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Note

High-performance liquid chromatography of polymyxin B sulfate and colistin sulfate

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Polymyxin B sulfate and colistin (polymyxin E) sulfate are potent antimicrobial antibiotics used chemotherapeutically in the treatment of infections caused by gram-negative bacteria, particularly the *Pseudomonas aeruginosa* and *Escherichia coli* pathogens[1]. Each antibiotic is a mixture of several closely-related polypeptides obtained from cultures of *Bacillus polymyxa*[2]. The major constituents of polymyxin B are polymyxin B_1 and B_2, while colistin is mainly composed of polymyxin E_1 and E_2 (Fig. 1).

Several assay methods have been reported for determining polymyxin B and colistin. Microbiological methods are officially accepted[3]. These, however, as well as numerous colorimetric procedures[4–6] described for polymyxin B sulfate, lack specificity and capability to demonstrate the composition of these multicomponent drugs. Recently, significant progress has been made in this direction by the application of the technique of high-performance liquid chromatography (HPLC) to the analysis of various polymyxin substances. Tsuji and Robertson[7] analyzed polymyxin B and colistin by gradient elution on a μBondapak C_{18} column using a mobile phase consisting of acetonitrile and methanol in a pH 2.0 phosphate buffer. Terabe *et al.*[8] reported the use of ion-pair reversed-phase chromatographic conditions to study the relationship between the sequence of elution and structure of 17 decapeptide antibiotics of the polymyxin group and of 9 octapeptide antibiotics. In this work, a laboratory-packed Nucleosil 5 C_{18} column was employed with a mobile phase consisting of tartrate buffer, acetonitrile, sodium 1-butanesulfonate and sodium sulfate. Also, the analysis of polymyxin B was reported by Fong and Kho[9] using an in-house packed Hypersil-ODS column and a multicomponent mobile phase consisting of tetramethylammonium chloride, sulfuric acid, potassium phosphate and acetonitrile in water.

These published HPLC chromatographic systems are associated with one or more of the following disadvantages that inhibit facile adoption of the methods for routine application: (a) expensive and complex gradient solvent delivery system; (b) complicated multicomponent mobile phases that compromise polymyxin component resolution and detection, (c) laboratory prepared columns that may not be packed reproducibly or efficiently and (d) long analysis times of approximately 25 min.

This paper reports the development of a simple, rapid, sensitive and specific HPLC method for the analysis of polymyxin B and colistin. Using a commercially available, prepacked Altex Ultrasphere ion-pair column and an acetonitrile–phos-

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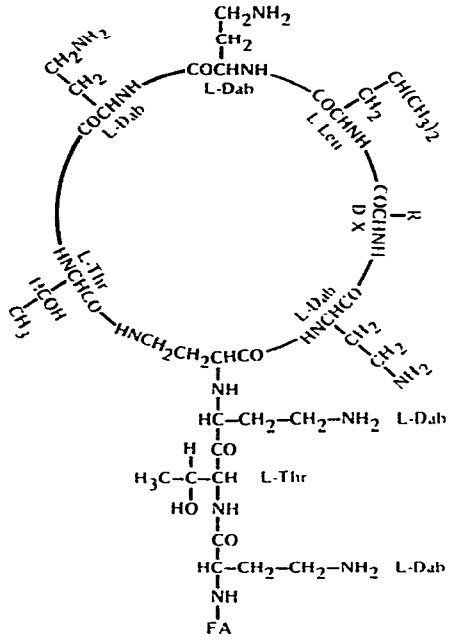


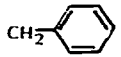
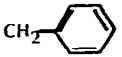
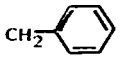
	FA	R	X
Polymyxin B_1	MOA	CH_2– 	Phe
Polymyxin B_2	IOA	CH_2– 	Phe
Polymyxin B_3	OA	CH_2– 	Phe
Polymyxin E_1	MOA	$CH_2CH(CH_3)_2$	Leu
Polymyxin E_2	IOA	$CH_2CH(CH_3)_2$	Leu

Fig. 1. Structures of known members of the polymyxin group. FA = fatty acid, MOA = (+)-6-methyloctanoic acid, IOA = isooctanoic acid (6-methylheptanoic acid), Phe = phenylalanine, Leu = leucine, Dab = α,γ-diaminobutyric acid, Thr = threonine.

phate buffer mobile phase, superior resolution of polymyxin components is achieved isocratically within an analysis time of 15 min. This method can be considered to be a suitable candidate for future routine quality control use.

EXPERIMENTAL

Polymyxin B sulfate reference standard (lot No. 8J272-61QCS) was supplied by Pfizer (Quality Control Division, Brooklyn, NY, U.S.A.). Polymyxin B sulfate reference standard (lot No. 0974-H) was purchased from the US Pharmacopeial Convention, (Rockville, MD, U.S.A.). The reference sample of colistin sulfate was kindly provided by Dr. F. A. Hochstein of Pfizer Diagnostic Division (Groton, CT,

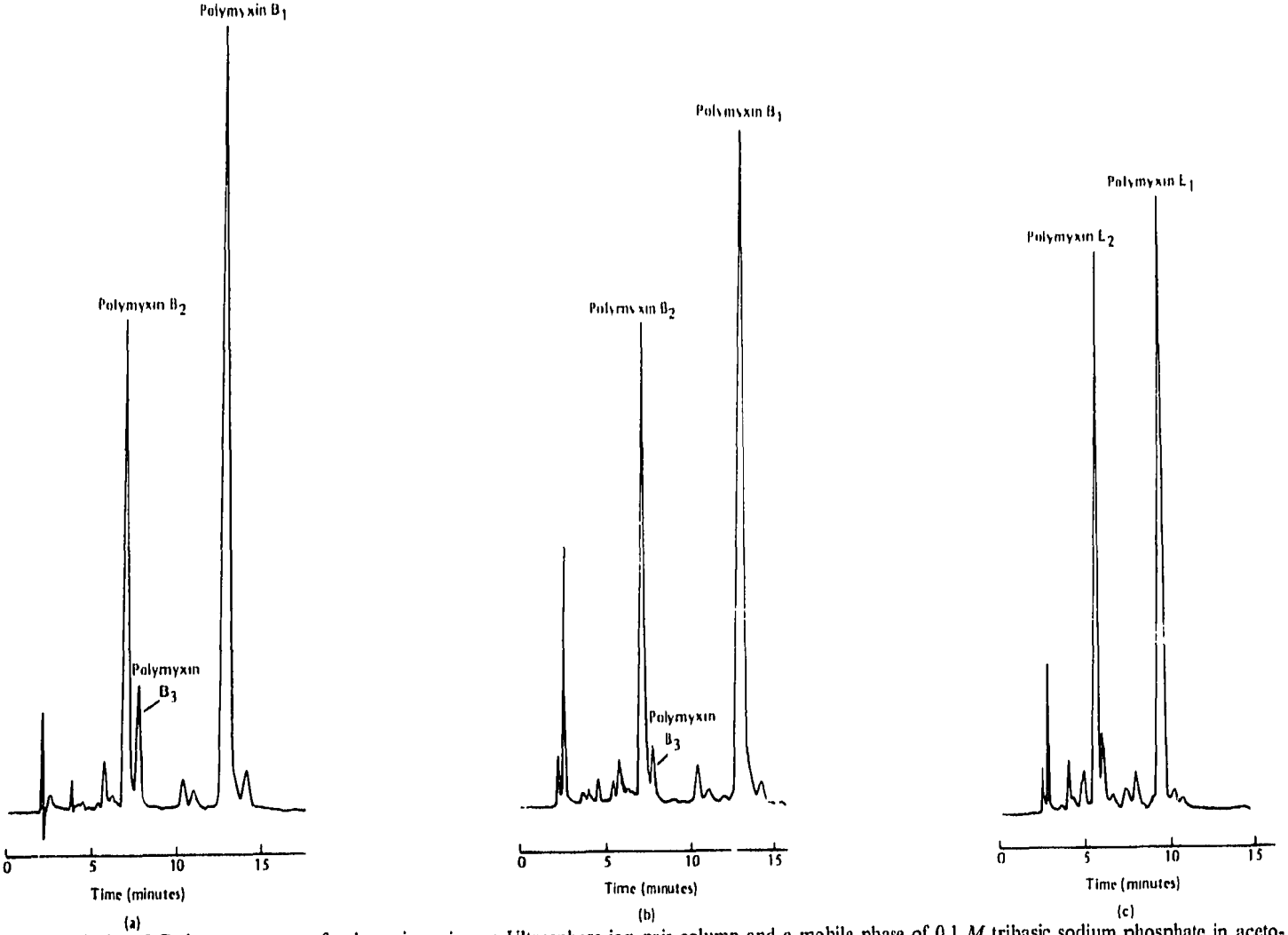


Fig. 2. Typical HPLC chromatograms of polymyxins using an Ultrasphere ion-pair column and a mobile phase of 0.1 *M* tribasic sodium phosphate in acetonitrile–water (23:77, v/v), pH 3.0. Samples (1.0 μg application): (a) USP polymyxin B sulfate (lot No. 0974-H). (b) Pfizer polymyxin B sulfate (lot No. 8J2 72-61QCS), (c) colistin sulfate. Detection at 200 nm and 0.1 a.u.f.s.

U.S.A.). Distilled water, acetonitrile distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and sodium phosphate tribasic (Mallinckrodt, Paris, KY, U.S.A.) were used without further purification.

Apparatus

A Waters M6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with Valco Model CV-UHPa-C-20 10-μl loop injection valve (Valco Instruments, Houston, TX, U.S.A.) and a Waters 450 variable-wavelength detector attached to a Varian A-25 single channel recorder (Varian Instruments Division, Palo Alto, CA, U.S.A.) were employed.

Column

An Altex Ultrasphere ion-pair column (4.6 × 250 mm, particle size 5 μm) distributed by Rainen was used as received.

Mobile phase preparation

The mobile phase was prepared by dissolving 38.0 g of sodium phosphate tribasic in a mixture of 700 ml of distilled water and 230 ml of acetonitrile. The solution was adjusted to pH 3.0 with phosphoric acid, diluted to 1 l with distilled water and filtered through a 5-μm, Type LS, Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use.

Chromatographic conditions

The column flow-rate was maintained at 1.0 ml/min (inlet pressure *ca.* 1500 p.s.i.). The column temperature was maintained at 27°C. The detector wavelength was either set at 200 nm or 185 nm using a sensitivity of 0.1 or 0.04 a.u.f.s., respectively, at a recorder input of 10 mV. The recorder chart speed corresponded to 10 in./h.

Procedure

Solutions containing varying amounts of either polymyxin B sulfate or colistin sulfate (0.01–0.1 mg/ml) were prepared in mobile phase and injected into the HPLC system for analysis.

RESULTS AND DISCUSSION

Employing the above chromatographic conditions, polymyxins B_1, B_2, B_3, and related components are well resolved in less than 15 min (Fig. 2a and b). Similarly, polymyxins E_1 and E_2 and other sample components of colistin sulfate are separated within 12 min (Fig. 2c). The Ultrasphere ion-pair column efficiency was satisfactory as evidenced by theoretical plates (N)* on the order of 25,000 per meter for both polymyxins B_1 and E_1. A resolution factor (R_s)** of 1.5 for polymyxins B_2 and B_3 demonstrated that resolution of these two components was complete. The coefficient

* $N = 5.54\ (t_R/W_{\frac{1}{2}})^2$, where N = number of theoretical plates, t_R = peak retention time, and $W_{\frac{1}{2}}$ = peak width at half peak height.

** $R_s = 2\ (t_{R2} - t_{R1})/(W_1 + W_2)$, where W = peak width at the base.

of variation for replicate injections of either antibiotic as measured by the major component peaks (*i.e.* polymyxins B_2 and B_1 or polymyxins E_2 and E_1) was better than $\pm 1.0\%$. Polymyxin B sulfate and colistin sulfate linearity was observed over a convenient analysis concentration range of 0.01 to 0.1 mg/ml. Sensitivity of detection for both polymyxin B sulfate and colistin sulfate was established to be approximately 30 ng per injection using a detector wavelength of 185 nm. A representative chromatogram is depicted in Fig. 3. The Ultrasphere ion-pair column life is easily in excess of six months under routine polymyxin B sulfate analysis conditions.

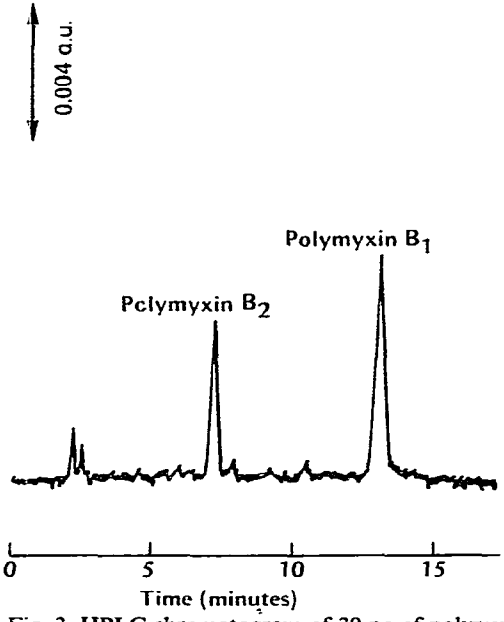


Fig. 3. HPLC chromatogram of 30 ng of polymyxin B sulfate at a 185 nm detector wavelength using an Ultrasphere ion-pair column and a mobile phase of 0.1 *M* tribasic sodium phosphate in acetonitrile–water (23:77, v/v), pH 3.0.

The HPLC conditions described above offer the following distinct advantages over previously reported HPLC chromatographic conditions:

(1) Rapid analysis time of 15 min or less employing isocratic elution.

(2) Simple, inexpensive two-component mobile phase which permits detection of nanogram quantities of polymyxins.

(3) Use of a reproducibly prepacked commercially-available HPLC column of high column efficiency and long lifetime.

The equivalency of the above described HPLC procedure with the microbiological assay is under study.

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