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A Simple HPLC Method for the Separation of Colistimethate Sodium and Colistin Sulphate

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Abstract

In this paper, a simple high performance liquid chromatography (HPLC) method for the determination of colistimethate sodium in the productions of synthesis from colistin E sulphate was established. A HPLC gradient which was: 20% B+80% A changed to 50% B+50% A in 10 min (A was 0.05% TFA aqueous solution and B was acetonitrile) was used and the separation was realized in 8 minutes. Moreover, this method was also used in the separation of colistin sulphate with good resolution. Compared to the methods reported previously, the present method was finished easier in a much shorter time which is 6 min. LC-MS was used to detect colistin sulphate and the result showed that the two compositions were colistin sulphate E1 and E2 as expected. Good separation and reproducibility were obtained.

Keywords: Colistimethate sodium; HPLC; Gradient; Colistin sulphate

Introduction

Colistin, also known as polymyxin E, a polymyxin antibiotic, comprised of colistin E_1 and colistin E_2, has good inhibitory effect for pathogen infection [1,2]. The characteristic lariat structure of colistin E_1 was proven necessary for antimicrobial activity [3]. Since the sulfate polymyxin compounds were separated from different types of bacillus polymyxa in 1947, they have been tested by vitro experiments. In P.aeruginosa, it has been proven that high-level resistance can arise from adaptation in the presence of colistin E_2 in vitro [4,5]. There is cross-resistance between colistin E_1 and colistin E_2 [6]. However its application has not been popularized because of its toxicity and other side effects. But the toxicity and side effects of colistimethate sodium (CMS) will be depressed without drug action diminishing. Colistimethate sodium is synthesized by treating the primary amine groups of the α, γ-diaminobutyric acid residues in colistin with formaldehyde followed by sodium bisul-fite [7]. There are two main compositions in the product which are colistimethate sodium E_1 (CMS E_1) and colistimethate sodium E_2 (CMS E_2). The structures of colistin sulphate and CMS are shown in Figure 1.

The studies of colistin in separation and determination are less than that in pharmacodynamics, especially as compared to CMS. There are several HPLC methods for the separation of colistin sulphate reported previously [8,9] in which the retention time of the colistin was more than 12 min. But for the determination of CMS, there has no direct HPLC method till now, while the usual analytical method is the microbiological assay. Jian Li etc. [10] separated the CMS in plasma and urine in conjunction with HPLC by hydrolyzing CMS using sulfuric acid. This method was very cumbersome and time consuming (retention time were 14 min and 12 min for colistin E_1 and colistin E_2, respectively). There is no HPLC method for direct determination of CMS reported, and HPLC method is not found in European Pharmacopoeia or in American Pharmacopeia. The present paper has developed a simple HPLC/ LC-MS method for the separation of colistimethate sodium and colistin sulphate.

Materials and Methods

Chemicals

Colistin sulphate was purchased from Zhejiang Qianjiang Biochemical Co. Ltd. Colistimethate sodium was synthesized by treating the primary amine groups of the α,γ-diaminobutyric acid residues in colistin with formaldehyde followed by sodium bisul-fite in our lab. Standard preparation of colistimethate sodium was purchased from USP Rockville. MD. LOT, NO 1147009. Acetonitrile and trifluoroacetic acid (TFA) were purchased from Kemio Chemical Reagent Co. Ltd. All these chemicals were analytical reagent grade except for the chromatographic grade acetonitrile. Triply distilled water was used for all experiments.

HPLC and LC-MS conditions

HPLC and LC-MS instrument: An 1100 HPLC system from Agilent Technologies (Shanghai, China) consisted of a quaternary pump with an online vacuum degasser, an autosampler with variable injection capacity from 0.1 µL to 100 µL and an UV detector was applied to chromatographic studies. A LC/MSD Trap XCT instrument with electrospray ionization (ESI) source which was bought from Agilent Technologies (Shanghai, China) was used.

Chromatographic separations of colistin sulphate and colistimethate sodium were achieved on the C_{18} column (Optimapak, 150mm × 4.6 mm i.d.). All sample solutions were filtered through a millipore membrane (0.45 µm) to remove particles and large aggregates. Agilent liquid chromatography system chemical software was used for data acquisition and integration. The UV detector was set at 214 nm and the temperature was ambient temperature. The sample injection volume of the autosampler was 5.0 µL.

HPLC procedure: 5 mg of colistin sulphate and colistimethate sodium were weighted separately and solved in 2.0 mL of 0.05% TFA

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aqueous solution, and then diluted with 8.0 mL of acetonitrile to get the concentration of 0.5 mg/mL, respectively. 5 μL of the samples were injected into the HPLC system, respectively. The chromatographic separation was performed using a linear gradient (20% B (acetonitrile) +80% a (0.05% TFA aqueous solution) changed to 50% B+50% an in 10 min).

This procdure was repeated 7 times to obtain the RSD of retention times, resolution, symmetry factor and theoretical plates.

LC-MS experiment

The LC/MSD Trap XCT ion trap mass spectrometer (Agilent technology, America) was equipped with an electrospray (ESI) source with a nebulizer spacer. The ESI settings were a capillary voltage of 3500V; a drying gas flow of 8 L/min at a temperature of 350ºC, and a nebulizer pressure of 25 psi. The trap parameters were set at a smart target of 200–2000 m/z. Positive mode was applied.

Colistin sulphate which was prepared in section 2.2.2 was diluted to 5 μg/mL. The mobile phase which was used in this section was the mixture of A (0.05% TFA aqueous solution), B (acetonitrile) and C (3% ammonia aqueous solution). The gradient was: 19.5% B + 77.5% A + 3% C changed to 48.5% B + 48.5% A + 3% C in 10 min.

Results and Discussion

Selection of mobile phase

In general, mobile phase that was used in the separation of colistin are usually acetonitrile and aqueous solution. Acetonitrile, methanol and avantin were also tested as mobile phase (B). Ion pair agents, for example, TFA, tetrabutylammonium bromide, 1-dodecanesulfonate acid sodium salt were tested as mobile phase (A) to mix with organic solvent (B) to separate the samples.

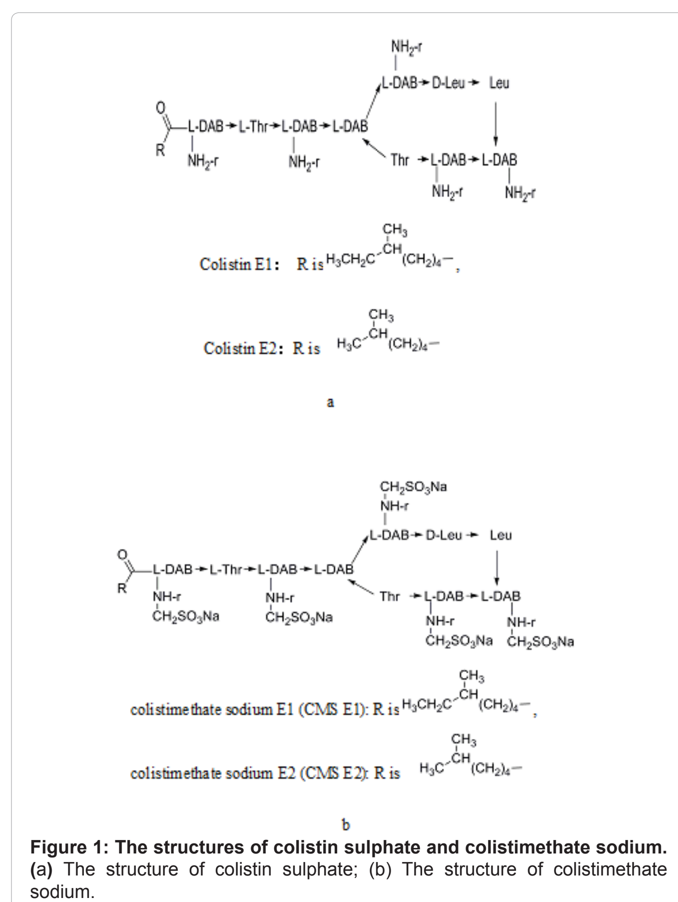

Figure 1: The structures of colistin sulphate and colistimethate sodium. (a) The structure of colistin sulphate; (b) The structure of colistimethate sodium.

No	Chromatographic condition	Parameters of absorption peaks	Estimate of the results
1	30%B+70%A changed to 45%B+55%A in 10 min	retention time: 1.946 resolution: 1.210 symmetry factor: 0.951 theoretical plate: 3408	The retention time is short, and a certain degree of separation is obtained.
2	27%B+73%A changed to 45%B+55%A in 10 min	retention time: 3.051 resolution: 1.365 symmetry factor: 0.996 theoretical plate: 4068	The retention time is short and a certain degree of separation is obtained.
3	20%B+80%A changed to 50%B+50%A in 10 min	retention time: 6.403 resolution: 1.610 symmetry factor: 0.993 theoretical plate: 4745	The retention time is proper, and good resolution is obtained.
4	30%B+70%A , isocratic elution, 0-15 min	The sample is remained in the column.	The sample is retained in the column
5	35%B+65%A, isocratic elution, 0-15 min,	retention time: 2.152 resolution: 1.214 symmetry factor: 0.966 theoretical plate: 2055	The retention time is too short, and the resolution is not good.

Table 1: The chromatographic conditions and results. A is 0.05% TFA aqueous solution and B is acetonitrile. The sample which was injected into the HPLC system in this study is CMS (0.5 mg/mL). The parameters are the values from measurements of CMS E_1.

According to the results, a mixture of acetonitrile and TFA aqueous solution could lead to a good separation. Therefore, the mixture of acetonitrile and TFA aqueous solution was selected as the mobile phase.

When 30% B+70% A were used as mobile phase, there was no chromatographic peak obtained. When 35% B+65% A were used as the mobile phase, the retention time of CMS E_1 was 2.152 min which is too short to get baseline separation with other peaks. Therefore, gradient elution was studied and the results were shown in Table 1. Moreover, the concentration of TFA in the water was studied and its optimal concentration was 0.05%.

The results in Table 1 showed the condition 3 was the best chromatographic condition for separation of colistimethate sodium. This gradient was also used to separate colistin sulphate, and good resolution (1.61) was obtained in a short time which was 6 min.

The RSD (n=7) of retention time and resolution were 0.02%, respectively. RSD (n=7) of symmetry factor and theoretical plates are 0.01%, respectively. Good reproducibility was obtained.

Chromatograms of the samples

Chromatograms of colistin sulphate and colistimethate sodium under the optimizing condition were shown in Figure 2. The chromatogram of colistimethate sodium was compared with the chromatogram of standard preparation and the second peak was CMS E1.

The LC-MS spectra of colistin sulphate

The colistin sulphate was also detected with LC-MS under the condition which was demonstrated in section 2.3. The spectra were shown in Figure 3. According to the results can we know that there were two components in the sample which were colistin sulphate E1 ($(M+2H)^{2+}$=585.5) and colistin sulphate E2 ($(M+2H)^{2+}$=578.5). The results of Figure 3 confirmed that the present method was a simple and rapid method to separate colistin sulphate from the mixture of the sulphates of polypeptides which produced by certain strains of *Bacillus polymyxa* var. *colistinus* or obtained by any other means.

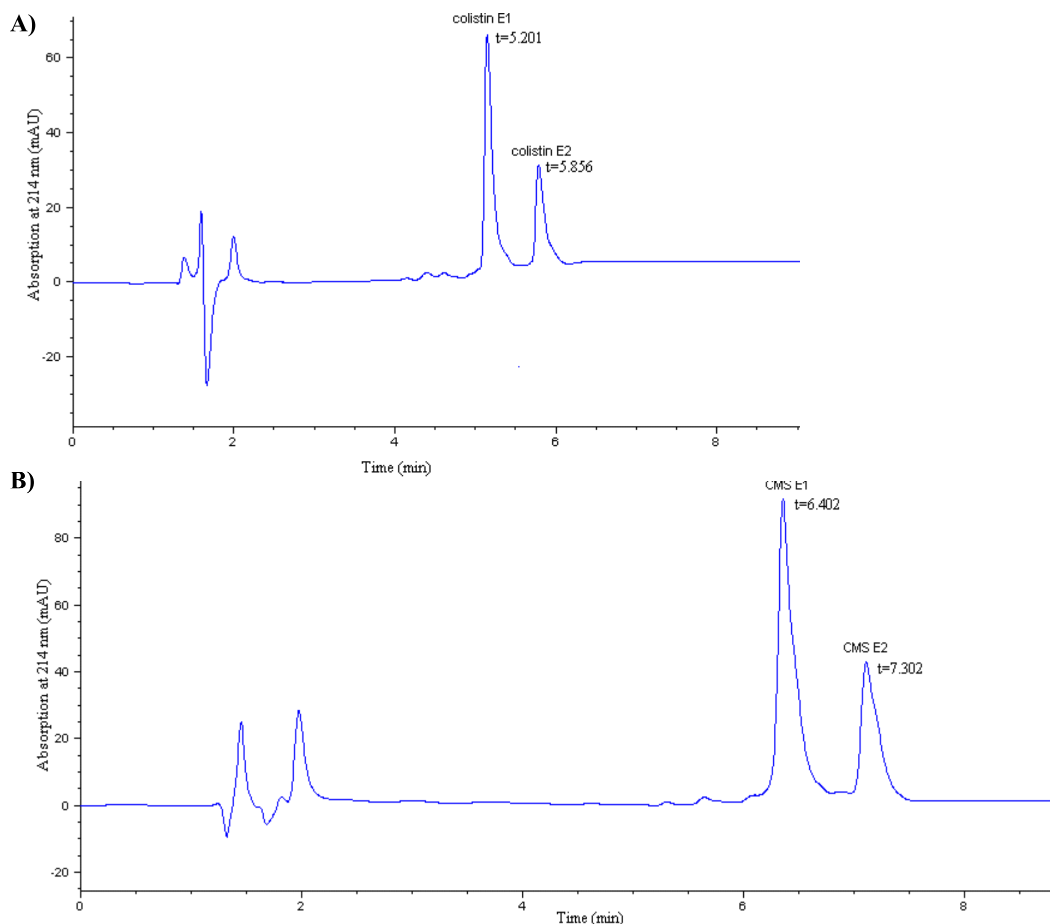

Figure 2: The chromatograms of colistin sulphate and colistimethate sodium (CMS). The liner gradient was: 20% B (acetonitrile) + 80% A (0.05% TFA aqueous solution) changed to 50% B+50% A in 10 min. 5 μL of the samples were injected into the HPLC system, respectively. The UV detector was set as 214 nm. (a) The chromatograms of colistin sulphate; (b) The chromatograms of CMS).

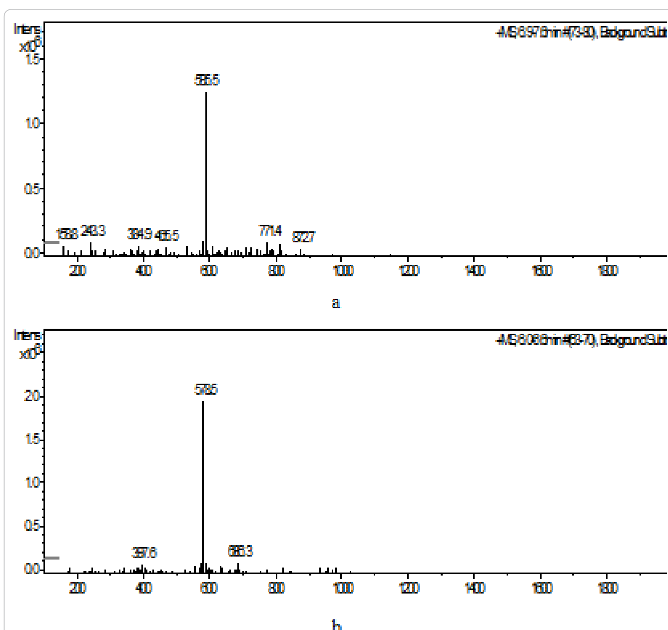

Figure 3: The LC-MS spectra of colistin sulphate. ESI and positive mode were used in this experiment. (a) The MS spectrum of colistin sulphate E1$(M+2H)^{2+}$ = 585.5; (b) The MS spectrum of colistin sulphate E2 $(M+2H)^{2+}$ = 578.5).

Conclusion

A very simple HPLC method for the separation of colistin sulphate and colistimethate sodium was established successfully. Gradient mode was used in this work to separate colistimethate sodium and colistin sulphate within 8 min. For CMS, this method can be used to separate the analogs of colistin sulphate and colistimethate sodium.

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