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# Development and validation of a high-performance liquid chromatography-fluorescence detection method for the accurate quantification of colistin in human plasma

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ABSTRACT

Recently, colistin has become one of the most important drugs for treating infections caused by multidrug-resistant Gram-negative bacteria. Therapeutic drug monitoring is recommended to ensure the safety and efficacy of colistin and to improve clinical outcomes. This study developed an accurate and sensitive high-performance liquid chromatography-fluorescence detection (HPLC-FLD) method for the quantification of colistin in human plasma. The sample preparation included protein precipitation using trichloroacetic acid (TCA) and methanol, followed by in-solid phase extraction (In-SPE) derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl). A Poroshell 120 EC-C18 2.1 × 100 mm (2.7 μm) column was used in the HPLC method with a mobile phase composed of acetonitrile (ACN), tetrahydrofuran (THF), and deionized (DI) water (82%, 2%, 16% (v/v), respectively). Polymyxin B1 was used as the internal standard. The total analysis time was 22 min under optimal separation conditions. The HPLC-FLD method was validated over a therapeutic range of 0.3–6.0 μg mL$^{-1}$. The intra-day and inter-day precisions for colistin A and colistin B were below 9.9% and 4.5% relative standard deviations, respectively. The accuracy test results were between 100.2 and 118.4%. The extraction recoveries were between 81.6 and 94.1%. The method was linear over the test range, with a 0.9991 coefficient of determination. The limit of detection was 0.1 μg mL$^{-1}$. The validated HPLC-FLD method was successfully applied to quantify the colistin concentrations in 2 patient samples for therapeutic drug monitoring.

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## 1. Introduction

Outbreaks of Gram-negative multidrug-resistant (MDR) bacterial infections, including infections by *Acinetobacter baumannii* and MDR *Pseudomonas aeruginosa*, have been reported in Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan, and Korea in recent years [1,2]. Colistin (polymyxin E), an old lipopeptide antibiotic with known nephrotoxicity and neurotoxicity, was withdrawn from clinical use in the 1970s, but is now being used to treat Gram-negative MDR strains [3]. It is increasingly used as a last-line therapy to combat multidrug-resistant Gram-negative bacteria. Colistin is administered parenterally as colistin methanesulfonate (CMS), which is an inactive prodrug. It is composed of at least 30 different components, with the general structure of a cyclic heptapeptide moiety and a side chain acylated with a fatty acid at the *N*-terminus [4–6]. The two main components are colistin A (polymyxin El) and colistin B (polymyxin E2), which are acylated with 6-methyloctanoic acid and 6-methylheptanoic acid, respectively (Fig. 1).

Due to the neurotoxicity and nephrotoxicity of colistin, it is important to monitor the plasma colistin levels for dose adjustment. According to The Clinical and Laboratory Standards Institute (CLSI), susceptibility for *P. aeruginosa* and *A. baumannii* is defined as an MIC of ≤2 mg L$^{-1}$ [7]. The suggested steady-state plasma concentration of colistin is between 1 and 5 μg mL$^{-1}$ [8]. However, the dosage regimen is still not clear for some special patient

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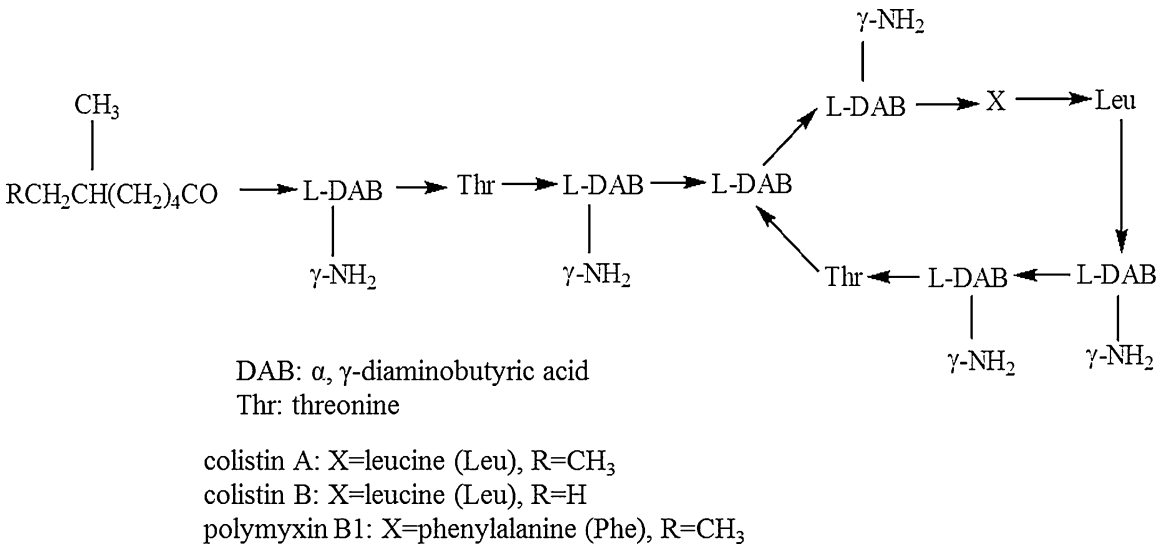


**Fig. 1.** Chemical structures of colistin A, colistin B and polymyxin B1 (internal standard).

populations, such as end-stage renal disease patients, and many recent studies have investigated the pharmacokinetic properties of colistin in these patient populations [9]. Several analytical methods have been established to fulfill the requirement for colistin concentration measurement. Developed methods include enzyme-immuno assay [10,11], microbiological assay [12,13], high-performance liquid chromatography-fluorescence detection (HPLC-FLD) [14–17], CE-laser-induced fluorescence (LIF) [18] and liquid chromatography–mass spectrometry (LC–MS) [4,19–22]. Because colistin is administered as an inactive prodrug, CMS, that is rapidly hydrolyzed to colistin, pharmacokinetic studies using biological assays that lack selectivity are unreliable [23]. Although, LC–MS provides high sensitivity and selectivity, it is still an expensive analytical platform and might not be available in many laboratories. The HPLC-FLD method developed by Li et al. [17] has been widely applied to the analysis of colistin [24–27]. However, our preliminary studies revealed that the lack of selectivity in this method can increase the quantification error.

In this study, we report an HPLC-FLD method for the accurate determination of the colistin concentrations in human plasma samples. We used in-SPE derivatization to simultaneously cleanup and derivatize human plasma samples. Finally, we applied the established method for the quantification of plasma samples obtained from patients undergoing colistin treatment.

## 2. Materials and methods

### 2.1. Standards and reagents

Colistin sulfate, polymyxin B, vancomycin, daptomycin and tecoplanin standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). The colistin standard contained 2 major components with a composition percentage of 26% and 54% for colistin A and colistin B, respectively. 9-Fluorenylmethyl chloroformate (FMOC-Cl purity ≥99.0%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Trichloroacetic acid TCA, hydrochloric acid (HCl), and sodium hydroxide (NaOH) were purchased from Sigma–Aldrich (Steinheim, Germany). LC-grade acetonitrile (ACN), tetrahydrofuran (THF), and sodium hydrogen carbonate were purchased from Merck (Darmstadt, Germany). LC-grade methanol (MeOH) and acetone were purchased from J.T. Baker (Phillipsburg, NJ, USA). Boric acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deionized (DI) water was purified using a Millipore Milli-Q system (Millipore, Billerica, MA, USA).

### 2.2. HPLC-FLD method

All of the LC analyses were performed using an HPLC system (HITACHI, Tokyo, Japan) equipped with a quaternary solvent pump (L-7100) and a degasser (DG-2410) (Uniflows, Tokyo, Japan). A fluorescence detector (L-2485) with an excitation wavelength of 260 nm and an emission wavelength of 315 nm was connected to an AID controller (D-7000) and data processing system (Hitachi, Tokyo, Japan). An Agilent Poroshell 120 EC-C18 2.1 mm × 100 mm (2.7 μm) column (Agilent Technologies, USA) connected to an Atlantis dC18 2.1 mm × 10 mm (3 μm) guard column (Waters, Ireland) with a flow rate of 0.5 mL $min^{-1}$ was used for compound separation. The mobile phase was composed of ACN, THF, and DI (82%, 2%, 16% (v/v), respectively). The injection volume was 25 μL.

### 2.3. Preparation of standard solutions and calibration standards

Stock solutions of colistin (1 mg $mL^{-1}$) and internal standard (IS) (20 μg $mL^{-1}$) were prepared by dissolving in 50% ACN. Working solutions of colistin were prepared from stock solutions by further diluting in 50% ACN. Calibration standards of colistin at concentrations 0.3 μg $mL^{-1}$, 0.5 μg $mL^{-1}$, 1 μg $mL^{-1}$, 2 μg $mL^{-1}$, 4 μg $mL^{-1}$, and 6 μg $mL^{-1}$ were prepared by spiking an appropriate amount of working solutions in blank human plasma obtained from healthy human volunteers. The spiked samples were further subjected to plasma pretreatment protocol.

### 2.4. Plasma sample pretreatment

Two hundred and fifty microliters of human plasma was added into a clean glass tube to which 25 μL of the internal standard (polymyxin B, 20 μg $mL^{-1}$) was added. After vortex-mixing, a mixture of 25 μL of 20% TCA, and MeOH (50:50, v/v) was added for protein precipitation. The sample was then centrifuged at 3000 rpm for 15 min, and 200 μL of the supernatant was transferred into a new glass tube. Then, 10 μL of 1 M NaOH was added to the glass tube. After vortex-mixing, a mixture of 250 μL of 0.01 M HCl and MeOH (50:50, v/v) was added. After vortex-mixing, the sample was subjected to SPE.

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