Separation of polymyxins by micellar electrokinetic capillary chromatography

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ABSTRACT

High-efficiency separations of basic polypeptides from the polymyxin group with equal or nearly equal mass to charge ratios were achieved by micellar electrokinetic chromatography in dynamically modified capillaries using a zwitterionic surfactant as an additive to the running buffer. The peptides are probably separated on the basis of differences in their partition coefficients to the surface of the pseudo-phase ion exchanger and to the lipophilic core of the micelles. Samples of polymyxins B, D and E (colistin) from different sources were examined.

INTRODUCTION

Polymyxins (Fig. 1) are a group of closely related decapeptide antibiotics produced by strains of *Bacillus polymyxa*. They have a general structure composed of a cyclic heptapeptide moiety and a side-chain consisting of a tripeptide acylated at the N-terminus. The polymyxins differ from each other in amino acid composition and each polymyxin consists of a mixture of polypeptides with an identical amino acid composition but with a different terminating fatty acid. Thus, each polymyxin may be subdivided corresponding to at least three fatty acid components: 6-methyloctanoic acid, 6-methylheptanoic acid and *n*-octanoic acid. The characteristic feature of polymyxins, in addition to the hydrophobic fatty acid moiety, is their strong basicity due to four or five unmasked amino groups from 2,4-diaminobutyric acid. The peptides in each group of the polymyxins (A, B, D and E) have equal or near-ly equal mass to charge ratios and therefore exhibit very similar electrophoretic mobilities.

The complex composition of the polymyxins has earlier been demonstrated by HPLC [1,2] and TLC [3], and a difference in the potency of polymyxin B_1 and B_2 fractions has been demonstrated by microbiological assays [4]. The fatty acids present in polymyxins have been measured by GC after hydrolysis [5]. Samples of polymyxins from different sources have been examined for potency and composition by use of microbiological and chemical assays [6]. Complete separation of the peptides has not been achieved with any of the above-mentioned methods. However, in order to identify these antibiotics, to examine their purity and to determine the relative contents of their components, a complete separation of the peptide will be very useful.

About 10 years ago, capillary electrophoresis (CE) was shown to be very suitable for separating complex peptide mixtures [7], especially when problems with adsorption to the capillary wall were taken into consideration [8]. The affinity of peptides toward the capillary wall may be reduced by appropriate pH adjustment [9,10], by the action of surface modifiers [11] and by the addition of surfactants to

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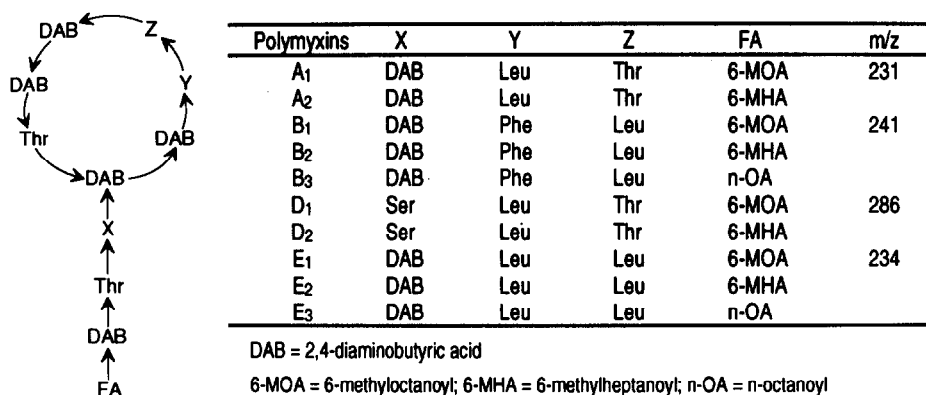
Polymyxins	X	Y	Z	FA	m/z
A₁	DAB	Leu	Thr	6-MOA	231
A₂	DAB	Leu	Thr	6-MHA	
B₁	DAB	Phe	Leu	6-MOA	241
B₂	DAB	Phe	Leu	6-MHA	
B₃	DAB	Phe	Leu	n-OA	
D₁	Ser	Leu	Thr	6-MOA	286
D₂	Ser	Leu	Thr	6-MHA	
E₁	DAB	Leu	Leu	6-MOA	234
E₂	DAB	Leu	Leu	6-MHA	
E₃	DAB	Leu	Leu	n-OA	

DAB = 2,4-diaminobutyric acid

6-MOA = 6-methyloctanoyl; 6-MHA = 6-methylheptanoyl; n-OA = n-octanoyl

Fig. 1. Structure of the polymyxins.

the running buffer [12]. Further, additions of sur-factants above their critical micelle concentration (CMC) also generate the possibility of an additional separation mechanism, micellar electrokinetic capil-lary chromatography, which can be beneficial in separating substances with similar electrophoretic mobilities. However, high concentrations of these surfactants are often necessary and, owing to the high ionic strength of the buffer, only lower volt-ages can then be applied. This problem has been overcome by the use of non-ionic or zwitterionic surfactants [13], which have no net charge and, therefore, do not contribute to the conductivity of the buffer.

Recently, it was shown that 3-(N,N-dimethylhex-adecylammonium)propanesulphonate (PAPS) dy-namically modifies the silica surface of the capillary as well as it forms micelles [14]. High-efficiency sep-arations of polymyxin B were achieved using PAPS as an additive to the running buffer.

In this study, micellar electrokinetic capillary chromatography was used to separate polymyxins A, B, D and E and to examine the composition of samples of polymyxins from different sources.

EXPERIMENTAL

Chemicals

3-(N,N-Dimethylhexadecylammonium)propane-sulphonate (PAPS), octanoic acid and Dowex 50W-X8 (100–200 mesh) were obtained from Fluka (Buchs, Switzerland). Sudan III was purchased from Merck (Darmstadt, Germany) and methane-

sulphonic acid from Sigma (St. Louis, MO, USA). Diazomethane solution was prepared by mixing 35 ml of diethylene glycol monoethyl ether (Fluka), 20 ml of diethyl ether and a solution of potassium hy-droxide (6 g) in water (10 ml). During the subse-quent distillation of the mixture, a solution of 21.5 g of N-methyl-N-nitroso-p-toluenesulphonamide (Aldrich, Steinheim, Germany) in about 200 ml of diethyl ether was added.

Polymyxins A, B, D and E sulphate, 6-methyl-heptanoic acid and 6-methyloctanoic acid were kindly provided by Dumex (Copenhagen, Den-mark). Polymyxin B sulphate was also obtained from Pfizer (Karlsruhe, Germany) and polymyxin E from Smit (Turin, Italy), Sumitomo Shoji Kaisha (Tokyo, Japan) and Warner-Lambert Research In-stitute (Morris Plains, NJ, USA).

CE apparatus

A Waters Quanta 4000 capillary electrophoresis system (Millipore, Milford, MA, USA) was used and on-column detection was performed by UV ab-sorption at 214 nm. Electropherograms were re-corded on a DP 700 data processor (Carlo Erba, Valencia, CA, USA).

A fused-silica capillary of 75 μm I.D. and 360 μm O.D. was obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capil-laries used was 60 cm and 52.4 cm to the detector.

Procedure

The fused-silica column was treated with 0.1 M sodium hydroxide solution for 30 min and subse-

quently with distilled water for 10 min before introducing the electrophoresis buffer for 10 min. When changing buffer composition, the capillary was rinsed for 10 min with 0.1 M sodium hydroxide and subsequently with distilled water for 5 min before introducing the new buffer.

For the preparation of the running buffer, 0.2 M sodium phosphate stock solution (pH 2.5) was used. The buffer system was composed of appropriate amounts of PAPS, buffer stock solution and distilled water without further readjustment of pH.

Sudan III in methanol (0.01%) was used as an indicator for the migration of the micelles.

Polymyxins A, B, D and E sulphate were dissolved in distilled water to a concentration of 0.5, 1.0 or 5.0 mM and kept refrigerated when not in use.

Sample injection was accomplished by hydrostatic injection (9.8 cm) for 5 or 10 s. All analyses were performed using an applied potential of 15 kV (90 μA).

Preparative HPLC

The chromatographic system consisted of a Merck Hitachi Model 655-A12 liquid chromatograph equipped with a Model 655 variable-wavelength UV monitor operated at 220 nm and a Rheodyne (Berkeley, CA, USA) Model 7120 injector with a 1.5-ml loop. The column (250 × 8 mm I.D.) was packed with Spherisorb ODS 2 (5 μm).

The experiments were carried out at ambient temperature using methanol–methanesulphonic acid–water (49.5:1.0:49.5, v/v/v) as the eluent [2] at a flow-rate of 3 ml/min. Polymyxin B or E sulphate was dissolved in the eluent to a concentration of 100 mg/ml and kept at about 4°C when not in use. A total amount of 4 mg of polymyxin B or E was injected each time and six major peaks were collected.

A sample from each isolated compound was injected into the capillary electrophoresis system in order to identify the peaks obtained in the electropherograms of polymyxin B and E.

The pooled fractions of each compound were, adjusted both before and after evaporation to half the volume, to neutral pH with sodium hydroxide solution and subsequently transferred on to a 5 mm I.D. cation-exchange column packed with 2 g of Dowex 50W-X8. The column was then washed with 3 ml of

distilled water and the peptide was eluted from the column using 7 ml of 2 M ammonia solution followed by 20 ml of distilled water. The ion-exchange column was regenerated with 3 ml of 4 M hydrochloric acid followed by 7 ml of distilled water.

The collected fraction was evaporated on a rotary evaporator for 15 min at ambient temperature to remove ammonia and then freeze-dried.

GC–MS

A few milligrams of the freeze-dried residue were hydrolysed with 1 ml of 6 M hydrochloric acid at 110°C for 20 h. The hydrolysate was extracted with three 2-ml portions of diethyl ether and the ethereal extract was dried over anhydrous sodium sulphate. With a slow stream of nitrogen at 25°C, the dried extract was evaporated to a final concentration of 10–20 ng/μl.

Fatty acid samples and standard solutions were derivatized with diazomethane solution. The methyl esters produced were analysed by injection on to a 30 m × 0.26 mm I.D. fused-silica DB-5 column (J&W, Folsom, CA, USA) with a film thickness of 0.25 μm and coupled to a Finnigan MAT (Bremen, Germany) Model 4515 B mass spectrometer operated in the electron impact ionization mode at 70 eV. The GC oven temperature was held at 35°C for 2 min, then increased linearly at 12°C/min to 260°C, where it was maintained for 30 min.

RESULTS AND DISCUSSION

Separation of polymyxins B_1, B_2 and B_3

The polymyxins B are a group of peptides having equal or nearly equal mass-to-charge ratios and therefore they exhibit very similar electrophoretic mobilities. Recently, the separation mechanisms involved in CE with PAPS was studied [14] and the ability of PAPS to separate these almost identical peptides was shown.

Fig. 2. shows the electropherograms of polymyxin B with no and with 30 mM of PAPS added to the running buffer. No separation of the peptides was obtained in plain buffer whereas the addition of 30 mM PAPS provided a significant increase in the separation of the peptides, resulting in at least ten peaks.

Based on a comparison of the electrophoretic mobilities of the peptides with those of the isolated

fractions and taking the identified free fatty acids into account, the following can be concluded: the peak labelled 3 in Fig. 2b corresponds to polymyxin B$_1$ with the 6-methyloctanoyl residue, peak 1 was identified as polymyxin B$_2$ with the 6-methylhepta-noyl residue and peak 2 appears to be due to poly-myxin B$_3$ with an *n*-octanoyl residue.

The electrophoretic mobilities of polymyxins B$_1$ and B$_2$ were calculated based on peaks 3 and 1, respectively, and plotted against the concentration of PAPS (Fig. 3). With increasing concentration of

Fig. 3. Electrophoretic mobility of (∗) polymyxin B$_1$ and (×) polymyxin B$_2$ *versus* the concentration of PAPS in the running buffer. Conditions as in Fig. 2.

PAPS, the electrophoretic mobility of the two pep-tides decreases, probably owing to increased inter-action with the micellar pseudo-phase, the concen-tration of which increases proportionally to that of PAPS. Simultaneously, the relative mobility of the two peptides increases.

Separation of polymyxins A, B, D and E (colistin)

Polymyxin A differs from polymyxin B in the substitution of Phe and Leu with Leu and Thr, re-spectively. Polymyxin D differs from the other poly-myxins investigated because a DAB group is re-placed by Ser. In consequence, the polymyxins D have a higher mass to charge ratio than the other polymyxins included in this study. Fig. 4a and b show the electropherograms of polymyxins A and D, each being separated into two main peaks. A tentative assignment of the peaks has been made.

Fig. 4c shows the electropherogram of the poly-myxins E (colistin), which differ from polymyxin B only in the substitution of a single amino acid. The assignments of the main peaks, E$_1$ (colistin A) with 6-methyloctanoyl, E$_2$ (colistin B) with 6-methylhep-tanoyl and E$_3$ with the *n*-octanoyl residue, were based on a comparison of the electrophoretic mo-

Time (min)

Fig. 2. Electropherograms of polymyxin B (0.5 m*M* in water) obtained (a) with no PAPS and (b) with 30 m*M* PAPS added to the running buffer. Capillary, 75 *μ*m I.D.; buffer, 0.2 *M* sodium phosphate (pH 2.5)–water (1:1, v/v); voltage, 15 kV; hydrostatic injection, 10 s. Peaks: 1 = polymyxin B$_2$; 2 = polymyxin B$_3$; 3 = polymyxin B$_1$.
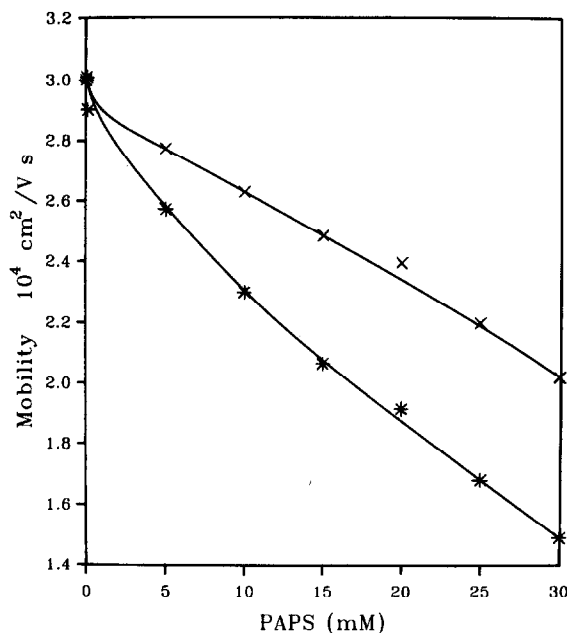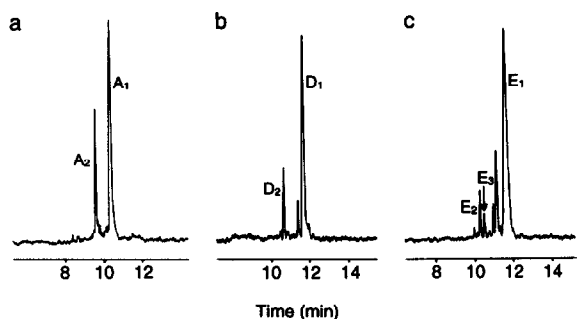
Fig. 4. Electropherograms of (a) polymyxin A, (b) polymyxin D and (c) polymyxin E. Sample concentrations, 1.0 mM; capillary, 75 μm I.D.; buffer, 0.2 M sodium phosphate (pH 2.5)–water (1:1, v/v) + 30 mM PAPS; voltage, 15 kV; hydrostatic injection, 5 s.

bilities of the peptides with those of the isolated fractions, and the identification of the free fatty acids.

Proposed separation mechanism

As described previously [14], above the CMC of PAPS, separation depends on the partitioning of solute molecules between the bulk solvent and the micellar pseudo-phase in the buffer solution. The separation also depends on the difference in electrophoretic mobility of the bulk solvent versus the mi-

celle. Micelles formed from zwitterionic surfactants have no net charge and are therefore expected to migrate with the electroosmotic flow. However, surfaces in contact with aqueous media are more often negatively than positively charged, because the smaller, less hydrated and more polarizing anions have a greater tendency to be specifically adsorbed than the cations [15]. Adsorption of anions on the micelles gives rise to mobility in an applied field and the micelles migrate towards the anode. This is illustrated with Sudan III, which elutes about 20 min after the electroosmotic flow marker.

In free solution, the peptides migrate with a speed given by the sum of their electrophoretic mobility and the electroosmotic flow. When zwitterionic surfactants are added to the buffer in high concentrations, the zwitterionic associations with the peptide molecules and with the silica capillary surface may both prevent peptide adsorption on the fused silica and help to break up peptide–peptide interactions. In the presence of PAPS at concentrations above its CMC, the peptides show ion-exchange partitioning to the surface of the micelles (Fig. 5). While associated with the micelles, the peptides migrate with the speed of the micelles.
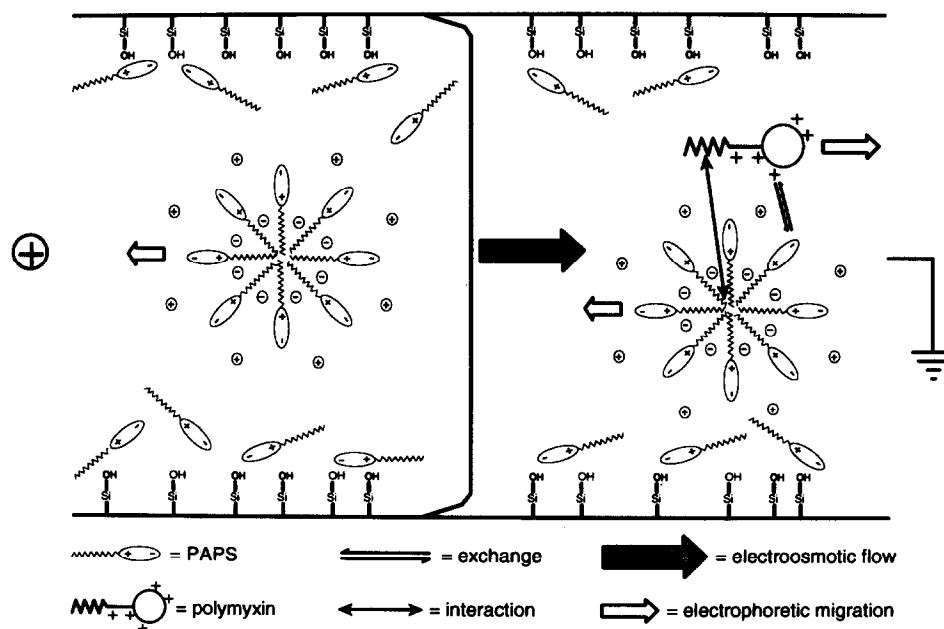
The hydrophobic moiety of the peptides may

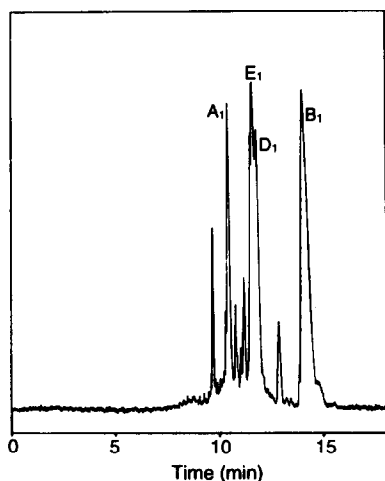Fig. 5. Schematic representation of the proposed separation mechanism of polymyxins.

Fig. 6. Electropherogram of polymyxins A, B, D and E mixed in equal amounts. Conditions as in Fig. 4.

show affinity for the inner core of the micelles (Fig. 5), while the remaining polar parts of the peptides stay outside. Consequently, polymyxins with an identical peptide moiety and with equal mass to charge ratios are eluted in order of increasing degree of hydrophobicity, 6-methylheptanoyl < n-octanoyl. The polymyxins having a 6-methyloctanoyl residue show a higher affinity for the micelles owing to the higher mass to charge ratio and to an increased degree of hydrophobicity of the fatty acid moiety. The peptides are therefore separated on the basis of differences in their partition coefficients to the surface of the pseudo-phase ion exchanger and to the lipophilic core of the micelles.

In plain buffer, the polymyxins migrate as single peaks in the order A < E < B < D (not shown), in accordance with the increasing mass to charge ratios. When PAPS is added to the buffer, the migra-
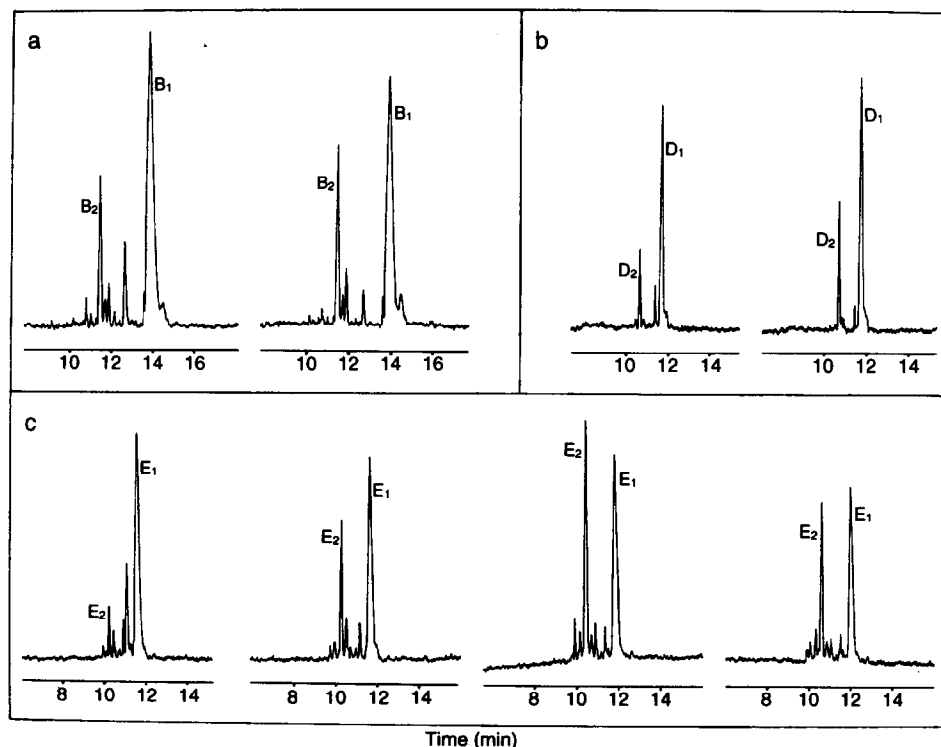

Fig. 7. Electropherograms of (a) two samples of polymyxin B, (b) two samples of polymyxin D and (c) four samples of polymyxin E from different sources. Conditions as in Fig. 4.

tion order of polymyxins B_1 and D_1 is reversed. The contribution of an amino acid residue to the order of elution can be evaluated based on a comparison of the migration times of polymyxins A_1, B_1, D_1 and E_1, which have an identical fatty acid group. As the polymyxins migrate in the order $A_1 < E_1 < D_1 < B_1$ (Fig. 6), the migration times increase in the order Thr < Leu and Leu < Phe. Polymyxin D_1 has a slower migration rate than polymyxin A_1, as expected because of a higher mass to charge ratio, but for the same reason polymyxin D_1 was also expected to have a slower rate of migration than polymyxin B_1 as observed in plain buffer. The probably explanation is that the replacement of DAB by Ser, Phe by Leu and Leu by Thr makes polymyxin D more polar than polymyxin B and therefore it shows less affinity for the micelles. Consequently, in the presence of PAPS at concentrations above its CMC, the order of migration of the polymyxins is determined by the mass to charge ratios and by the polarity of the peptides.

Polymyxins from different sources

Two samples each of polymyxins B and D and four samples of polymyxin E from different sources were separated by CE (Fig. 7). The elution profile of each sample was characteristic and sufficiently distinct to identify the samples by manufacturer. The variations between the relative peak areas of the main peaks in the electropherograms of the four samples of polymyxin E from different sources are very high, but correspond to results found by GC and HPLC analysis [6].

CONCLUSIONS

The method developed is useful for both qualitative and quantitative measurements on polymyx-

ins. As some of the major components of each polymyxin may possibly be present as impurities in samples of other polymyxins, the method is also valuable for purity testing of the polymyxins.

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