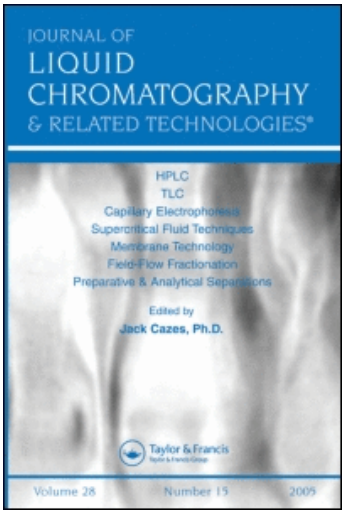

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IMPROVED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF CYCLIC POLYPEPTIDE ANTIBIOTICS---POLYMYXINS B ---AND ITS APPLICATION TO ASSAYS OF PHARMACEUTICAL FORMULATIONS

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ABSTRACT

An isocratic high performance liquid chromatographic (HPLC) method for the analysis of polymyxins B_1 and B_2 is described. The method uses a 25 cm Hypersil-ODS column, a mobile phase containing 22.5% acetonitrile (v/v) in an aqueous phase with tetramethylammonium chloride (TMAC), a flow rate of 1.09 ml/minute and a wavelength of 220 nm for detection. Complete resolution of B_1 and B_2, and their separation from all other components and/or impurities have been achieved in less than 23 minutes. The capability of the method for the determination of the polymyxin content in pharmaceutical preparations has also been demonstrated.

INTRODUCTION

Polymyxins, a group of closely related cyclic decapeptides, have been known as effective antibiotics since the period between 1947 and 1950. They have been considered mostly as 'minor antibiotics', although they are actually the most active inhibitors of the growth of gram-negative bacteria (1).

Recently, improvements in analytical and synthetic methods of polypeptides have allowed the various aspects of the chemistry of

this group of antibacterial substances to be revealed (1-4). Several reviews (5-8) exist dealing mainly with biological and clinical aspects.

Various analytical methods such as countercurrent distribution (9,10), column chromatography (11,12), thin layer chromatography (13), and gradient elution high performance liquid chromatography (HPLC) (14,15) have been used to separate various components of polymyxins. A proposed colorimetric assay method using an α-amino carboxylic acid with sodium hypobromite for determination of polymyxins suffers from non-specificity and incapability of differentiating various components of polymyxins (16). A microbiological assay method (17) has been adopted as the official analytical method, but there is limitation also.

The two components of polymyxin B, namely B_1 and B_2 have been resolved by gradient elution HPLC (15). But a survey of the literature shows that no simple, isocratic HPLC procedure, which is highly desirable for laboratory automation of routine assays, has ever been reported. The purpose of this paper is to report our current development in this area.

EXPERIMENTAL

Instrumentation

The HPLC system employed in this work included a Laboratory Data Control Constametric II Model pump (Riviera Beach, Fla.), a Micromeritics Auto-injection system (Norcross, Ga.), a Schoeffel Spectroflow SF770 variable wavelength UV detector (Westwood, N.J.), a Linear Instruments double-channel chart recorder (Irvine, Ca.) and a Spectra-Physics System IV integration system (Santa Clara, Ca.).

Reagents and Materials

Acetonitrile and chloroform, distilled-in-glass grade, were obtained from Burdick and Jackson Lab. Inc., (Muskegon, Mich.). Sulfuric acid, reagent grade, was obtained from J. T. Baker (Phillipsburg, N.J.). Tetramethylammonium chloride (TMAC), was

purchased from Eastman Organic Chemicals (Rochester, N.Y.). Hypersil-ODS (5 μm) bulk packing material was purchased from Shandon Southern Instruments, Inc. (Sewickley, Pa.). Empty stainless steel column, 316 grade, was obtained from Alltech Assoc. (Arlington Heights, Ill.). Polymyxin B Sulfate, USP Reference Standard was obtained from USP (Rockville, Md.). Samples of a liquid formulation containing polymyxin B sulfate were supplied by Ayerst, McKenna & Harrison, Inc.,(Montreal, Quebec, Canada). All other chemicals and solvents used in this work, unless otherwise stated, were obtained from Burdick and Jackson Labs.

PROCEDURE

(i) Column - A Hypersil-ODS (5 μm) column (250 x 3 mm I.D.) was slurry-packed in-house using a homemade packer operated in the downward mode. Slurry was prepared in methanol by sonifying about 2.2 gm of the packing material for 15-20 minutes. This was quickly transferred into the packing reservoir with a 20 ml syringe, followed by a quick fill-up of the packing reservoir with hexane. The packing solvent was hexane pressurized to 8,000 psi with a Haskel pump (Burbank, Ca.). The column was conditioned with hexane at 8,000 psi for 30 minutes, then washed with methanol, water, and finally equilibrated with the mobile phase for about 90 minutes at the flow rate used for this work.

(ii) Mobile Phase - This was prepared as follows: 2 ml concentrated sulfuric acid and 5 gm of tetramethylammonium chloride (TMAC) were measured into a 1000 ml volumetric flask, which was then filled with distilled water to the mark. The resulting pH of the aqueous solution was 1.6. The pH could be readjusted to higher values by adding a concentrated K_2HPO_4 solution, if needed. The mobile phases containing 20, 22.5, 25 and 30% acetonitrile obtained by measuring the appropriate volumes of acetonitrile and aqueous phase separately, and then mixed thoroughly, followed by degassing in an ultrasonic bath (Branson).

(iii) Reference Standard Solution - A stock solution of polymyxin B sulfate reference standard was prepared by weighing out

approximately 36 mg of the USP reference standard accurately in a 10 ml volumetric flask and dissolved with water. Stepwise dilutions were then made from this stock solution by pipetting 5 ml of the stock solution into a 10 ml volumetric flask, and diluting to the mark with water. The most dilute standard solution prepared for this study was 56.5 IU/ml, which was found to be the lower limit of detection with the experimental set-up at 220 nm. More will be discussed later. For the purpose of assaying the samples, a given standard solution would be extracted twice with chloroform, using 10 ml per extraction.

(iv) Sample Preparation - The samples were in the form of a glycerin solution which made it impossible to be pipetted for sample volume measurements. Instead, the density of a given sample was determined. The sample volume was then computed from the sample weight, which was accurately determined on an analytical balance. The sample solutions were prepared by diluting approximately 15 ml of the liquid sample in a 50 ml volumetric flask with water to the mark. Shaking and sonifying were necessary in order to obtain a homogeneous sample workup.

A 15 ml portion of sample solution was transferred into a 50 ml centrifuge tube containing 10 ml chloroform. This was shaken on a mechanical shaker for 5 minutes followed by centrifugation at high speed (e.g., 2,000 rpm) for 5 minutes. This extraction procedure was repeated with another 10 ml of fresh chloroform. The reason for doing this duplicate extraction was that most of the other ingredients, which were present in the liquid formulation to a much higher concentration than polymyxin B, would interfere with the first component of the polymyxin B, making quantitation less accurate.

RESULTS AND DISCUSSION

Figure 1 shows a HPLC separation of polymyxins B_1 and B_2 on a 15 cm Hypersil-ODS (5 μm) column with a mobile phase containing 20% (v/v) acetonitrile in the aqueous phase (pH ~ 1.6). The excellent resolution of polymyxins B_1 and B_2, which was obtained

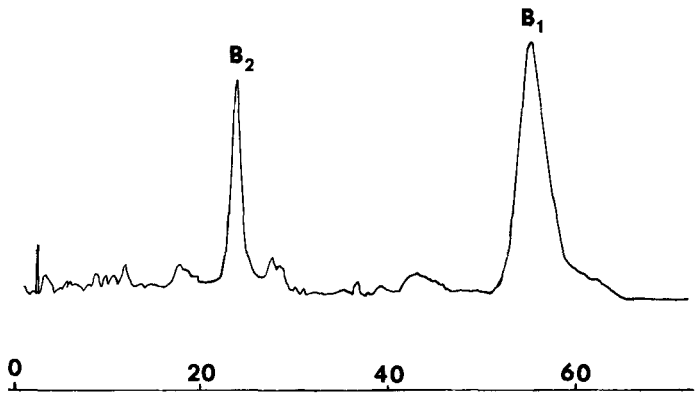

Figure 1: HPLC separation of polymyxins, B_1 and B_2. Columns: Hypersil-ODS (5 μm), 150 x 4 mm I.D.; Mobile phase: 20% (v/v) CH_3CN in aqueous phase (aqueous phase composition: 0.2% (v/v) H_2SO_4, 0.5% (w/v) TMAC in water, pH~1.6). Flow rate = 1.2 ml/min. Detection wavelength = 210 nm. Perkin-Elmer, LC-55 detector at 0.2 AUFS. Sample amount: 27.3 μg on column.

isocratically, was encouraging but time consuming (about 60 minutes). By adjusting the amount of the organic modifier in the mobile phase, the analysis time has been shortened. Figure 2(a) shows a HPLC separation with 25% (v/v) acetonitrile in the mobile phase and a 25 cm long Hypersil-ODS column. It was shown that much sharper peaks and shorter retention times were achieved. Note that the analysis time was much reduced also, from about 60 minutes to less than 18 minutes though the column length was increased from 15 cm to 25 cm.

It is worth mentioning that the polymyxin B sulfates, as chromatographed, showed a strong and sensitive dependence on the concentration of the organic modifier in the eluent. It was reported recently that the nature of the water/acetonitrile ratio

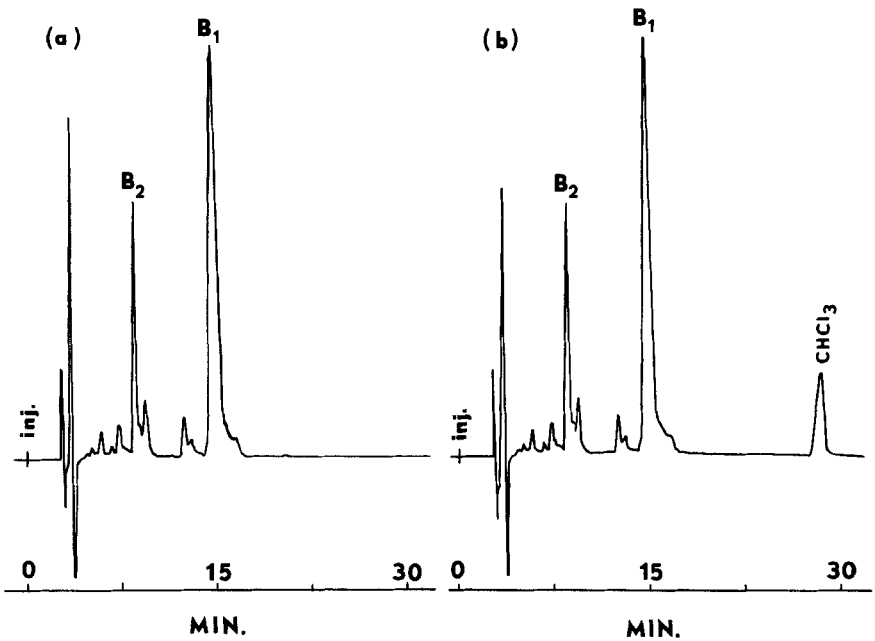

Figure 2: HPLC separation of polymyxins, B_1 and B_2. Column: Hypersil-ODS (5 μm), 250 x 3 mm I.D.; Mobile phase: 25% (v/v) CH_3CN in aqueous phase (aqueous phase composition: 0.2% (v/v) H_2SO_4, 0.5% (w/v) TMAC in water, pH~1.6). Flow rate = 1.09 ml/min. at 3,000 psi. Detection wavelength = 220 nm at 0.4 AUFS. Concentration of standard solution = 5915 IU/ml. (a) Non-extracted standard, and (b) extracted standard, note the extra peak due to chloroform.

in the eluent was very critical to the separation of minute quantities of impurities in nonapeptides by reverse phase HPLC (18). A more systematic study of the effects of pH, ionic strength and concentration of organic modifier on the retention and chromatographic behavior of short-chain peptides on a bonded

peptide stationary phase was also reported (19). It was not surprising that these cyclic decapeptides behaved in a predictable manner, but the large change in magnitude of the retention or capacity ratios relative to that of acetonitrile in the mobile phase should be noted (see Table I).

In fact, any concentration of acetonitrile below 20% would produce undesirably large capacity ratios and thus hours might be needed to elute the polymyxins. On the other hand, the small capacity ratios of polymyxins B_1 and B_2 at 30% acetonitrile or higher concentrations implied that all components of interest would appear near the solvent front. It was within this narrow, yet useful, range of acetonitrile concentrations that a concentration of 22.5% acetonitrile was chosen to improve the resolution of both polymyxins B_1 and B_2 from other components or impurities that are often found in polymyxins. Figure 3 shows typical HPLC chromatograms of an extracted standard and an extracted sample. The analysis time was about 23 minutes.

The retention of chloroform in this system was also very sensitive to the concentration level of organic modifiers, as evidenced in Figures 2(b) and 3(a). Theoretically, the chloroform peak could overlap or interfere with either the B_1 or B_2 peak, depending on the concentration of acetonitrile in the mobile phase.

TABLE I

Dependence of Capacity Ratios of Polymyxins B_1 and B_2 on the Concentration of Acetonitrile in Mobile Phase

Concentration of Acetonitrile in Mobile Phase (v/v)	Capacity Ratios, k'	
	B_1	B_2
20%	32.31	12.97
22.5%	7.75	3.25
25%	4.63	2.19
30%	0.91	0.55

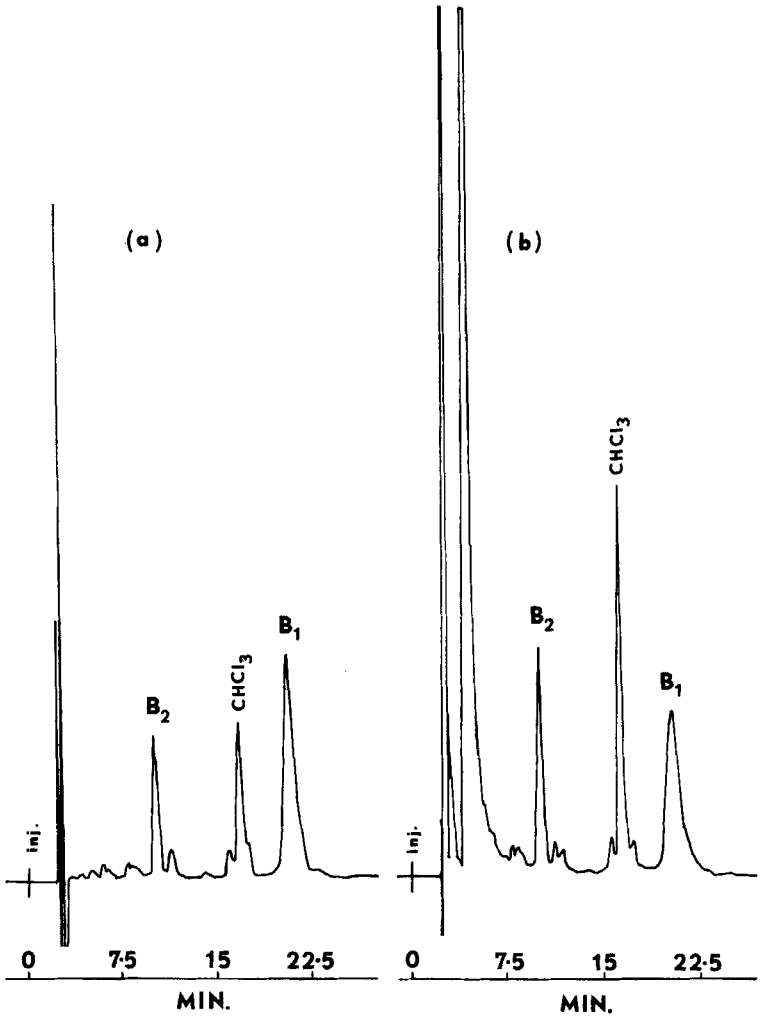

Figure 3: Typical HPLC chromatograms of extracted reference standard (a), and extracted sample (b). Column: Hypersil-ODS (250 x 3 mm I.D.); Mobile phase: 22.5% (v/v) CH_3CN in aqueous phase (aqueous phase composition: 0.2% (v/v) H_2SO_4, 0.5% (w/v) TMAC in water, adjusted to pH 2.7 with 0.5M K_2HPO_4). Flow rate = 1.09 ml/min. Detection: 220 nm at 0.1 AUFS.

This precaution should be taken in order to achieve complete resolution of B_1 from B_2, and their separation from all other components present in the sample workup.

The use of hydrophilic anions such as phosphates and citrate to decrease the retention of peptides was reported recently (19-21). It was shown that ion-pairing of a cationic reagent with the carboxyl groups of the peptides in solution would lead to modification of their chromatographic behavior (22). Tetraalkylammonium salts were used in the improvements of HPLC procedures of polar pharmaceuticals (23,24). It is our experience that the retention of peptides may be undesirably long if only water-acetonitrile or water-methanol mixtures were used as mobile phases. This was also true with these cyclic decapeptides.

In order to evaluate the linearity and calculate the lower limit of detection with this procedure, standard solutions from 56.5 to 28911.2 IU/ml were carried through the described procedure. Results were plotted as the sum of the peak areas of B_1 and B_2 versus the concentration of polymyxins. The plot was linear over the concentration range examined, with a correlation coefficient, $\gamma = 0.9999$ (Figure 4). The lower limit of detection was estimated to be 56 IU/ml or 6.9 µg/ml at 220 nm of a variable wavelength UV detector. Based on the UV spectrum of polymyxin B sulfate in water, a maximum was found at about 195 nm, which is very common for compounds containing amide linkages. Even at 210 nm, the UV absorbance is more than double that at 220 nm. With the system described here, it was shown possible to detect the polymyxins at 205 nm with no problem in zeroing the detector. In other words, the lower limit of detection could be lowered by about ten-fold, if needed.

CONCLUSION

The method described here represents the first isocratic HPLC procedure ever reported in the literature for the resolution of polymyxins B_1 and B_2 and their separation from all other components and/or impurities commonly present in a polymyxin bulk powder.

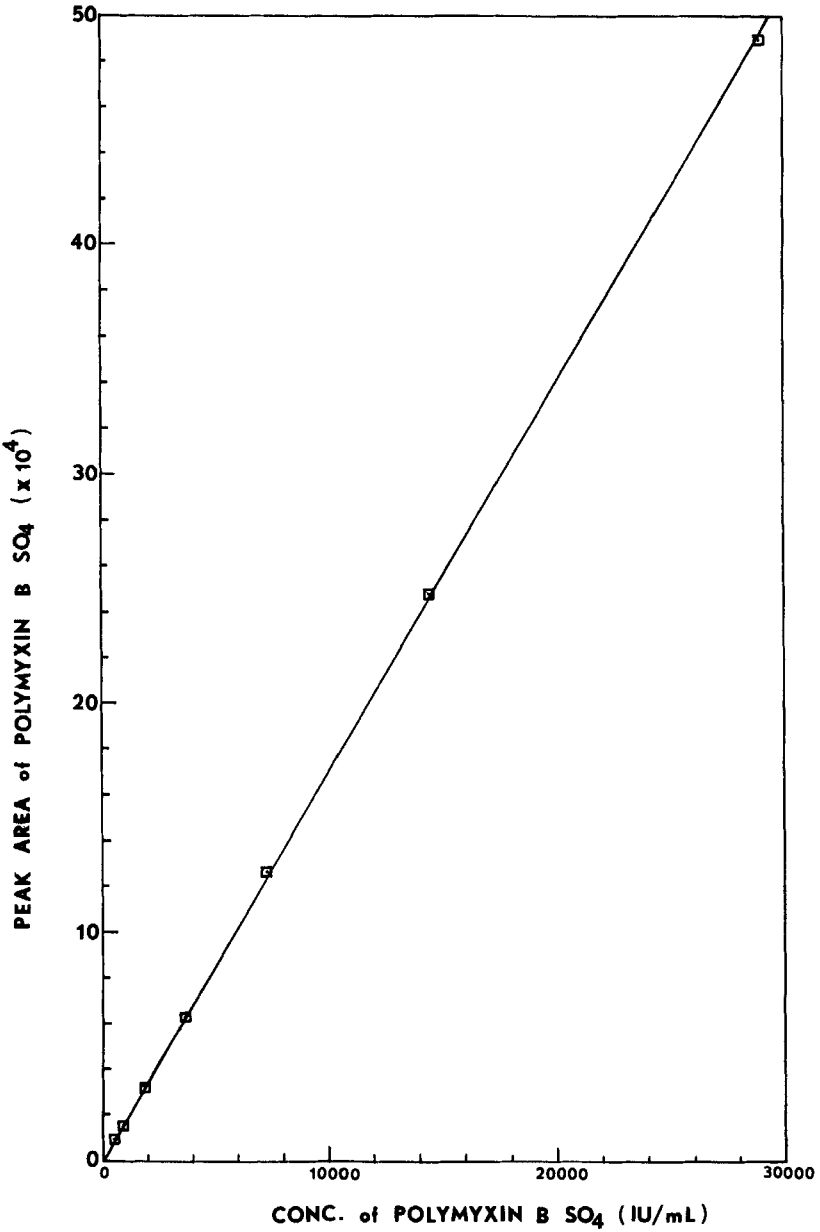

Figure 4: Plot of concentration of reference standard versus total area of polymyxins B_1 and B_2. The linearity is demonstrated by the excellent correlation coefficient of 0.9999.

The method is accurate, simple, selective and sensitive for determining the polymyxin content of pharmaceutical preparations. An isocratic HPLC method is preferred over a gradient one due to the fact that laboratory automation can easily and inexpensively be accomplished with an isocratic system. Finally, it should be pointed out that the detection limit could be lowered further if the preparation of fluorescent derivatives is to be pursued.

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