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A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloroformate in solid-phase extraction cartridges and reversed-phase high-performance liquid chromatography

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Abstract

A simple, selective and sensitive high-performance liquid chromatographic (HPLC) method is described for the determination of colistin in human plasma. Derivatization with 9-fluorenylmethyl chloroformate was performed in the same solid-phase extraction C_{18} cartridge used for sample pre-treatment, followed by reversed-phase HPLC with fluorimetric detection. Quantification was achieved using the ratio of the summed peak areas of colistin A and B derivatives to that of the derivative of netilmicin (internal standard). Linear calibration curves were obtained within the concentrations of colistin sulfate from 0.10 to 4.0 mg/l in plasma. Accuracy was within 10% and reproducibility (RSD) was less than 10%. Crown copyright © 2001 Published by Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Colistin; 9-Fluorenylmethyl chloroformate

1. Introduction

Colistin (polymyxin E), an antibiotic produced by *Bacillus polymyxa* subsp. *colistinus* [1], consists of a cyclic heptapeptide and a side-chain of three amino acids acylated at the N-terminus by a fatty acid (Fig. 1). It is a complex mixture of at least 30 different components [2]. The two main components are colistin A (polymyxin E1) and colistin B (polymyxin E2), which differ only in the fatty acid side chain.

Early clinical experience with the use of colistin in the 1960s for the treatment of serious infections with Gram-negative bacteria was characterized by adverse effects [3]. Consequently, it was superseded by potentially less toxic antibiotics. Recently, however, there has been a resurgence of interest in its use for the treatment of infections caused by some Gram-negative bacteria, especially *Pseudomonas aerugin-*

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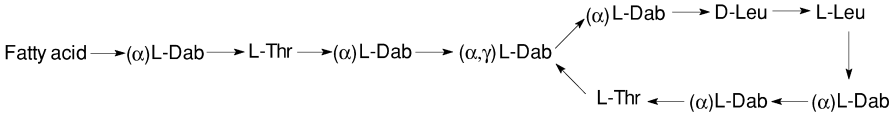

Fig. 1. Chemical structures of colistins A and B. Fatty acid=6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B. Thr: Threonine; Leu: leucine. Dab: α,γ-Diaminobutyric acid. α and γ indicate the respective $-NH_2$ involved in the peptide linkage.

osa, which possess resistance to many of the more commonly prescribed antibiotics [4]. For patients with cystic fibrosis, one of the most common life-shortening genetic disorders in populations of European origin, respiratory infections with *P. aeruginosa* present a significant problem as many strains of this organism are resistant to aminoglycosides and other anti-Pseudomonal antibiotics. Two different compounds for clinical use are produced from colistin, colistin sulfate for oral and topical use, and colistin methanesulfonate (also known as colistin sulfomethate) for parenteral and aerosol therapy. To form colistin methanesulfonate, the primary amine groups on five of the Dab residues (Fig. 1) are derivatized with methane sulfonate moieties. Careful monitoring of plasma colistin levels is recommended because of its potential toxicity [5].

Numerous methods for the assay of colistin have been developed, based on microbiological [6], thin-layer chromatographic (TLC) [7], immunological [8], capillary electrophoretic [9] and high-performance liquid chromatography (HPLC) methods [2,10–14]. The microbiological method has been widely used for clinical monitoring of colistin concentration. Unfortunately, the microbiological method lacks sensitivity, selectivity and requires considerable time (21 h) [6]. The majority of HPLC methods [2,10–12] have been applied to the separation of the components of colistin in batches of raw material. The analysis of colistin in biological fluids by HPLC poses problems because it has very weak ultraviolet absorption and no native fluorescence. Hence, it cannot be quantified in biological fluids with high sensitivity by conventional techniques without derivatization with UV-absorbing or fluorescent reagents.

To the best of our knowledge, only two HPLC methods for the assay of colistin in biological material have been reported, and both used orthophthalaldehyde (OPA) as the derivatizing reagent [13,14]. Because of the instability of OPA derivatives, reaction conditions need to be carefully controlled and automated on-line derivatization is usually required [15]. To minimize those problems, Decolin et al. employed a switching HPLC system for the sequential derivatization and assay of colistin isolated from bovine milk and four different tissues [13]. Le Brun et al. [14] reported a method, based on the method of Decolin et al. [13], for the analysis of colistin in serum, urine and sputum using colistin methanesulfonate as the reference standard. It should be noted that OPA only reacts with primary amine groups and there are no primary amine groups in colistin methanesulfonate. Therefore, in the method reported by Le Brun et al. [14], it is possible that the derivatives with OPA were formed with the primary amine groups of the hydrolysis products of colistin methanesulfonate.

In the present report, a new method is described in which colistin was extracted from human plasma onto a solid-phase extraction (SPE) C_{18} cartridge and derivatized in the same cartridge with 9-fluorenylmethyl chloroformate (FMOC-Cl). Elution from the cartridge was followed by HPLC analysis of the fluorescent derivatives. The method was accurate, reproducible and sensitive.

2. Experimental

2.1. Apparatus

The HPLC system comprised a LC-10AS pump, an SIL-10AD*vp* autoinjector (Shimadzu, Kyoto, Japan) and either an SPD-6A UV spectrophotometric detector (Shimadzu) or a 470 scanning fluorescence detector (Waters, Milford, MA, USA) connected to a multi-instrument data acquisition and data process system (Maxima 820, Waters). Mass spectral analysis was performed with a Perkin-Elmer Sciex API 365 tandem mass spectrometer system (MDS Sciex, Concord, Canada) with an ion-spray source con-

nected to a Harvard syringe pump. All the analyses were carried out at ambient temperature. SPE cartridges (C_{18}, 100 mg) were supplied by J.T. Baker (Phillipsburg, NJ, USA) and Waters.

2.2. Chemicals and reagents

Colistin sulfate and FMOC-Cl were purchased from Sigma (St. Louis, MO, USA), netilmicin sulfate from Schering-Plough (Madison, NJ, USA), ceftazidime from Glaxo Wellcome (Boronia, Australia), meropenem from Zeneca (Macclesfield, UK), aztreonam from Bristol-Myers Squibb (Noble Park, Australia), piperacillin from Lederle Labs. (Baulkham Hills, Australia), ciprofloxacin from AG Bayer (Pymble, Australia), tobramycin from Eli Lilly (West Ryde, Australia), ticarcillin from SmithKline Beecham (Dandenong, Australia), and triethylamine (HPLC grade) from Prolabo (Paris, France). Trichloroacetic acid, boric acid, sodium hydrogen carbonate and sodium hydroxide of analytical grade, and acetonitrile, methanol, tetrahydrofuran and acetone of HPLC grade were all obtained from BDH Laboratory Supplies (Poole, UK). Water was purified by a Millipore Milli-Q system (Millipore, Bedford, MA, USA).

The carbonate buffer (1%, w/w, pH 10) was prepared by dissolving in water the appropriate amount of sodium hydrogencarbonate and adjusting pH with sodium hydroxide (10%, w/w). All solutions were stored at 4°C.

2.3. Plasma sample pre-treatment

The internal standard (20 μl of 5 mg/l netilmicin sulfate in water) was mixed with 250 μl plasma in a 1.5-ml polypropylene tube (Techno-plas, Adelaide, Australia). After adding 50 μl of methanol–10% trichloroacetic acid (50:50, v/v), the mixture was vortex-mixed for 1 min, and centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was transferred to another 1.5-ml polypropylene tube and mixed with 10 μl of 1 *M* sodium hydroxide. After being vortex-mixed for 1 min, 250 μl of methanol–0.01 *M* hydrochloric acid (50:50, v/v) was added followed by vortex-mixing for 1 min. The final solution was delivered to the conditioned SPE cartridge.

2.4. Derivatization in solid-phase extraction cartridge and HPLC analysis

SPE C_{18} cartridges (J.T. Baker) were conditioned with 1 ml of methanol, and equilibrated with 1 ml of carbonate buffer. These steps were performed using a vacuum manifold (Supelco Visiprep 24, Bellefonte, PA, USA) without allowing the cartridges to run dry. All of the final solution from the plasma pretreatment was transferred to a cartridge. After washing the cartridge with 1 ml carbonate buffer, 30 μl FMOC-Cl (100 m*M* in acetonitrile) was added. Following 10 min of reaction, the cartridge was dried by drawing air through under vacuum. The derivatives were eluted with 900 μl acetone, and the eluate mixed with 600 μl boric acid solution (0.2 *M*). After vortex-mixing for 2 min, 50 μl was injected onto a 250×4.6 mm I.D. steel column prepacked with 5 μm Ultrasphere C_{18} (Beckman, Berkeley, CA, USA). A mobile phase of acetonitrile–tetrahydrofuran–water (87:4:13, v/v) was degassed by sonication prior to use and pumped at a flow-rate of 1 ml/min. Fluorescence detection was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm.

2.5. Validation of the colistin assay

2.5.1. Confirmation of identity of the colistin derivatives

An aliquot of colistin sulfate in water (1 g/l) was chromatographed on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–35 m*M* triethylamine (17:83, v/v), adjusted to pH 2.5 with 85% phosphoric acid [13]. The flow-rate was set at 1.5 ml/min and detection was by UV absorption at 210 nm. The eluate fractions corresponding to the two dominant peaks were collected and freeze–dried overnight (Virtis Sentry, New York, NY, USA).

Desalting of the collected HPLC eluates was performed on an SPE C_{18} cartridge (Waters) previously activated with 1 ml of methanol and 1 ml of water. After loading the eluate fractions reconstituted in 3 ml water, the cartridge was washed with 5 ml of water, followed by elution of the components with 1 ml of acetonitrile. Formic acid (0.25%, w/w, 1 ml) was added prior to mass spectral analysis. The electrospray mass spectrometer was operated in the

positive ionization mode and data were collected and processed by Multiview 1.4 operating on a Macintosh G3 computer system.

To identify the peaks from chromatographic analysis of the FMOC derivatives, the collected eluate, corresponding to each of the two main components of colistin, was reacted with FMOC-Cl. In a 10-ml polypropylene test tube (Sarstedt, Adelaide, Australia), 20 μl of the eluate was mixed with 500 μl carbonate buffer and 500 μl FMOC-Cl (1.0 m*M* in acetonitrile), and left to react for 10 min. Excess FMOC-Cl was reacted with 30 μl glycine (0.1 *M*) and, after a further 2 min, boric acid solution (0.2 *M*, 600 μl) was added. An aliquot (10 μl) of the solution was chromatographed on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–tetrahydrofuran–water (87:4:13, v/v) at a flow-rate of 1 ml/min. The derivatives were detected using fluorescence, as described above.

2.5.2. Specificity

Seven antibiotics, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin and ticarcillin, which are commonly co-administered during therapy with colistin, were used to investigate the specificity of the method. These antibiotics (at a concentration of 8.0 mg/l) were individually derivatized and chromatographed as described for colistin in Section 2.4 above.

2.5.3. Linearity, reproducibility and accuracy

Appropriate volumes of three working standards of colistin sulfate in water (1.0, 10 and 50 mg/l) were used to prepare calibration standards in drug-free plasma (obtained from the Red Cross, Adelaide, Australia) containing colistin sulfate at concentrations of 0.10, 0.40, 0.80, 1.0, 2.0, 3.0, and 4.0 mg/l. A linear calibration curve was constructed from the relationship between the ratios of the summed peak areas of colistins A and B to that of netilmicin (internal standard) and concentrations of colistin sulfate. Reproducibility and accuracy were assessed by (1) the intra-day assay with six consecutive analyses of quality control samples of 0.20 mg/l and 2.0 mg/l colistin sulfate in human plasma (the quality control samples were prepared independently), and (2) the inter-day assay with three consecutive analyses of the same quality control samples on separate occasions. The limit of quantification was determined by measuring the concentration of colistin sulfate in six samples of human plasma prepared independently from the calibration standards at a concentration of 0.10 mg/l.

3. Results and discussion

In general, pre-column derivatization with fluorescent reagents provides a simple and sensitive analysis for compounds with poor or no UV absorbance or native fluorescence. FMOC-Cl has been shown to be an ideal reagent for fluorescent derivatization of primary and secondary amines [16]. It reacts rapidly and quantitatively under mild conditions and the resulting derivatives are stable at room temperature for several days. Also the fluorescent derivatives could be detected at the low picomole level [15]. We have developed a new simple, efficient and sensitive method for measuring the concentrations of colistin in human plasma using derivatization with FMOC-Cl, followed by reversed-phase HPLC.

3.1. Derivatization of colistin isolated from human plasma in an SPE cartridge

SPE is an attractive technique that reduces the consumption of, and exposure to, solvent, disposal costs and extraction time. In an effort to simplify sample pre-treatment for the assay of β-phenylethylamine in urine, Pilar et al. described a method utilizing derivatization on solid-phase extraction supports [17]. Decolin et al. reported a method using C_{18} SPE cartridges to extract colistin from bovine tissues [13]. We modified the method for the isolation of colistin from human plasma using an SPE C_{18} cartridge and developed a new method for the efficient derivatization of colistin with FMOC-Cl in the same cartridge.

For the conditioning and equilibration of cartridges, it was found that different volumes of methanol and carbonate buffer had no effect on the recovery and derivatization of colistin. Therefore, 1 ml methanol and carbonate buffer were used routinely to pre-treat the cartridges prior to the addition of plasma. Washing the cartridges with 1 ml carbonate buffer after loading the sample was successful in

removing interfering components originating from plasma. Deproteinization of plasma prior to application on the SPE cartridge resulted in substantially cleaner chromatograms.

FMOC-Cl must be added to the SPE cartridges in as small as possible volume of acetonitrile, to avoid elution of underivatized and derivatized colistin from the cartridge. In our method, addition of FMOC-Cl (100 m*M*) in 30 μl acetonitrile did not cause any breakthrough of underivatized or derivatized colistin from the cartridges. Superior results were obtained with SPE cartridges having a small internal diameter and particle size for the packing. This is probably due to the greater retentive capacity of the packing for the analytes, trapping them in a very narrow zone at the top of the cartridges and allowing the small volume of the derivatizing reagent to react with the majority of the adsorbed colistin.

Within the range of concentrations of colistin sulfate used to construct a calibration curve, different concentrations of FMOC-Cl in acetonitrile were tested. With lower concentrations (<10 m*M*) the fluorescence responses were very low, probably because of the low reaction efficiency. With high concentrations (>150 m*M*), the reaction efficiency decreased and unwanted peaks appeared near those of the colistin derivatives, possibly because the excess reagent reacted with endogenous compounds from plasma. The effect was magnified at low concentrations of colistin sulfate (<0.20 mg/l).

The derivatization reaction generates derivatives less polar than colistin and, hence, acetone was used to ensure efficient elution of the derivatives from the cartridge. The collected extracts were acidified by dilution with 0.2 *M* boric acid, in which the FMOC derivatives of colistin were found to be stable for at least 3 days at ambient temperature (Fig. 2). The derivatives of colistins A and B displayed almost identical stability. The derivative of netilmicin (internal standard) was quite stable even after 2 weeks at room temperature. In the absence of boric acid, the derivatives degraded more than 40% overnight at room temperature. Phosphoric acid and hydrochloric acid were ineffective in stabilising the derivatives, suggesting their stability was not related to pH alone.

Fig. 3 shows representative chromatograms of blank human plasma, the same plasma spiked with 0.10 mg/l colistin sulfate and a sample taken from a

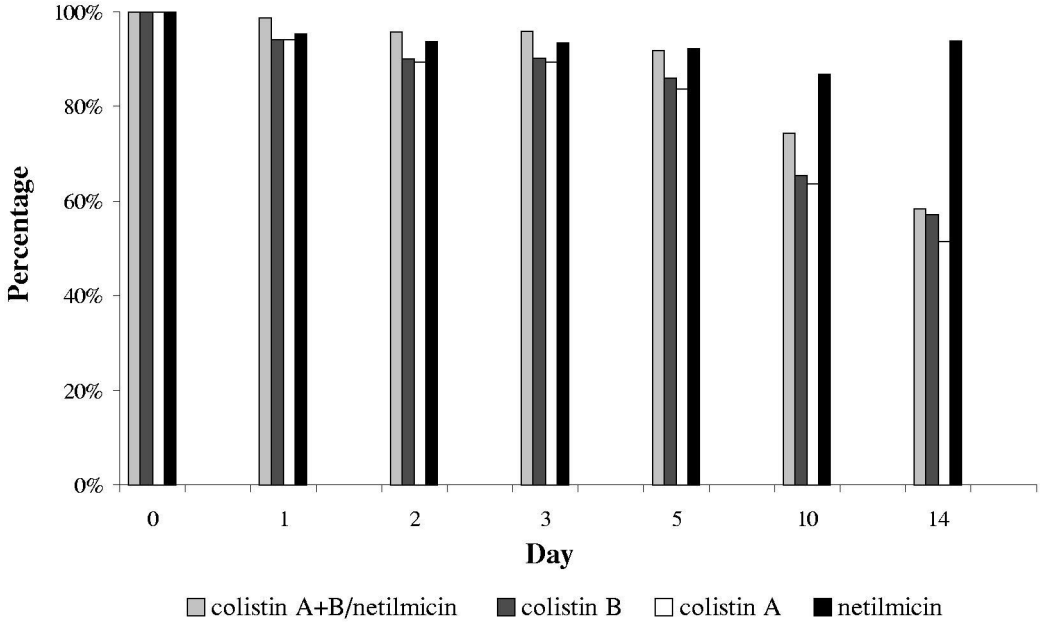

Fig. 2. The stability of the derivatives of colistin and netilmicin in a solution of acetone–0.2 *M* boric acid (9:6) at room temperature. Values are expressed as a percentage of peak area at time zero.

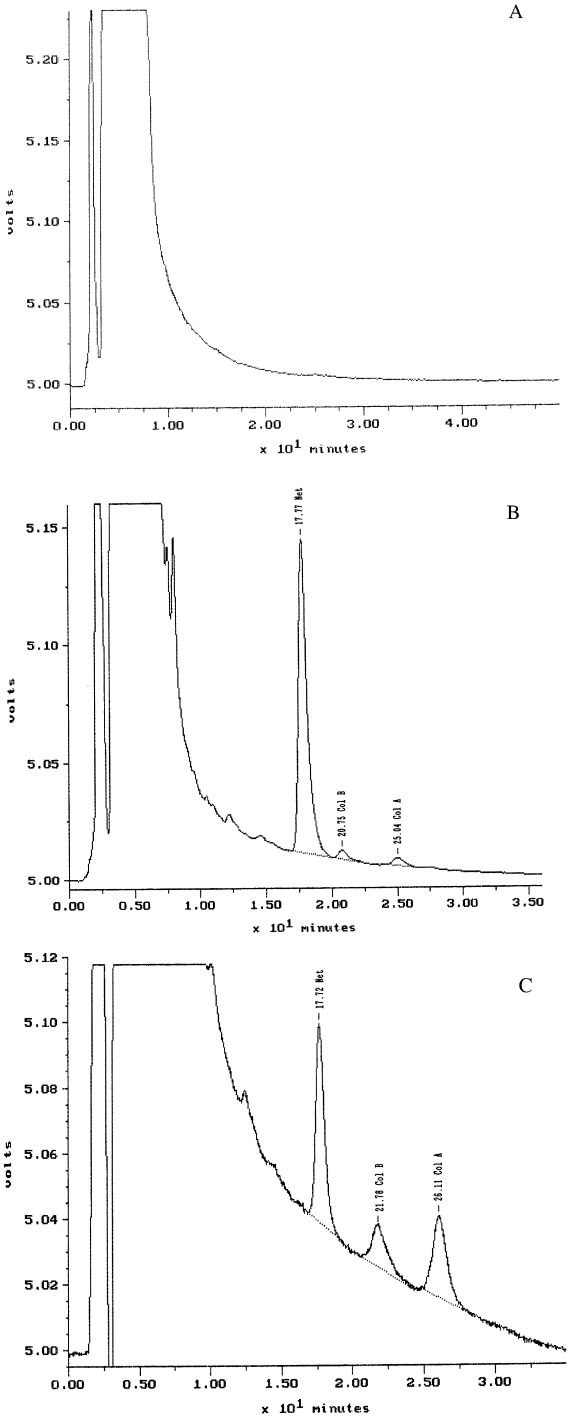

Fig. 3. Typical chromatograms obtained with fluorescence detection for blank human plasma (A), the same plasma spiked with 0.10 mg/l colistin sulfate (B), and a sample from a patient with cystic fibrosis (C). For experimental details, see text.

patient with cystic fibrosis 1 h after intravenous bolus administration of 1.54 mg/kg colistin methanesulfonate (Parke-Davis, Australia). The derivatives of netilmicin, colistin B and colistin A were well separated. The identity of the derivatives of colistin B and A was confirmed as described in the following section. There was no chromatographic interference to the derivatives of netilmicin, colistin A and B from endogenous compounds or reaction by-products.

3.2. Validation of the colistin assay

3.2.1. Confirmation of identity of the colistin derivatives

The two major derivative peaks were identified indirectly. With UV detection at 210 nm, two main components of colistin were well resolved on the Ultrasphere C_{18} column with a mobile phase of acetonitrile–35 m*M* triethylamine (17:83, v/v), adjusted to pH 2.5. Phosphoric acid in the freeze–dried eluates was removed by SPE C_{18} cartridges (Waters) prior to mass spectral analysis, otherwise the signals from phosphate predominated and no peaks from the derivatives of colistin were observed.

The positive ion electrospray mass spectral analysis of the reference sample of colistin (Sigma) showed there are two main components in the sample of colistin mixture (Fig. 4A). The mass spectra of the individual components collected by HPLC and dissolved in formic acid solution, contained ions with *m*/*z* 1156.0 (Fig. 4B) and 1170.0 (Fig. 4C), respectively. These were consistent with the protonated molecular ions of colistin B (theoretical mass 1155.76) and colistin A (theoretical mass 1169.77). Hence, in the component analysis of underivatized colistin, the first peak corresponded to colistin B and the second to colistin A.

There were also only two major peaks in the HPLC chromatograms of the FMOC derivatives of colistin, suggesting only one derivative was produced for each component. Their identification was performed by reacting colistin A or B with FMOC-Cl separately. The first peak corresponds to the derivative of colistin B and the second to that of colistin A (Fig. 3).

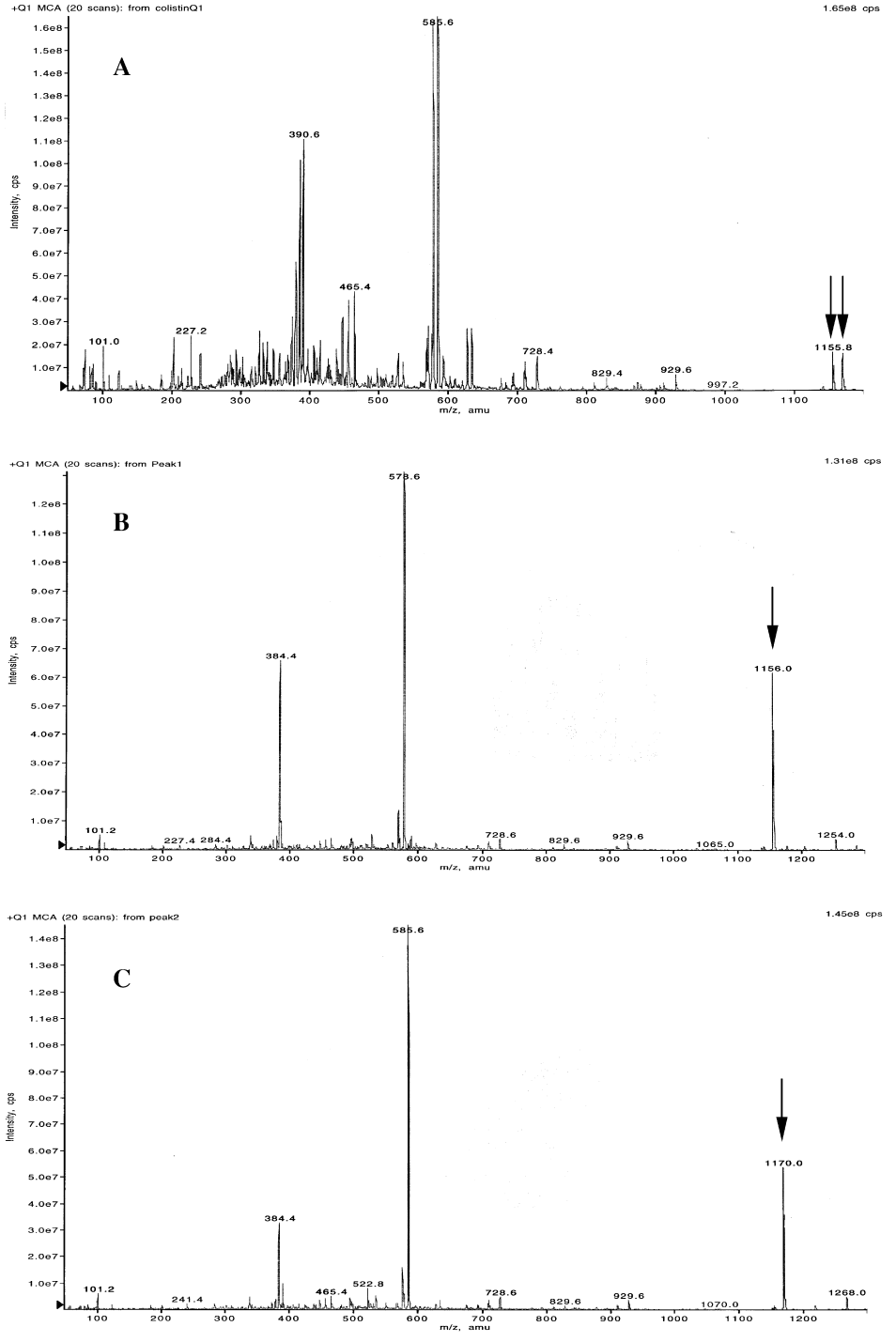

Fig. 4. Mass spectra of the colistin sulfate mixture (A), colistin B (B), and colistin A (C) obtained by electrospray ionization MS (ESI-MS). Arrows show the protonated molecular ion $[M+H]^+$ of colistin A and B.

3.2.2. Specificity

In patients with cystic fibrosis, ceftazidime, meropenem, aztreonam, piperacillin, ciprofloxacin, tobramycin or ticarcillin, may be co-administered with colistin to treat infections caused by *P. aeruginosa*. None of the compounds interfered with the chromatographic analysis of the derivatives of colistin and netilmicin.

3.2.3. Linearity, reproducibility, and accuracy

An internal standard (netilmicin) was used in our method to correct for potential losses of sample during sample pretreatment and errors that may be caused by instrumental variations. This is the first report employing an internal standard for the assay of colistin in biological matrices.

High coefficients of determination ($r^2 > 0.998$) were obtained for calibration curves with concentrations ranging from 0.10 to 4.0 mg/l. The linear regression equation for the relationship between the ratios of the summed peak areas of colistins A and B to that of netilmicin (internal standard) and the concentrations of colistin sulfate had a mean ($\pm$SD) slope of 0.789 ± 0.032 and an intercept of -0.010 ± 0.007 ($n=6$). A recovery of $107 \pm 12\%$ ($n=3$) was calculated when comparing the responses from plasma with those from water spiked at 1.0 mg/l colistin sulfate. The limit of quantification was 0.10 mg/l, at which concentration the mean value measured was 0.10 mg/l and the relative standard deviation (RSD) 9.14% ($n=6$). Inter-day reproducibility and accuracy were determined by conducting assays on 3 consecutive days of plasma containing 0.20 and 2.0 mg/l colistin sulfate as quality controls on separate occasions; determination of intra-day reproducibility and accuracy involved six assays of the same quality controls. The results, shown in Table 1, confirm the reliability of the method for measuring concentrations of colistin sulfate (0.10–4.0 mg/l) in human plasma.

4. Conclusion

We have developed and validated a novel HPLC method for the assay of colistin in human plasma. The method involves extraction of colistin from plasma onto SPE cartridges, followed by rapid and quantitative derivatization under mild conditions on the same cartridge with FMOC-Cl. The method is simple, convenient, and avoids the need for automatic equipment for derivatization and subsequent column switching. To our knowledge, this is the first report of an HPLC method for the analysis of colistin in human plasma using FMOC-Cl as the derivatizing reagent.

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Table 1
Reproducibility and accuracy for the assay of colistin sulfate in human plasma

	Intra-day ($n=6$)		Inter-day ($n=3$)	
	0.20 mg/l	2.0 mg/l	0.20 mg/l	2.0 mg/l
Mean concentration (mg/l)	0.18	2.0	0.18	2.1
RSD (%)	4.5	3.6	8.4	2.2

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