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PREPARATIVE-SCALE SEPARATION OF POLYMYXINS WITH AN ANALYTICAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM BY USING DISPLACEMENT CHROMATOGRAPHY

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SUMMARY

Polymyxin antibiotics were separated by using reversed-phase chromatography in the displacement mode with columns generally employed in analytical separations. By using an aqueous solution of dodecyloctyldimethylammonium chloride as the displacer more than 100 mg of commercial polymyxin B sulfate could be separated into its constituents on a 250 × 4.6 mm column packed with 5-μm octyl-silica. The results are compared to those obtained by elution chromatography and some of the main features of displacement chromatography are discussed.

INTRODUCTION

Polymyxin antibiotics have been separated by paper chromatography[1], counter-current distribution[2], thin-layer chromatography (TLC)[3,4] and recently by high-performance liquid chromatography (HPLC) using reversed-phase chromatography[5–11]. Preparative-scale separations with large-bore columns (I.D. 10 mm) packed with silica-bonded hydrocarbonaceous stationary phases as well as with macroreticular styrene–divinylbenzene copolymers were also reported[6–8]. All chromatographic separations were carried out in the conventional elution mode that yields individual —preferably well separated— peaks of the sample components.

We wish to report that upon using the displacement mode of chromatography, preparative-scale separation of a mixture containing polymyxin B_1 and B_2 can be accomplished also with standard (250 × 4.6 mm) columns generally employed in analytical HPLC.

Although Tiselius[12] has clearly recognized the essential features and great potential of displacement chromatography the technique has yielded to elution "analysis" as prime mode of chromatographic separations[13]. Despite early success of Porath[14–16] in using displacement chromatography for the purification of complex biological substances such as ACTH and bacitracin, lack of sufficiently efficient columns and selective sorbents having uniform surface as well as of methods for convenient monitoring of the column effluent precluded the technique from unfolding its full potential.

A recent work from our laboratory[17], however, has demonstrated that due to

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recent advances in HPLC instrumentation and column technology the displacement mode of chromatography may offer significant advantages and facilitates preparative scale separations with analytical columns and customarily available HPLC instrumentation. In the process of displacement chromatography, the column is first loaded with the feed mixture to be separated by using a carrier solvent that is a very weak eluent under the conditions employed. Thereafter the solution of a displacer, which is adsorbed to the stationary phase stronger than any of the feed components and is present at sufficiently high concentrations, is pumped through the column. The sample components are thereafter displaced from the stationary phase surface and move down the column preceding the displacer front. When the conditions are appropriate the components of the mixture become separated as adjacent bands and form a "displacement train" in which all bands move with the same velocity as the displacer front. Upon development of the displacement train *isotachic* conditions[17] are reached and maximum separation is obtained. Fractions of column effluent containing the contiguous solute bands are collected and analyzed by an appropriate technique such as HPLC or TLC to estabilish the location of the individual component bands and their boundaries.

The process employed in this study was essentially the same as described for other applications of reversed-phase chromatography in the displacement mode[17].

EXPERIMENTAL

Materials

Polymyxin B sulfate (Cat. No. 5291, Lot No. 101,558) was obtained from Calbiochem-Behring (LaJolla, CA, U.S.A.). Octyldodecyldimethylammonium chloride (50% solution in water and 2-propanol) HPLC grade, triethanolamine (certified), phosphoric acid 85%, methanol and acetonitrile, HPLC grade, were supplied by Fisher (Fair Lawn, NJ, U.S.A.). 1-Butanesulfonic acid sodium salt was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Distilled water was prepared by a Branstead apparatus.

Solutions

0.05 *M* solution of octyl dodecyl dimethylammonium chloride in water containing 10% (v/v) acetonitrile was prepared by dissolving 3.62 ml of the reagent solution in 100 ml of distilled water containing 10% acetonitrile.

0.05 *M* triethanolammonium phosphate, pH 2.2, containing 0.0025 *M* 1-butanesulfonic acid was mixed with acetonitrile to contain 25% (v/v) organic solvent and used as the eluent in analytical HPLC.

Columns

A 5-μm octyl-silica column (250 × 4.6 mm) home-packed with LiChrosorb RP-8 (MCB, Cincinnati, OH, U.S.A.) was used as the fractionator column for displacement chromatography. HPLC analysis of the fractions was performed with 5-μm octadecyl-silica column home-packed with LiChrosorb RP-18 (MCB). In some preliminary experiments, a column of the above dimensions that was packed with large-pore diphenyl-silica (Whatman, Clifton, NJ, U.S.A.) was also employed.

Apparatus

A flow sheet of the fractionator unit is shown in Fig. 1. The reservoirs for water, the solution of octyl dodecyl dimethylammonium chloride and methanol used as the carrier, displacer and regenerant, respectively, are connected via a four-way valve to a Model No. 110A solvent metering pump (Altex, Berkeley, CA, U.S.A.). The feed was introduced by a Model No. 7010 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 1.5-ml feed loop. The column effluent was monitored by a Model LC 55 variable-wavelength detector (Perkin Elmer, Norwalk, CT, U.S.A.) and a Model SR-206 dual-pen strip chart recorder (Heath, Benton Harbor, MI, U.S.A.). Fractions containing 0.5 ml of the effluent were collected with an Ultrarack II, No. 2070, fraction collector (LKB, Rockville, MD, U.S.A.).

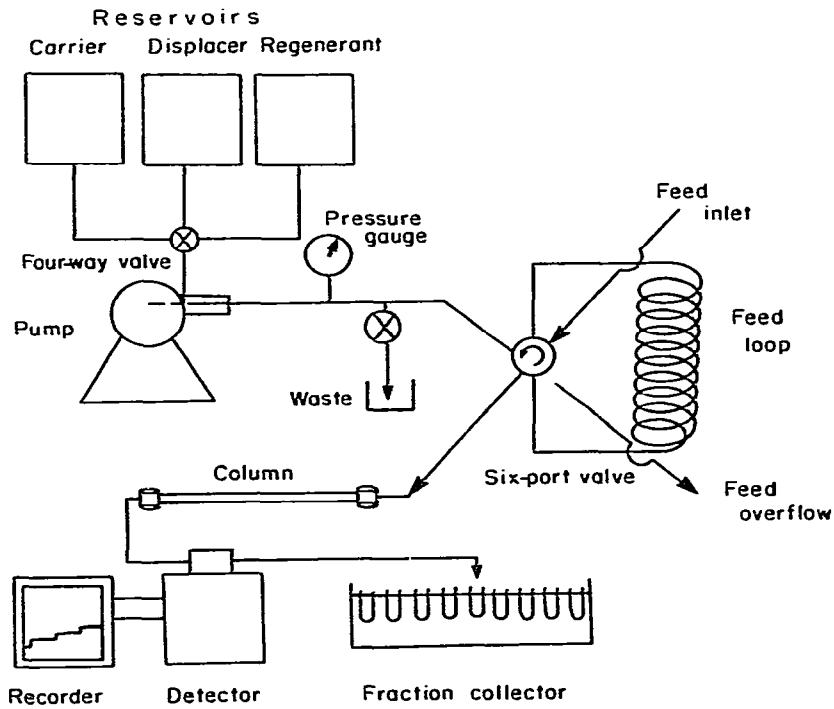


Fig. 1. Flow sheet of the apparatus used for fractionation by displacement chromatography.

Procedures

Fractionation by displacement chromatography. The 1.5-ml feed loop of the sample injector shown on the flow sheet in Fig. 1 was filled up with the solution of the mixture to be separated. At the same time, the reservoir of the displacer solution was connected to the pump by turning the four-way valve. Upon opening the valve to "waste" the displacer solution was pumped into the system to fill up the line upstream of the feed valve. After closing the valve to "waste" and turning the feed valve into feed position the displacer solution swept the feed into the column at a flow-rate of 0.1 ml/min. As the displacer solution was moving through the column, separation of

the feed components into juxtaposed concentration zones in the effluent occurred. Fractions of the column effluent were collected at every 5 min and analyzed later as described below. The collection of fractions began after turning the feed valve. After the displacer front emerged from the column, the four-way valve was adjusted so that the reservoir of methanol (regenerant) was connected to the pump. Methanol was pumped through the system at a flow-rate of 1.2 ml/min for 1 h to remove the displacer. Thereafter the four-way valve was adjusted to connect the reservoir of water (carrier) to the pump and water was pumped at the same flow-rate for 0.5 h in order to return the chromatographic system to its initial stage.

Analysis. The fractions were analyzed by a HPLC analyzer unit containing the same major components as the fractionator. Since isocratic elution with 0.05 *M* triethanolammonium phosphate buffer containing 25% (v/v) acetonitrile and 0.0025 *M* 1-butanesulfonic acid was used, only one reservoir was needed for the analytical liquid chromatograph. The volume of sample loop in analytical work was 20 μl. The column effluent was monitored with the variable-wavelength detector at 220 nm.

RESULTS AND DISCUSSION

The chemical structure of the two major components present in commercial polymyxin B sulfate is depicted in Fig. 2. As impurities there are frequently present colistin A and B which contain C_8 and C_7 fatty acid chains, respectively, in a molecular structure very similar to that of polymyxins except that D-phenylalanine is replaced by D-leucine.

Preliminary experiments have shown that chromatographic systems containing silica gel and a relatively non-polar eluent do not have sufficient selectivity to bring about the separation of polymyxin B_1 and B_2. On the other hand, reversed-phase chromatography has been found to be eminently suitable to separate the two homologues in agreement with findings reported in the literature[3–11].

A typical analytical separation of the sample is depicted in the chromatogram

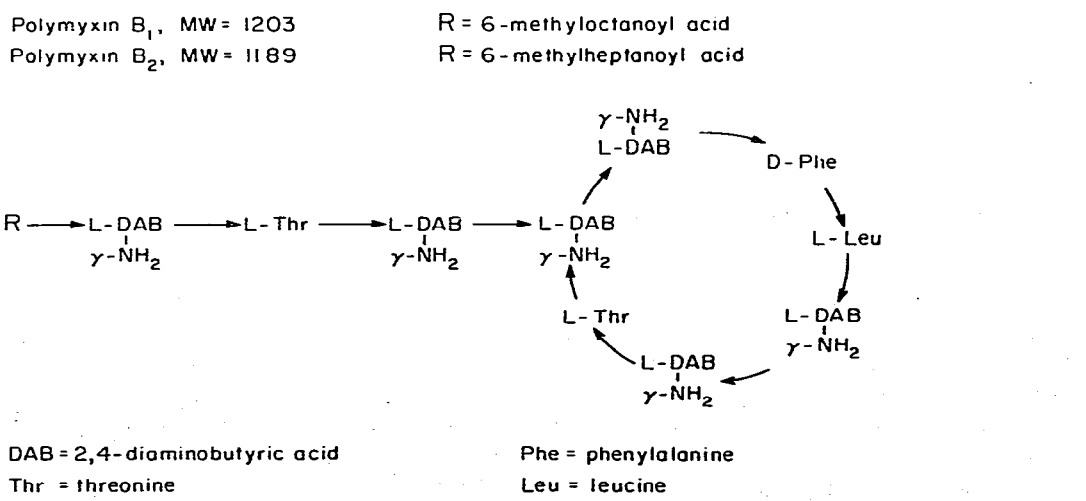
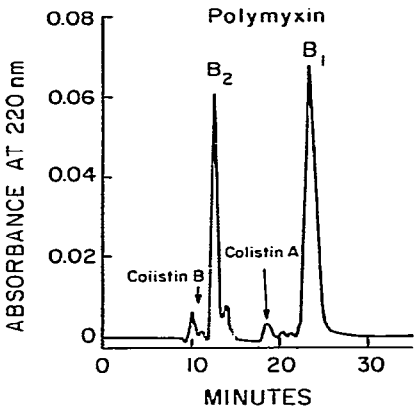


Fig. 2. Chemical structure of polymyxin B's.



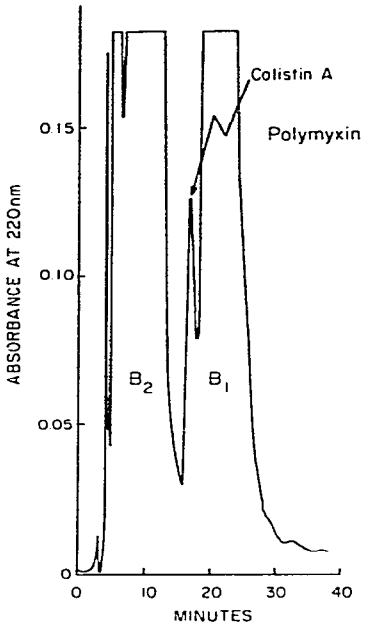


Fig. 3. Analytical separation of polymyxins by reversed-phase chromatography in the elution mode. Column, 5 μm LiChrosorb RP-18, 250 × 4.6 mm; eluent, 0.05 *M* triethanolammonium phosphate buffer, pH 2.2, containing 0.025 *M* 1-butanesulfonic acid and 25% (v/v) acetonitrile; flow-rate, 1.0 ml min^{-1}; column inlet pressure, 2800 p.s.i.; temperature, 22°C; detector setting at 220 nm, 0.08 a.u.f.s.; amount of polymyxin B sulfate injected, 60 μg.

Fig. 4. Chromatogram illustrating the effect of column overloading in elution chromatography of polymyxins. Conditions are the same as in Fig. 3 except the amount of sample was 2 mg and the detector sensitivity was 0.2 a.u.f.s. at 220-nm setting.

shown in Fig. 3. Upon increasing the sample load, however, the separation efficiency rapidly deteriorates. An elution chromatogram obtained with the analytical column at a sample load of 2 mg is shown in Fig. 4. It appears that at a higher load the purity of the product falls below permissible limits and this observation lends support to the need for preparative columns (I.D. $\geqslant 1$ cm) reported in the literature[7,8,11] for the separation of milligram quantities of polymyxins.

The potential of the displacement mode in preparative chromatography is demonstrated in Fig. 5 by the separation of relatively large amounts of polymyxin B_1 and B_2 with an analytical column generally used in HPLC. Column dimensions were the same as those used in analytical work (see Figs. 3 and 4), but the stationary phase was octyl-silica instead of octadecyl-silica. The displacement diagram shown in Fig. 5 was drawn through points obtained by determining the concentrations of the individual components by HPLC in the fractions of the effluent. Monitoring the column effluent by a detector yields a chromatogram that shows only concentration steps but not the boundaries of the adjoining solute bands. Lack of a rapid and convenient method for analyzing the composition of the effluent greatly impeded the development of displacement chromatography in the past.

Comparison of Figs. 3 and 5 strikingly demonstrates the differences between

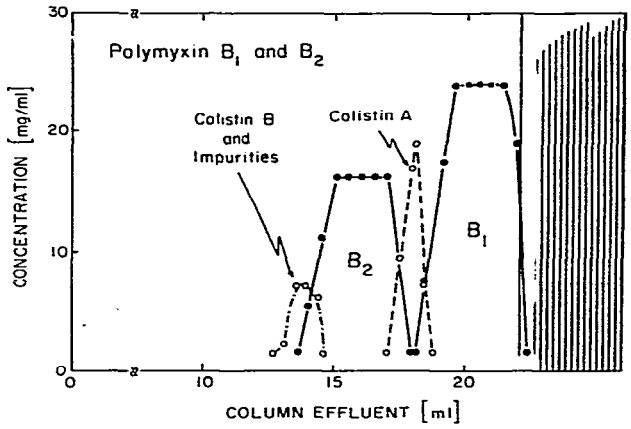


Fig. 5. Preparative separation of polymyxins by reversed-phase chromatography in the displacement mode using an analytical liquid chromatograph. Column, 5 μm LiChrosorb RP-8, 250 × 4.6 mm; carrier, water containing 10% (v/v) acetonitrile; sample volume, 1.5 ml; displacer, 0.05 *M* octyldodecyldimethylammonium chloride in water containing 10% acetonitrile; flow-rate of displacer, 0.1 ml min^{-1}; temperature, 23°C; fraction size, 0.5 ml; analysis of fractions by HPLC; regenerant, methanol; amount of polymyxin B sulfate in feed 150 mg. The shaded zone in the displacement diagram represents the displacer.

elution and displacement chromatography and the superiority of the latter in preparative-scale separations at least for the case presented here. Elution chromatography yields good separation of all components including the colistin impurities as long as the sample concentration is sufficiently low to maintain linear elution conditions as shown in Fig. 3. Upon overloading the column the efficiency of separation is reduced so that preparative chromatography in the elution mode mandates the use of columns having large diameter.

On the other hand the same chromatographic system in the displacement mode can be used for the separation of preparative amounts of such a relatively simple mixture. We have found that under conditions given in Fig. 5 up to 300 mg of polymyxin B sulfate can be separated with an efficiency comparable to that exhibited in Fig. 5. The high loading capacity in displacement chromatography is the result of the better utilization of the column and equipment than possible in linear elution chromatography where not only the solute concentration has to be low but also a large fraction of the column volume between the peaks is idle.

The theory of displacement chromatography is well established[17–19] and the properties of the fully developed displacement train are readily explained on the basis of the adsorbtion isotherms of the feed components and displacer. For successful displacement it is required that the isotherms have a concave down shape as it is characteristic for Langmuir isotherms. So far the isotherms of relatively simple organic compounds on alkyl-silica bonded phases have been found to be Langmuirian in our laboratory. At the same time results obtained in displacement chromatography with polymyxin antibiotics are evincive that the isotherms of these substances have also a concave down shape at least under conditions described for their separation.

It should be noted that the separated feed components emerge from the column in the water–acetonitrile mixture used as the carrier at relatively high concentrations (see Fig. 5), so that the recovery of products is much easier than in elution chromato-

graphy where solute concentrations in the effluent are usually much lower and the eluent may contain modifiers that have to be removed from the product in a subsequent separation step.

The selection of a suitable displacer may be the most difficult part of establishing the conditions for displacement chromatography[17]. The displacer has to bind to the stationary phase stronger than any of the components of the mixture to be separated. In addition it has to be sufficiently soluble in the carrier, it should not form a complex with any of the feed components and it should be conveniently removable from the column with an appropriate regenerant. In our study *n*-propanol, *n*-butanol, phenol and N,N-dimethylcyclohexylamine were tried as potential displacers in combination with aqueous acetonitrile carriers at various concentrations. With these substances, however, isotachic conditions were not obtained by using the column and flow-rate stated in Fig. 5. In many instances partial elution occurred due to the insufficient affinity of the substance to the stationary phase so that no *bona fide* displacement chromatography took place. Finally, a large chain quaternary amine was selected from recognizing that both hydrophobic and silanophilic interactions are involved in the binding of polymyxins to the silica-bonded hydrocarboneous stationary phase[20,21]. Unless the isotherms of all compounds involved are known, the choice of an appropriate displacer requires trial and error. On the basis of our experience a substance that has functional groups similar to those of feed components and has significantly stronger affinity to the stationary phase than the substances to be separated is a good displacer candidate provided it has adequate solubility in the carrier.

Reversed-phase chromatography offers high selectivity for polymyxins due to a felicitous combination of solvophobic and silanophilic binding mechanisms to the stationary phase of such molecules that have a long alkyl chain and abundance of amino groups[20,21]. Columns packed with naked silica yielded separation of the mixture neither in the elution nor in the displacement mode. Whereas on bonded phases having relatively small hydrocarbonaeous ligates such as diphenyl-silica, polymyxins were more strongly retarded in the elution mode probably due to the high accessible silanol concentration, the separation of the components in displacement chromatography was less efficient than that shown in Fig. 5 under comparable conditions. Nevertheless experiments with a given chromatographic system and sample mixture by using elution chromatography can generally be useful in designing separation by displacement chromatography although the information thus obtained is not sufficient to choose the appropriate displacer and to predict the outcome of the separation.

CONCLUSIONS

Preparative-scale separation of complex biological molecules such as polymyxin antibiotics was successfully carried out with analytical columns by using reversed-phase chromatography in the displacement mode. Octyl dodecyl dimethylammonium chloride was used in aqueous solutions as the displacer. The results suggest that the displacement mode of chromatographic separations by using HPLC instrumentation offers an efficient means to separate 100-mg quantities with analytical columns packed with microparticulate stationary phase.

The technique appears to be particularly useful for the separation of sub-

stances having similar chemical properties such as peptides and nucleic acid fragments. Besides preparative applications displacement chromatography with narrow-bore packed columns may be eminently suitable for tandem operation of a liquid chromatograph and mass spectrometer.

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