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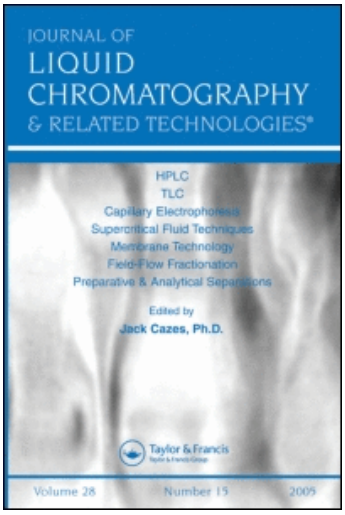

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
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To cite this Article Kristensen, Helle Kjærgaard and Hansen, Steen Honoré(1993) 'Micellar Electrokinetic Chromatographic Separation of Basic Polypeptides with Equal Mass to Charge Ratio Using Dynamically Modified Silica Capillaries', Journal of Liquid Chromatography & Related Technologies, 16: 14, 2961 — 2975
To link to this Article: DOI: 10.1080/10826079308019626
URL: http://dx.doi.org/10.1080/10826079308019626

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JOURNAL OF LIQUID CHROMATOGRAPHY, 16(14), 2961-2975 (1993)

MICELLAR ELECTROKINETIC CHROMATOGRAPHIC SEPARATION OF BASIC POLYPEPTIDES WITH EQUAL MASS TO CHARGE RATIO USING DYNAMICALLY MODIFIED SILICA CAPILLARIES

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ABSTRACT

A method using 3-(N,N-dimethylhexadecylammonium) propanesulfonate (PAPS) as an additive to the buffer for performing micellar electrokinetic chromatography in dynamically modified silica capillaries has been developed. PAPS is a zwitterionic surfactant. The advantage of zwitterions is that they do not increase the conductivity of the buffer. Therefore these surfactants can be added to the buffer at high concentrations while still allowing the application of high voltages and the use of capillaries of larger internal diameter. The separation mechanisms involved in CE with PAPS were investigated and found to be a combination of electrophoresis and hydrophobic interactions. The critical micelle concentration of PAPS was determined to be 25 μM. Above this concentration, an increase in electroosmotic flow due to dynamic surface

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modification was observed. High-efficiency separations of basic polypeptides with equal or nearly equal mass to charge ratios have been achieved using PAPS as an additive to the running buffer. The influence of pH on the separation has been examined.

INTRODUCTION

One of the major advantages of capillary electrophoresis (CE) is its capability of separating macromolecular compounds with high efficiency. However, peptides and especially proteins often exhibit high affinity toward the capillary wall (1). This is primarily due to electrostatic inter-actions between silanol groups on the inner capillary wall and protonated amino groups in the molecule, and it often results in a significant decrease in separation efficiency.

Several approaches have been taken to counteract adsorption and thereby improve separation efficiency and reduce peak tailing. Among these is the use of buffers in the pH range 8–11 (2), where the capillary wall and many peptides and proteins are electronegative and therefore repel one another. Buffers with low pH were used by McCormick (3) in order to protonate the silanol groups.

Other approaches have been to deactivate silanol groups by the action of surface modifiers, such as polyacrylamide (4), methylcellulose (4), polyethylene glycol (5) or maltose (6), which also modify or suppress the electroosmotic flow.

Green and Jorgenson (7) minimised adsorption of proteins onto fused silica by the addition of alkali-metal salts to the buffers. Recently, Emmer et al. (8) used a cationic fluorosurfactant as an additive to the running buffer. For the same purpose, an alternative method has been introduced and involves the addition of ethylene glycol to the protein samples (9).

High ionic strength buffers (10) and high concentrations of zwitterionic buffer additives (11) have been found effective in reducing the interactions of proteins with fused silica capillary surfaces and therefore enhancing separation efficiency.

The addition of ionic surfactants to the running buffer may also generate electrokinetic chromatography and, consequently, improve selectivity. The

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concentration of e.g. sodium dodecyl sulfate was linearly related to the capacity factor after the critical micelle concentration (CMC) was reached (12). Unfortunately, these buffers suffer from high conductivity and therefore require the use of low voltages or small internal diameter capillaries. This problem has been overcome by the use of non-ionic or zwitterionic surfactants. With one of these added to the running buffer, separation of heptapeptides with a single amino acid substitution was achieved (13).

In this paper we describe the use of long chain alkyl dimethyl-ammonium propanesulfonates for the formation of micelles in the running buffer as well as for the dynamic modification of the capillary surface. This method has been optimised for highly efficient separation of some basic polypeptides having equal or nearly equal mass to charge ratios.

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EXPERIMENTAL SECTION

Chemicals

3-(N,N-Dimethylhexadecylammonium) propanesulfonate (PAPS), 3-(N,N-dimethyltetradecylammonium) propanesulfonate (MAPS), 3-(N,N-dimethyldodecylammonium) propanesulfonate (DAPS), aniline, benzyl alcohol and p-cresol were obtained from Fluka (Buchs, Switzerland). Sudan III and benzoic acid were purchased from Merck (Darmstadt, Germany), phenol from Riedel-de Haën (Seelze, Germany) and ß-phenylethylamine from Aldrich (Steinheim, Germany).

Polymyxin B sulfate was kindly provided by Dumex Ltd. (Copenhagen, Denmark).

CE-apparatus

The Waters Quanta 4000 Capillary Electrophoresis System (Millipore, Milford, MA, USA) was used and on-column detection was performed by UV absorption at 214 nm. Electropherograms were recorded on a DP 700 Data Processor (Carlo Erba Instruments, Valencia, CA, USA).

Fused silica capillaries with the dimensions 25–100 μm I.D. and 360 μm O.D. were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total length of the capillaries used was 60 cm and 52.4 cm to the detector.

Procedure

The fused silica column was treated with 0.1 M sodium hydroxide for 30 min and subsequently 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.

0.2 M sodium phosphate stock solutions at pH 2.5, 4.0, 6.0 or 8.0 were used. The buffer system was composed of appropriate amounts of the zwitterion investigated, buffer stock solution and distilled water without further readjustment of pH.

A mixture consisting of the following 6 test substances: 0.4% aniline (pK_a 4.7), 0.05% ß-phenylethylamine (pK_a 9.8), 0.014% benzyl alcohol, 0.013% phenol (pK_a 9.9), 0.016% p-cresol (pK_a 10.2) and 0.07% benzoic acid (pK_a 4.2) was used to study the separation mechanisms involved and will subsequently be referred to as the test mixture.

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

Polymyxin B sulfate was dissolved in distilled water to obtain concentrations of 0.5 and 5.0 mM and kept refrigerated when not in use.

Sample injection was accomplished by hydrostatic injection (9.8 cm) for 1–10 seconds.

RESULTS AND DISCUSSION

Several ionic surfactants have been shown to improve selectivity of separation in CE when added to the running buffer. However, high concentrations of these surfactants are often necessary, especially in order to separate closely related species, and owing to the high ionic strength of the buffer only lower voltages can be applied.

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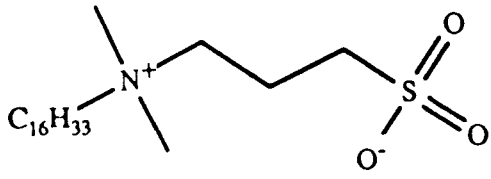

Figure 1. Structure of PAPS.

Zwitterions, on the contrary, have no net charge and do not contribute to the conductivity of the buffer. Thus they can be added to the running buffer at high concentrations while still allowing the application of high voltages to the capillary.

Recently, PAPS (fig. 1) has been investigated in HPLC (14) where it was shown that PAPS adsorbs to silica surfaces and gives rise to a reversed-phase effect. This additional separation mechanism due to PAPS provides changes in selectivity.

In the present investigations, PAPS was added to the running buffer, 0.2 M sodium phosphate pH 2.5 – water (1:1, v/v), increasingly up to a concentration of 30 mM. Figure 2 shows the electropherograms of the test mixture containing the two cations, aniline and ß-phenylethylamine, the three neutral compounds, benzyl alcohol, phenol and p-cresol, and benzoic acid with no PAPS added and with 25 μM of PAPS added to the running buffer. The addition of 25 μM of PAPS results in baseline separation of the three neutral substances and, concomitantly, leads to an increase of the electroosmotic flow velocity from 1.5 mm/s to 2.5 mm/s. Methanol and D_2O were used as electroosmotic flow markers. These results indicate that the zeta potential, in fact, has increased. In the HPLC experiments (14), it was shown that the adsorption of PAPS to the silica surface did not decrease the cation-exchange capacity of the system.

An abrupt change in electrophoretic mobility of benzoic acid, p-cresol and phenol is observed in a plot of the electrophoretic mobility against the surfactant concentration in the low μM range (fig. 3). These results are in accordance with those found in HPLC (14), where the reversed-phase effect only was obtained at PAPS concentrations above the CMC. One can expect these test substances to interact more strongly with the micelles

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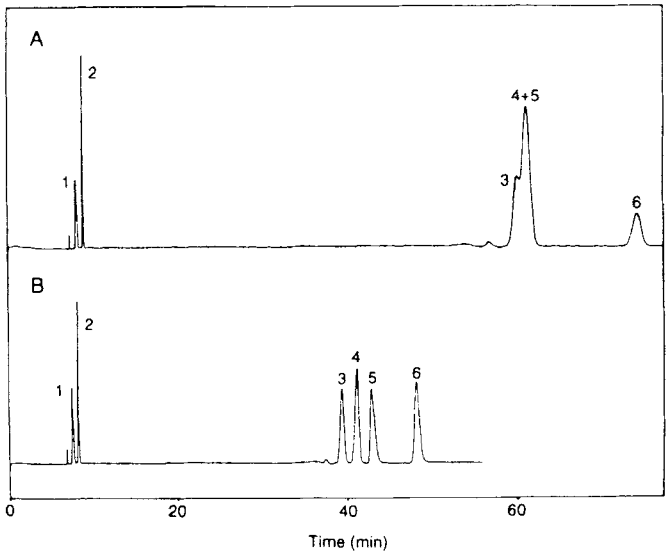

Figure 2. Electropherograms of six test substances obtained (A) with no PAPS and (B) with 25 μM of PAPS added to the running buffer. Capillary, 75 μm I.D.; buffer, 0.2 M sodium phosphate pH 2.5 – water (1:1, v/v); voltage, 15 kV; hydrostatic injection, 5 s. (1) Aniline, (2) ß–phenyl–ethylamine, (3) benzyl alcohol, (4) phenol, (5) p–cresol, (6) benzoic acid.

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than with the free surfactant molecules. The curves in figure 3 can therefore be used to estimate the CMC of PAPS at about 25 μM in this buffer solution, which is similar to the value given in (15).

When looking at the electrophoretic mobility of the test substances *versus* the concentration of PAPS in the mM range, a small decrease is observed for the two cations. The electrophoretic mobilities of benzoic acid, p–cresol and phenol all increase steadily above the CMC of PAPS, while the electrophoretic mobility of benzyl alcohol only shows a small increase. However, the appearance of the relative electrophoretic mobilities between benzyl alcohol, phenol, p–cresol and benzoic acid has changed considerably compared to the μM range.

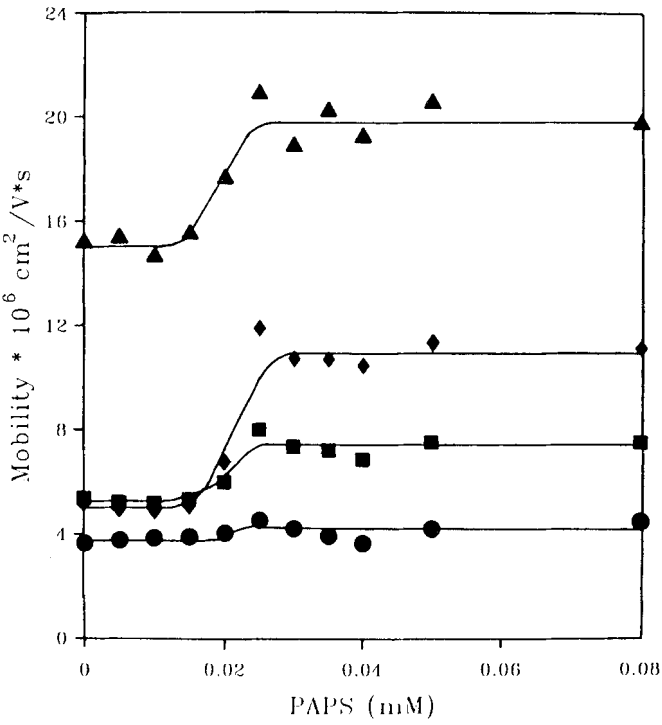

Figure 3. Determination of CMC of PAPS. Electrophoretic mobility of test substances *versus* the concentration of PAPS in the running buffer. Conditions as in fig. 2. (●) Benzyl alcohol, (■) phenol, (◆) p-cresol, (▲) benzoic acid.

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Proposed Separation Mechanism of PAPS

PAPS adsorbs to the surface of silica when added to the buffer in concentrations above the CMC. This dynamic modification provides the capillary wall with a lipophilic surface as well as the ammonium propane sulfonate groups. The zwitterionic surfactants increase the double layer thickness of the capillary wall and the zeta potential primarily due to the strong anionic character of the sulfonate groups. This, in turn, leads to an increase in electroosmotic flow. The curve obtained when plotting the electroosmotic flow coefficient against the concentration of PAPS exhibits a

similar abrupt change around 25 μM of PAPS as observed with the electrophoretic mobilities of the test substances (fig. 3).

Above the CMC of PAPS, separation depends on both the partitioning of solute molecules between the bulk solvent and the micellar pseudo phase in the buffer solution, and also the difference in electrophoretic mobility of the bulk solvent *versus* the micelle. 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 charged than positively charged, because the smaller, less hydrated and more polarising anions have a greater tendency to be specifically adsorbed than the cations (16). Adsorption of anions onto the micelles gives rise to a 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.

The three neutral substances are separated at and above the CMC of PAPS due to hydrophobic interactions with the inner core of the micelles. The test substances elute in order of increasing degree of hydrophobicity with migration times between those of methanol and Sudan III. Apparently, benzoic acid exhibits a weak charge at pH 2.5 and therefore elutes after the three neutral compounds both without and with PAPS added to a concentration above it's CMC in the running buffer.

The selectivity of migration of aniline and ß-phenylethylamine is not influenced by the addition of PAPS above the CMC. But their migration times are almost constant, and a decrease in electrophoretic mobility is observed at high concentrations of PAPS. This compared with the increased electroosmotic flow, indicates that the cations interact with the surface of the micelles.

Separation of Decapeptide Antibiotics

The method has been applied for the separation of polymyxins, which are a group of closely related decapeptide antibiotics produced by strains of *Bacillus polymyxa*. The polymyxins B (fig. 4) have a general structure composed of a cyclic heptapeptide moiety and a side chain consisting of a

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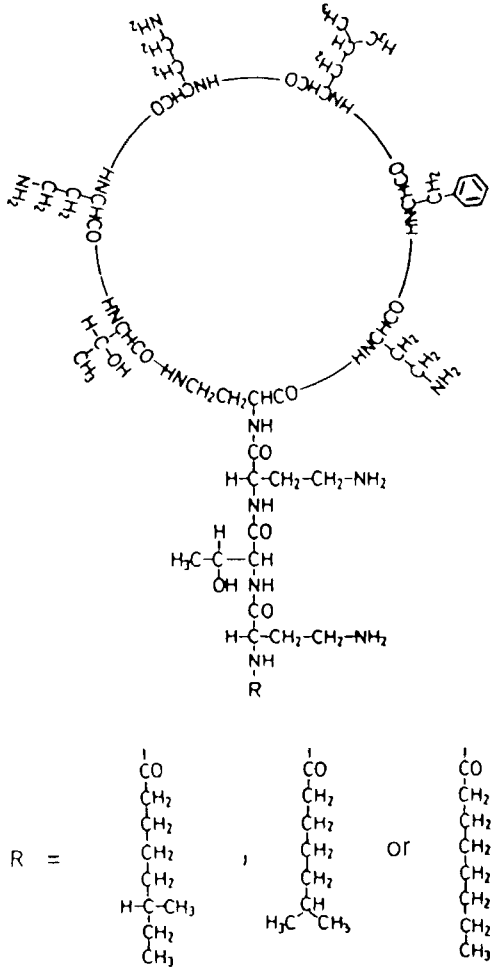

Figure 4. Structure of polymyxins B. R represents 6-methyloctanoyl (polymyxin B_1), 6-methylheptanoyl (polymyxin B_2) or n-octanoyl (polymyxin B_3).

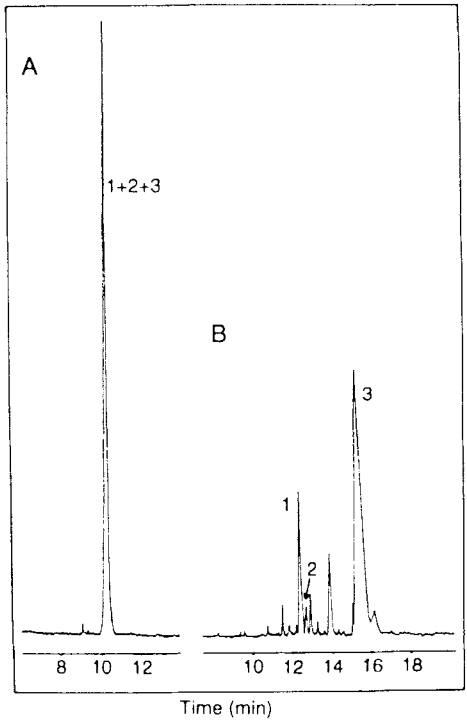

Figure 5. Electropherograms of polymyxins B obtained (A) with no PAPS and (B) with 30 mM of PAPS added to the running buffer. Capillary, 75 μm I.D.; buffer, 0.2 M sodium phosphate pH 2.5 – water (1:1, v/v); voltage, 15 kV; hydrostatic injection, 10 s. (1) polymyxin B_2, (2) polymyxin B_3, (3) polymyxin B_1.

tripeptide with a fatty acyl residue. The characteristic feature of polymyxins B, besides the hydrophobic fatty acid moiety, is their strong basicity due to 5 unmasked amino groups from 2,4-diaminobutyric acid. These peptides have equal or nearly equal mass to charge ratios and therefore exhibit very similar electrophoretic mobilities. In consequence, they are very suitable as test substances in order to study the ability of PAPS to separate almost identical peptides.

Figure 5 shows the electropherograms of polymyxin B with no PAPS added and with 30 mM of PAPS added to the running buffer. No separation

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of the peptides was obtained in plain buffer while addition of 30 mM PAPS provided a significant increase in separation of the peptides resulting in at least ten peaks. The peaks corresponding to polymyxins B_1, B_2 and B_3 have been identified using preparative HPLC and GC–MS (17).

The electrophoretic mobilities of polymyxin B_1 and B_2 were calculated based on peak 3 and 1, respectively, and plotted against the concentration of PAPS. With increasing concentration of PAPS, the electrophoretic mobility of the two peptides decreases. Simultaneously, the relative mobility of the peptides increases as the electrophoretic mobility of polymyxin B_1 decreases faster than that of polymyxin B_2.

Similar separations of the peptides in polymyxin B have been obtained using the surfactants, DAPS and MAPS, as additives to the running buffer. These two zwitterionic surfactants differ from PAPS with respect to the length of the alkyl chain. As they have shorter chain than PAPS, their CMC's have shifted to higher concentrations compared to PAPS. Consequently, around 40 mM of DAPS or 30 mM MAPS is required to achieve separations of the peptides similar to those obtained using 25 mM of PAPS.

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Proposed Separation Mechanism for the Peptides

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 at concentrations above their CMC, the peptides show ion–exchange partitioning to the surface of the micelles. While associated with the micelles, the peptides migrate with the speed of the micelles. As the peptides have different partition coefficients to the ion exchanger, the surface of the micelles, an efficient separation is obtained.

The hydrophobic moiety of the peptides may show affinity for the inner core of the micelles, while the remaining polar parts of the peptides stay outside. Consequently, the two peptides, polymyxin B_2 and B_3, with equal mass/charge ratios are eluted in order of increasing degree of hydrophobicity and a separation is achieved. Polymyxin B_1 has a different partition coefficient to the ion exchanger due to a higher mass to charge

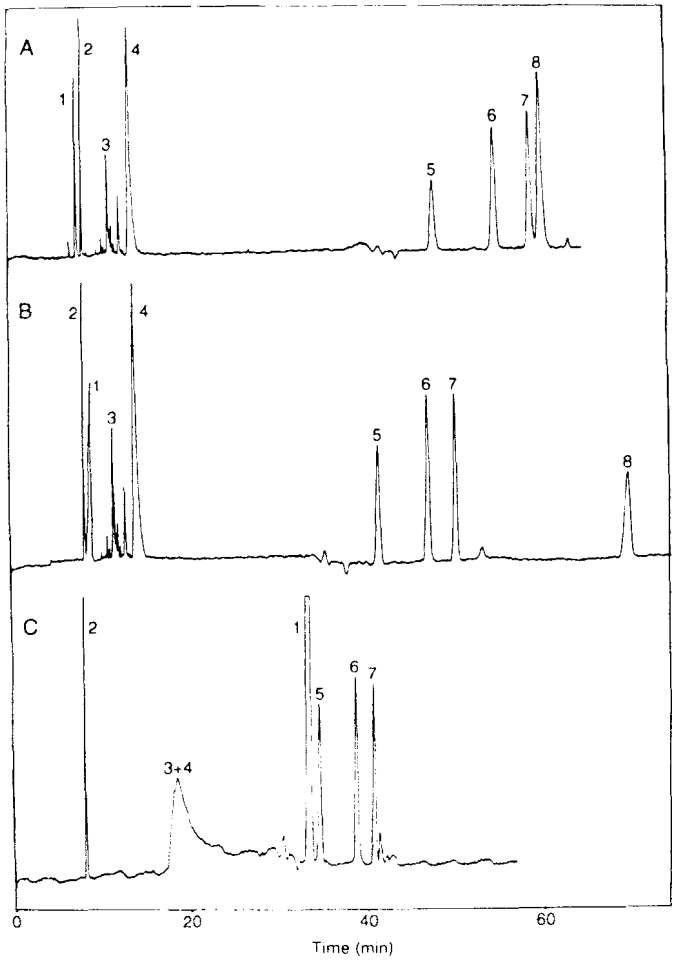

Figure 6. Electropherograms of polymyxins B and six test substances obtained at (A) pH 2.5, (B) pH 4.0, (C) pH 6.0 and (D) pH 8.0. Capillary, 75 μm I.D.; buffer, 25 mM PAPS in 0.2 M sodium phosphate pH 2.5, 4.0, 6.0 or 8.0 – water (1:1, v/v); voltage, 15 kV at pH 2.5, 4.0 and 6.0 and 10 kV at pH 8.0; hydrostatic injection, 2 s. (1) Aniline, (2) ß–phenylethylamine, (3) polymyxin B_2, (4) polymyxin B_1, (5) benzyl alcohol, (6) phenol, (7) p–cresol, (8) benzoic acid.

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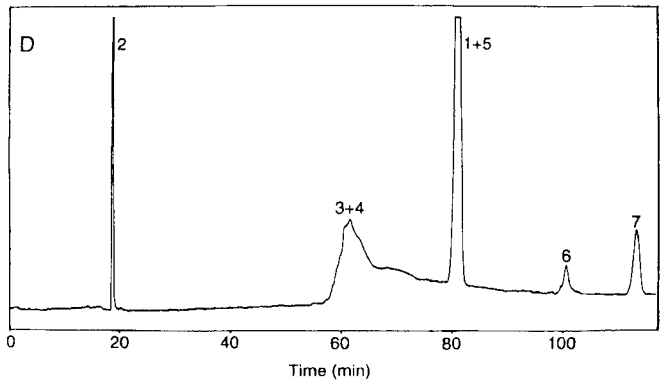

Figure 6 (continued)

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ratio. The other peaks observed in the electropherogram in fig. 5B are due to substitution of amino acids in polymyxin B resulting in other peptides, which exhibit different partition to the micelles.

Influence of pH

In order to investigate the effect of pH on the separation, separations were performed at pH 2.5, 4.0, 6.0 and 8.0, respectively, and the obtained electropherograms are shown in fig. 6. A mixture of the peptides in polymyxin B and the six test substances were used as test solution.

The peptides in polymyxin B are still separated at pH 4. At pH 6, the peptides interact with the surface of the capillary due to an increase in ionisation of the free silanol groups which will be present at the wall because the dynamic modification with PAPS only provides partial coverage of the silica surface (14). In consequence, zone broadening and no separation of the peptides are observed. The migration times of the peptides have increased, although the electroosmotic flow increases with increasing pH.

At pH 4, the order of aniline and ß-phenylethylamine is reversed, and at pH 6, aniline (pK_a 4.7) is no longer ionised and migrates as a neutral

substance. Benzoic acid is not observed in the electropherogram obtained at pH 6 where it migrates as a negatively charged ion.

Benzyl alcohol, phenol and p-cresol are neutral in the pH interval investigated. The electrophoretic mobilities of these non-ionic test substances are almost constant throughout the pH range 2.5-8.0.

ACKNOWLEDGEMENT

This work was supported by The Alfred Benzon Foundation, The Lundbeck Foundation and The Danish Natural Science Research Council, grant no: 11-8439-1.

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Received: October 14, 1992
Accepted: February 16, 1993

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