103 and 6.60 mg/L. Accuracy (% relative error) and precision (% coefficient of variation), pooled for all assay days and matrices ($n = 84$), were −6.85% (8.17%) at 0.248 mg/L, 1.73% (6.15%) at 2.48 mg/L and 1.54% (5.49%) at 4.95 mg/L, and within acceptable ranges at all concentrations examined. Further, the presence of high bacterial concentrations or of commonly co-administered antibiotics in the samples did not affect the assay. The accuracy, precision and cost-efficiency of the assay make it ideally suited to quantifying polymyxin B in samples from *in vitro* pharmacodynamic models.

1. Introduction

Polymyxin B is a cationic lipopeptide antibiotic with activity against Gram-negative bacteria. It was first isolated from *Paenibacillus polymyxa* in 1947 and consists of a cyclic heptapeptide linked *via* a tripeptide chain to a fatty acyl tail [1]. Concerns about nephro- and neuro-toxicity led to waning clinical use of polymyxins in the 1960s, as newer and supposedly safer classes of antibiotics such as aminoglycosides became favored by clinicians [2,3]. However, with increasing incidence of infections caused by multidrug-resistant (MDR) Gram-negative bacteria, there has been a recent resurgence in use of polymyxin B as a last-line therapy due to its activity against many of these MDR strains [4].

Presently, there is a dearth of information to guide clinicians in the optimal use of polymyxin B [1,4,5] and further pharmacological investigations are urgently required to preserve its antibacterial activity by optimizing dosage regimens and minimizing the emergence of resistance. Such investigations are commonly conducted using *in vitro* experimental models [6–9]. Accurate and precise quantification of polymyxin B in microbiological media is therefore critical for determining the pharmacokinetic/pharmacodynamic relationships that underpin both the antimicrobial activity of polymyxin B and emergence of bacterial resistance [10–12].

Both polymyxin B and polymyxin E (colistin) (Fig. 1), which differ by a single amino acid residue in the heptapeptide ring, are mixtures of several structurally related compounds. Polymyxin B1 and polymyxin B2 are the main constituents of polymyxin B while the corresponding major components of colistin are colistin A and colistin B; for both cases, these respective constituents generally account for over 85% of the total [13–15].

Quantification of polymyxins is complicated by their low UV absorption and lack of native fluorescence [16]. Several high-performance liquid chromatography (HPLC) and liquid chromatography–triple quadrupole mass spectrometry (LC–MS/MS) assays for polymyxins in plasma and other biological

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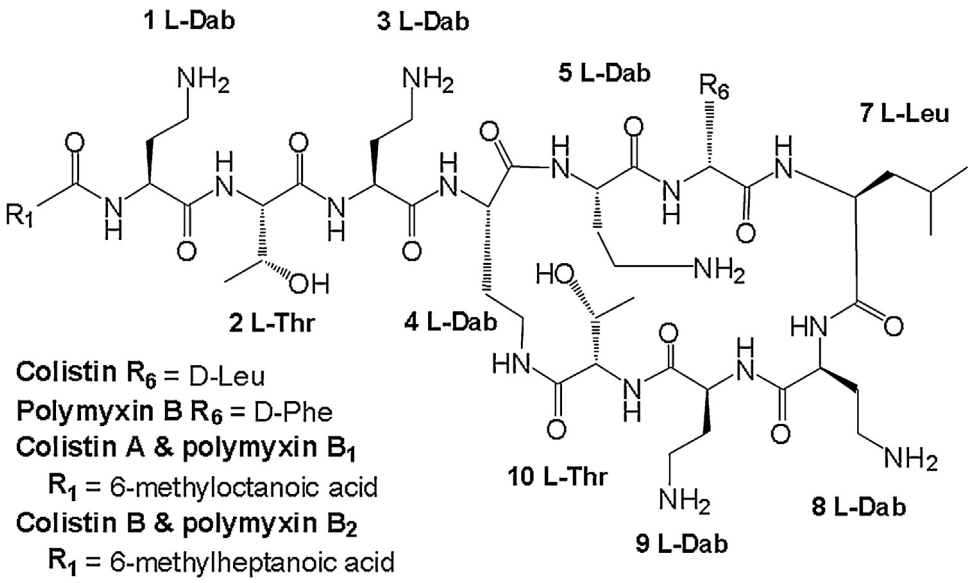

Fig. 1. The chemical structure of polymyxin B and colistin.

matrices have been reported [17–24]. However, to our knowledge, no liquid chromatography–mass spectrometry (LC–MS) assay for polymyxin B has been reported for cation-adjusted Mueller-Hinton broth (CAMHB) or tryptone soya broth (TSB), two microbiological growth media commonly used for antimicrobial susceptibility testing and *in vitro* infection models. In the present report we describe an accurate and reproducible LC–MS method for the quantification of polymyxin B in both of the above-mentioned growth media utilizing colistin as an internal standard.

2. Experimental

2.1. Apparatus

A Shimadzu (Kyoto, Japan) LC–MS system was used to obtain positive ion electro-spray mass spectra for the quantification of polymyxin B. This system consisted of a DGU-20A3 degasser, LC-20AD pump, SIL-20AC HT auto-sampler and CTO-20A column oven connected to an LCMS-2010EV single quadrupole mass spectrometer.

2.2. Materials and reagents

Mueller-Hinton broth powder was obtained from Oxoid (Basingstoke, Hampshire, England) and reconstituted with water in accordance with the manufacturer's instructions. This broth was cation adjusted to 11.64 mg/L Mg^{2+} and 23.41 mg/L Ca^{2+} with magnesium chloride (Sigma–Aldrich, St. Louis, MO, USA) and calcium chloride (Univar, Redmond, WA, USA) before sterilization. Polymyxin B sulfate was obtained from BetaPharma (Branford, CT, USA) and colistin sulfate from Sigma Aldrich. Formic acid was purchased from Ajax Finechem (New South Wales, Australia); HPLC-grade acetonitrile was from Merck (NJ, USA) and analytical-grade sodium hydroxide from Sigma Aldrich. Rifampicin (Sigma–Aldrich), imipenem/cilastatin (Merck Sharpe and Dohme, Whitehouse Station, NJ, USA), doripenem (Janssen Pharmaceuticals, Titusville, NJ, USA), cefepime (Omegapharm, Victoria, Australia) and tigecycline (Wyeth, Madison, NJ, USA) were obtained for assessing the specificity of the polymyxin B assay.

2.3. Sample pre-treatment

A 10 μL aliquot of the colistin internal standard solution (33.0 mg/L colistin base in acetonitrile/water [50:50, v/v]) was added to 100 μL of polymyxin B-containing growth medium (see Section 2.5) in a 1.7-mL polypropylene microcentrifuge tube (Quantum Scientific, Radnor, PA, USA). A 20-μL aliquot of sodium hydroxide in water (1.0 M) was then added to each sample and the tube contents were vortex mixed for ~2 s. Following the addition of 400 μL formic acid in acetonitrile (1:100, v/v), samples were vortex mixed for ~2 s and allowed to stand at room temperature for 10 min prior to centrifugation at 20,800 × *g* for 10 min. Subsequently, 150 μL of the supernatant was loaded into polypropylene auto-sampler vials for analysis by LC–MS.

2.4. LC–MS analysis

Samples were maintained at 4 °C within the auto-sampler and an injection volume of 10 μL was used. Chromatographic separation was achieved using a Phenomenex (Torrance, CA, USA) Synergi™ Hydro-RP column (80 Å, 125 mm × 4.00 mm) maintained at 40 °C. The mobile phase consisted of 0.5% aqueous formic acid (v/v, solvent A) and 0.5% formic acid in acetonitrile (v/v, solvent B). The flow rate was 0.4 mL/min with the following linear gradient elution program: 0% solvent B for 36 s, increasing to 13.0% over 1.4 min and maintained for 30 s before decreasing to 8% over 30 s and subsequently increasing to 33.5% over 8 min. This was followed by a 1.0-min flush at 95% solvent B and re-equilibration at 0% solvent B for 2.8 min at an increased mobile phase flow rate of 0.5 mL/min. Following chromatographic separation, multiple ion monitoring was used to detect the $[M+3H]^{3+}$ ions of polymyxin B (polymyxin B1 *m*/*z* 401.85, polymyxin B2 *m*/*z* 397.20) and colistin (colistin A *m*/*z* 390.55, colistin B *m*/*z* 385.95). The interface voltage was 4.5 kV, with a curved desolvation line temperature of 200 °C and voltage of 0 V.

2.5. Linearity, precision and accuracy

For preparation of calibration curve samples, stock solutions of polymyxin B (sulfate) were prepared in water and diluted to the required working solution concentrations in acetonitrile and water