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Electrochromatographic contributions in capillary electrophoresis of biomolecules

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Abstract

In this study, electrically driven separations of charged biomolecules including ribonucleotides and peptides were investigated using various surface modified columns. These columns included cationic, neutral and anionic polymeric-coated columns. Experiments demonstrated that for neutral polymer-coated columns, solutes eluted according to their electrophoretic mobilities. Using surface charged columns, the elution of charged solutes was determined by their electrophoretic mobilities, electroosmotic flow rates and interactions between charged solutes and the charged column inner wall. Retention factors were used to investigate the interactions between solutes and the column inner wall. Column efficiency measurements for charged solutes on charged surface columns were also examined, and the results were compared with those obtained using neutral polymer-coated columns. It was found that separations of multivalent biomolecules were difficult using charged surface columns because of either strong adsorption or high electrophoretic mobilities. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Previous capillary electrophoresis (CE) column technology studies have been mainly focused on reducing sample–wall interactions and eliminating electroosmotic flow (EOF) created by the fused-silica wall [1–5]. It is generally thought that interactions of solutes with the column inner wall only affect the column efficiency in CE, and the separation depends only on the difference in electrophoretic mobilities of the solutes.

Selectivity in chromatographic separations is mainly achieved by partition or adsorption interactions between solutes and the stationary phase. Recently, capillary electrochromatography (CEC) has aroused high interest because high column efficiencies similar to CE and selectivities of chromatography can be obtained simultaneously [6–8]. Capillary columns packed with microparticles are typically used in CEC.

Open tubular columns have also been investigated for selective separation of neutral solutes in CEC, however, unsatisfactory column efficiency was obtained [9]. Narrow bore (~10 μm I.D.) capillary columns modified using sol–gel technology have been used to improve this situation [10], however,

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the use of extremely narrow bore columns is too limiting with respect to sample capacity.

Most biomolecules are charged, and they are only stable in certain pH ranges. The interactions of charged analytes in CE are relatively complex. Solutes can be driven by the electrical field to produce movement, the direction and rate of which depends on the sign and number of charges on the solute. If a significant EOF exists in the column, it also forces the solute to flow along the column. The direction and rate of this flow are determined by the charge properties on the capillary column inner wall or packed particle surfaces on which EOF is produced. Furthermore, charged solutes interact with the charged capillary inner wall or charged particle surface. The suitability of charged surfaces for separations of charged samples has needed further investigation.

In this study, various charged inner column surfaces were prepared for electrically driven separations of charged biomolecules. Fused-silica capillary columns of 50 μm I.D. were used. Modifications of the capillary column inner wall were carried out yielding cationic, anionic and neutral surfaces. Ribonucleotides and peptides were used as test solutes to investigate the contributions of various migration and separation mechanisms.

2. Experimental

2.1. Materials and instrumentation

Ribonucleotide and peptide standards, polyethylenimine (PEI, 50% water solution), 1,4-butanediol glycol diglycidyl ether (BUDGE), 2-acrylamido-2-methylpropanesulfonic acid (AMSA), dibasic sodium phosphate, monobasic potassium phosphate and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). A neutral polyalkyloxide (Ucon 75-H-90 000) was purchased from Alltech (Deerfield, IL, USA). Acetonitrile, 1,4-dioxane, dicumyl peroxide (DCP) and hexamethyldisilazane (HMDS) were purchased from Aldrich (Milwaukee, WI, USA). Fused-silica capillary tubing (50 μm I.D.×360 μm O.D.) was purchased from Polymicro Technologies (Phoenix, AZ, USA). Deionized water for the preparation of the buffer solutions, as well as for rinsing capillaries, was obtained from a Milli-Q water system (Millipore, Millford, MA, USA).

A Model CES-1 CE system (Dionex, Sunnyvale, CA, USA) was used without any modification in this study. An optical window was generated by burning off a short section of polyamide coating on the outside of the column. The samples were detected by on-column UV absorbance at 254 nm. Data were collected using a Model SP4290 integrator (Spectra-Physics, San Jose, CA, USA).

2.2. Preparation of columns

Prior to coating the fused-silica columns with various polymers, they were rinsed with 5 ml of 1 *M* NaOH, 5 ml of 1 *M* HCl, 5 ml of water, 5 ml of methanol and 5 ml of methylene chloride for 20 min each. The whole rinsing process took approximately 2 h. The columns were finally purged with nitrogen gas for 10 min.

2.2.1. Cationic capillary surface

Polymeric PEI (Fig. 1A) was used to prepare capillary columns having a cationic surface. The

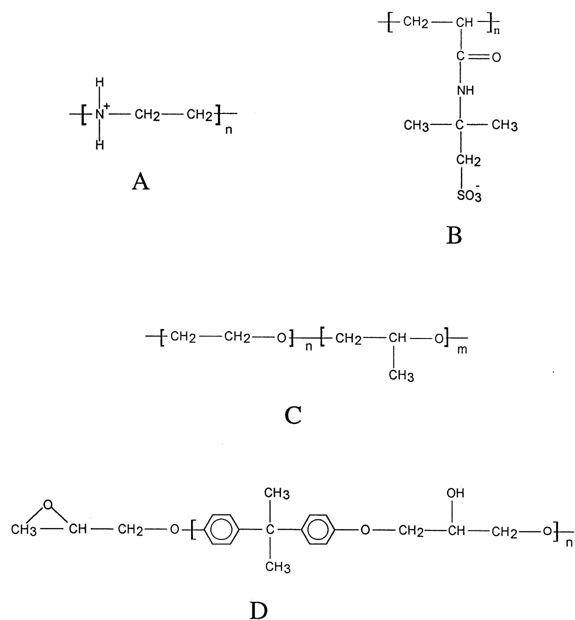

Fig. 1. Structures of polymers used for fused-silica capillary column surface modifications.

coating procedure is based on the work of Pearson and Regnier [11] with some modifications. A 30% (w/v) solution of PEI in methanol and 10% (w/v) solution of BUDGE in 1,4-dioxane were prepared. The coating was performed by passing 5 ml of the PEI solution through the column and crosslinking overnight with BUDGE–dioxane solution at room temperature.

2.2.2. Anionic capillary surface

AMSA (Fig. 1B) was used to prepare capillary columns having an anionic surface. The coating was performed by passing 5 ml of 30% AMSA solution through the column and removing the water by rinsing with 10 ml of methanol solution and then with 10 ml of methylene chloride. Crosslinking was performed by slowly purging ATB headspace vapors through the column with helium at 80°C for 30 min.

2.2.3. Ucon-coated surface

A neutral capillary column surface was prepared by coating with neutral Ucon 75-H-90 000 (Fig. 1C). The coating procedure was followed as described elsewhere [12]. Briefly, capillary columns were statically coated using a mixture of Ucon (1–8 mg ml^{-1}) in methylene chloride and dicumyl peroxide (0.2–0.5 mg ml^{-1}). After the solvent was evaporated, the coated capillary column was purged with nitrogen for 20 min and sealed at both ends with an oxyacetylene flame without allowing air to enter the column. Then the sealed capillary was heated from 40°C to 180°C at 4°C min^{-1} and held at 180°C for 60 min to carry out the crosslinking reaction.

2.2.4. Epoxy-coated surface

A second neutral capillary column surface was prepared using an epoxy resin (Fig. 1D). A dynamic coating method was used [13]. Briefly, 1% (w/v) epoxy in methylene chloride was passed through the column for 5 min and then the column was left for approximately 10 min to dry.

3. Results and discussion

3.1. Surface characteristics of modified columns

A rather quick test to determine whether or not the column surface modification was successful is to measure the EOF direction and magnitude. For untreated fused-silica columns, a negatively charged surface results when in contact with typical buffer solutions. The relationship between EOF and pH on untreated columns has been measured by numerous workers [13]. In this study, DMSO was used as a neutral marker to determine the EOF values produced by various surface modified columns, and negatively charged solutes were used to investigate the interactions with the column surface.

For both Ucon-coated or epoxy resin-coated columns, the EOF values were reduced to less than $3.3\cdot10^{-5}$ cm^2 s^{-1} V^{-1} from $4.8\cdot10^{-4}$ cm^2 s^{-1} V^{-1} for the untreated fused-silica column at pH 6. When lowering the pH to 3.0, similar EOF values (~$2.3\cdot10^{-5}$ cm^2 s^{-1} V^{-1}) were obtained for the untreated, epoxy-coated and Ucon-coated columns. At this low pH, few silanol groups were ionized. When a 15 kV voltage was imposed across a 50 cm long column, the velocity of the buffer solution produced by this EOF was approximately 0.0067 cm s^{-1}. This low flow is not significant in practical CE.

For the PEI-coated columns, the direction of EOF was reversed in comparison to that of an untreated fused-silica column. This suggests that a positively charged surface was formed. A large current (>100 μA) was measured when using the same buffer conditions as for the untreated fused-silica capillary columns. In order to decrease the current and, simultaneously, adjust the conditions for CEC operation, acetonitrile was added to the buffer solution. At pH 3.0, an EOF of $4.8\cdot10^{-5}$ cm^2 s^{-1} V^{-1} was detected from the cathode to the anode, even when the acetonitrile content was as high as 60% (v/v). This value is twice that provided by the untreated fused-silica capillary columns with no acetonitrile in the buffer solutions. When a 15 kV voltage was imposed across a 50 cm long column, the velocity of the buffer solution produced by the EOF was ~0.015 cm s^{-1}.

AMSA-coated columns provided an EOF of $2.4\cdot10^{-4}$ cm^2 s^{-1} V^{-1} with direction from the anode to the cathode when using a mixture of acetonitrile–buffer solution (60:40, v/v) at pH 3.0. This direction of flow was the same as that for untreated fused-silica columns. However, more than 10-times larger EOF was obtained with the coated column compared

to that obtained for untreated fused-silica columns with no acetonitrile in the buffer solution. A solution flow-rate of ~0.12 cm s^{-1} driven by the EOF was obtained for a 50 cm long column and 15 kV voltage.

In addition to electrophoretic mobility and EOF, the migration time of a charged solute can be affected by interactions with the charged column surface. As illustrated in Fig. 2, $F_{attraction}$ is the force of attraction between the negative solutes and the positive charged wall, $F_{repulsion}$ is the force of repulsion between the negative solutes and the negative charged wall, μ_{eof} is the EOF velocity, and μ_{ep} is the apparent electrophoretic mobility. However, interaction between the charged solute and capillary wall can be affected by the formation of a double layer near the charged capillary wall [14]. Tailoring of the surface charge was one early method of controlling the adsorption of selected proteins [15].

3.2. Separation of negatively charged biomolecules using columns with charged surface coatings

On the neutral Ucon-coated column, the ribonucleotides eluted in the order of triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP), as illustrated in Fig. 3. This elution order is in agreement with the magnitudes of the electrophoretic mobilities of the solutes. ATP has the highest electrophoretic mobility (three negative charges) and AMP has the smallest electrophoretic mobility (one negative charge). Table 1 lists the mobilities of the three test ribonucleotides at pH values of ~3.0.

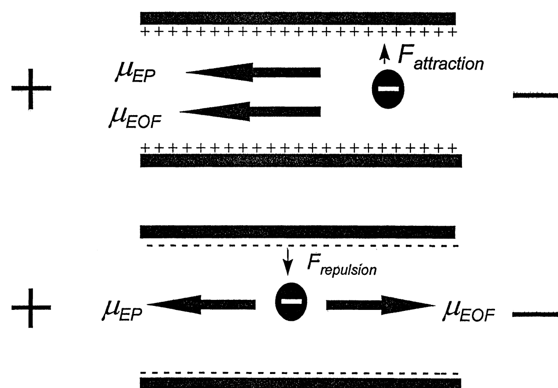

Fig. 2. Selected interactions of charged solutes in capillary columns with charged surfaces.

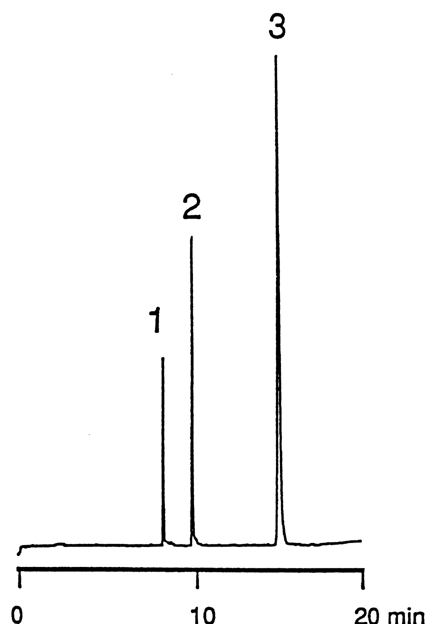

Fig. 3. Capillary electropherogram of test ribonucleotides using a capillary column coated with a neutral polymer. Conditions: 45 cm×50 μm I.D. fused-silica capillary column coated with Ucon, 40 m*M* phosphate buffer (pH~3.0), −20 kV applied voltage, UV detection (254 nm). Peaks: 1=ATP, 2=ADP, 3=AMP.

It is noteworthy that even though the EOF can be reduced to a negligible value by coating the column with neutral polymer, the properties of the coated polymer can also affect the separation. Fig. 4 shows a comparison of CE separations of the ribonucleotides UTP and CTP. Using the Ucon-coated column, they are baseline separated. However, using the epoxy resin-coated column, no separation was

Table 1
Mobilities and retention factors of test ribonucleotides on a PEI-coated column[a]

Solutes	μ_{AP} (cm s^{-1})	μ_{EP} (cm s^{-1})	k
ATP	0.059	0.140	1.64
ADP	0.093	0.123	0.50
AMP	0.052	0.073	0.70

[a] Conditions: 50 cm×50 μm I.D. fused-silica capillary column coated with PEI, 15 kV, pH 3.0, 0.015 cm s^{-1} EOF. Other conditions as in Fig. 4.

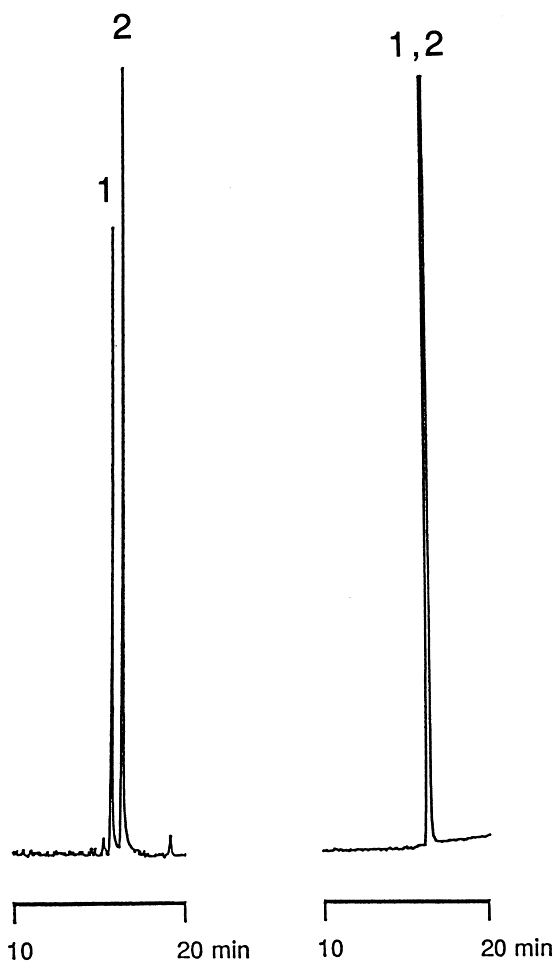

Fig. 4. Capillary electropherograms of UTP and CTP using fused-silica capillary columns modified with (left) neutral Ucon polymer and (right) epoxy resin. Conditions: 45 cm×50 μm I.D. capillary columns, 40 m*M* phosphate buffer, −10 kV applied voltage, UV detection (254 nm). Peaks: 1=UTP and 2=CTP.

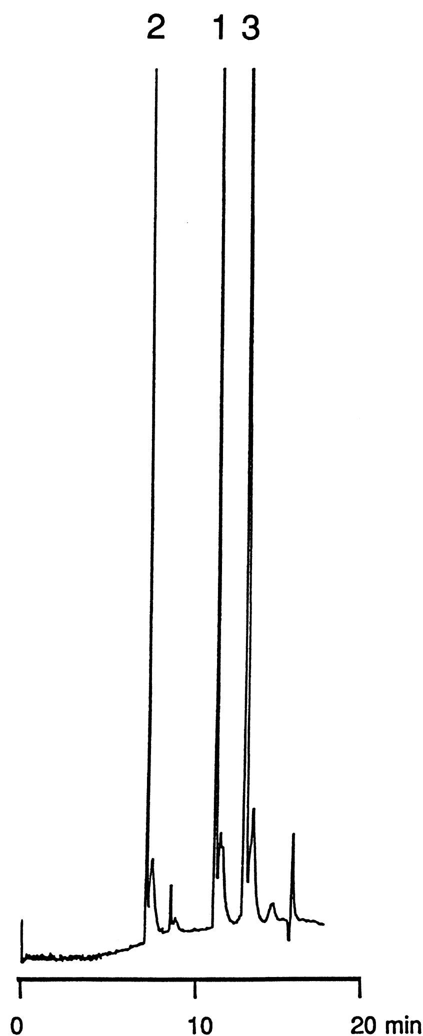

Fig. 5. Capillary electropherogram of test ribonucleotides using a capillary column coated with a cationic polymer. Conditions: 45 cm×50 μm I.D. fused-silica capillary column coated with PEI, acetonitrile–phosphate (60:40, pH~3.0), −20 kV applied voltage, UV detection (254 nm). Peaks: 1=ATP, 2=ADP, 3=AMP.

obtained. Comparing the structures of the Ucon and epoxy polymers (Fig. 1C,D), it can be surmised that the presence of benzene rings in the epoxy resin is probably the cause of different selectivity. Proper choice of coatings appears to be necessary to obtain the desired CE separation. On the PEI-coated column, the ribonucleotides eluted in the direction from cathode to anode. However, the elution sequence was ADP, ATP and AMP, as illustrated in Fig. 5.

In order to understand the contributions of solute–wall interaction on elution (or retention), the retention factor concept was used. In CE, the retention factor can be expressed as follows:

$$\mu_{AP} = (\mu_{EP} - \mu_{EOF}) \cdot \frac{1}{1+k} \qquad (1)$$

where μ_{AP} is the measured apparent mobility of the solute and μ_{EP} is the electrophoretic mobility of the solute. When the EOF is in the same direction as the

electrophoretic mobility, a positive sign is used, while the opposite is true when the EOF is in the opposite direction as the electrophoretic mobility [1].

The calculated retention factors for the test ribonucleotides on a PEI-coated column are listed in Table 1. From the results, it can be seen that ATP with its three negative charges has a much higher retention factor than ADP or AMP when using a positive charged column surface. It is not clear why AMP has a slightly higher retention factor than ADP. The interaction of the charged solutes and the column inner wall altered the elution order of the solutes and improved the separation, as is illustrated in Figs. 3 and 5. The small impurities in the sample were separated when using the positively charged capillary column.

For the negatively charged columns using AMSA, the direction of EOF was opposite to that of the electrophoretic mobilities of the negatively charged solutes, as illustrated in Fig. 2. It was found that the EOF determined the elution of AMP, while elution of the more highly charged ADP and ATP was controlled by the electrophoretic mobilities of the solutes, even though a low pH of 3.0 and a high acetonitrile content (60%) were used. From the data in Table 1, it can be seen that the mobilities of the solutes were 0.140 and 0.123 cm s^{-1} for ATP and ADP, respectively. The velocity of the solution driven by the EOF was only 0.120 cm s^{-1} for the AMSA-coated columns, which is slightly smaller than the electrophoretic mobilities of ADP and AMP. We could not elute ADP and ATP on the AMSA-coated columns.

Column efficiencies of $5.0 \cdot 10^5$, $2.8 \cdot 10^5$ and $1.6 \cdot 10^5$ plates were achieved on neutral Ucon-coated columns for ATP, ADP and AMP, respectively. These efficiencies can be considered to be typical of a pure CE process. For PEI-coated columns, the interaction between the capillary column inner wall and the solutes altered the elution sequence of the solutes, however, no significant losses in column efficiency were observed. For ATP, ADP and AMP, column efficiencies of $1.8 \cdot 10^5$, $2.1 \cdot 10^5$ and $1.6 \cdot 10^5$ plates were obtained, respectively. This result suggests that even with solute–wall interactions leading to a retention factor as high as 1.64, column efficiency was not sacrificed.

3.3. Separation of positively charged biomolecules using columns with different surface charges

After studying the negatively charged molecules in great detail, we further investigated the effects of different surface charges on the separation of positively charged biomolecules. Peptides were used in these experiments as test solutes. The peptides selected carried more charges than the ribonucleotides and, therefore, they imposed a greater challenge for separation. Our results show that by using a neutral Ucon treated surface, all three peptides were baseline resolved and the separation efficiency was quite high. The first peak to elute was Gly–Gly–Trp–Arg (five positive charges) with $2.2 \cdot 10^5$ plates, the second peak was Arg–Gly–Asp (four positive charges) with $2.7 \cdot 10^5$ plates, and the third peak was Ala–Gly–Ser–Glu (four positive charges) with $1.1 \cdot 10^5$ plates. All three peptides were eluted in

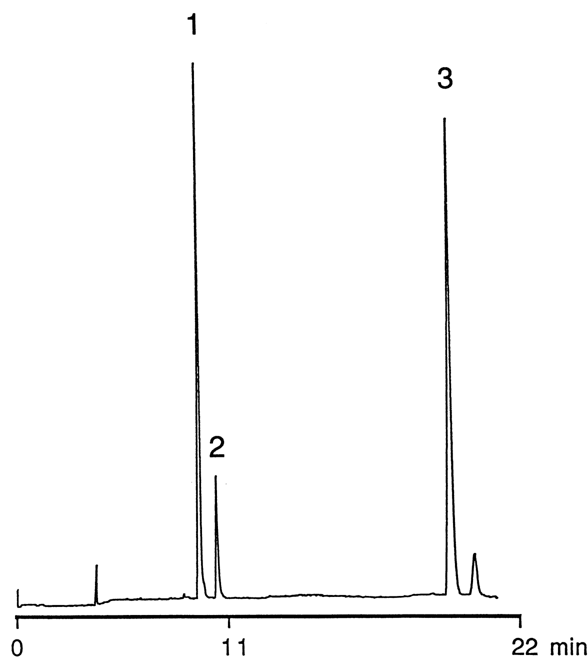

Fig. 6. Capillary electropherogram of peptides using a capillary column coated with a neutral polymer. Conditions: 45 cm×50 μm I.D. fused-silica capillary column coated with Ucon, 20 m*M* Tris·HCl buffer (pH~4.8), 20 kV applied voltage, UV detection (215 nm). Peaks: 1=Gly–Gly–Try–Arg, 2=Arg–Gly–Asp, 3=Ala–Gly–Ser–Glu.

the direction from anode to cathode within 22 min (Fig. 6).

When the peptides were separated on the AMSA-coated column, it was found that the last two peaks began to overlap. Severe peak tailing was also noted, as is shown in Fig. 7. This demonstrates that even when the column surface is the same charge as the test solutes, there can still be strong interaction between the sample and the wall. The peptides still migrated from the anode to the cathode, which further confirms our conclusion that for multivalent molecules, electrophoretic mobility plays the decisive role in the direction of elution of the solutes. However, the separation efficiencies for the peptides were rather poor. The retention times using the AMSA-coated column were much faster than those obtained using the Ucon-coated column. However, after several runs, the current started to decrease and peak tailing became more severe. On the PEI-coated column, unstable electrical current was obtained and no peaks were observed at either the cathode or the anode.

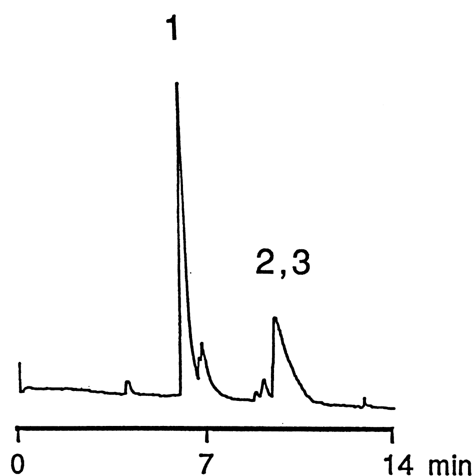

Fig. 7. Capillary electropherogram of peptides using a capillary column coated with an anionic polymer. Conditions: 45 cm×50 μm I.D. fused-silica capillary column coated with ASMA, acetonitrile–Tris·HCl buffer (pH 4.8), 20 kV applied voltage, UV detection (215 nm). Peaks: 1=Gly–Gly–Try–Arg, 2=Arg–Gly–Asp, 3=Ala–Gly–Ser–Glu.

4. Conclusions

By modifying the chemical composition of the capillary surface, different interactions were introduced between the charged solutes and the capillary walls, which affected solute migration times and even elution orders. However, no loss in column efficiency was found in some cases, while low efficiencies and poor peak shapes were observed in others. While the mechanisms for these observations are not entirely clear, surface interactions with solute clearly affect the elution of analytes, and strongly suggest an electrochromatographic contribution in certain cases.

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