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Microemulsion electrokinetic chromatography of proteins

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Abstract

Microemulsion electrokinetic chromatography was used to separate a test mixture of proteins effectively. The separation was carried out in a 42.5 cm (to the detector)×50 μm I.D. fused-silica capillary using a microemulsion system consisting of 80 m*M* heptane, 120 m*M* SDS, 900 m*M* butanol in 2.5 m*M* borate buffer, pH 8.5–9.5. Optimum separation conditions were investigated with respect to the running voltage, temperature, pH and the composition of microemulsion. Results were compared with those obtained in micellar electrokinetic chromatography and capillary zone electrophoresis. The examined method is practical and successfully applied to the assay of genetically engineering pharmaceuticals, recombinant human granulocyte macrophage colony stimulating factor injection and recombinant human granulocyte colony stimulating factor injection.

Keywords: Microemulsion electrokinetic chromatography; Proteins

1. Introduction

Capillary electrophoresis (CE) is a powerful tool for protein separation. Electrokinetic chromatography (EKC) is a branch of CE, which is widely used for the separation of hydrophobic molecules. Microemulsion electrokinetic chromatography (MEEKC) with sodium dodecyl sulfate (SDS), 1-butanol, heptane and water solution has emerged only recently. It was first introduced by Watarai [1] in 1991 for the separation of fluorescent aromatic compounds; Terabe et al. [2] compared MEEKC with micellar electrokinetic chromatography (MEKC) in terms of the separation selectivity, efficiency and the effect of the surfactant fraction; Ishihama and co-workers used MEEKC to evaluate the hydrophobicity scale based on the migration index for neutral solutes [3], anionic solutes [4] and cationic solutes [5]; Gluck et al. [6] used MEEKC as a screening tool for the indirect determination of octanol–water partition coefficients based on the retention factors of MEEKC; Boso et al. [7] used this system for the separation of fat- and water-soluble vitamins; Vomastova et al. [8] separated steroids and Fu et al. [9] separated antipyretic analgesic ingredients; Sandra et al. [10] used MEEKC for the resolution of hop bitter acids. Recently, Miksik et al. [11] separated diphenylhydrazones of dicarbonyl sugars and Debusschere et al. [12] separated cardiac glycosides.

In MEEKC, the separation medium is a microemulsion, i.e., a transparent solution consisting of a surfactant, a co-surfactant, oil and water. The structure of an oil in water emulsion is similar to that of the micelle except that the microemulsion has an oil droplet as a core. The surfactant and co-surfactant are located on the surface of the droplet in order to stabilize it.

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It is well known that proteins consist of amino acids with different hydrophobicity. There are eight amino acids with non-polar side-chains, and proteins with these amino acids have various hydrophobicity. In this study, we demonstrate the possibility of MEEKC in protein separation, and investigate factors influencing MEEKC separation, in terms of separation ability and efficiency. Comparison with MEKC and capillary zone electrophoresis (CZE) was also studied.

2. Materials and methods

2.1. Materials

Ribonuclease A (isoelectric point, pI=9.45), carbonic anhydrase II (pI=5.90), β-lactoglobulin A (pI=5.10) and myoglobin from horse heart (pI=7.0) were purchased from Sigma (St. Louis, MO, USA). SDS was purchased from Serva (Heidelberg, Germany); pentane, heptane, octane, nonane, decane, butanol, propanol, pentanol, disodium phosphate and Sudan III were obtained from Shanghai Guangming Chemical Reagent Plant (Shanghai, China). Recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) and recombinant human granulocyte colony stimulating factor (rhG-CSF) injections were obtained from the commercial company in Nanjing (Jiangsu, China). All buffers were prepared in ultra-pure water made by a Milli-Q Water System (Millipore, Bedford, MA, USA).

2.2. Capillary electrophoresis

MEEKC experiments were carried out with a Waters Quanta 4000E capillary electrophoresis unit using BASELINE 810 software. The capillaries were untreated fused-silica capillaries, 50 cm (42.5 cm to the detector)×50 μm I.D., purchased from Supelco (Bellefonte, PA, USA). Stock solutions of standards were prepared as 8, 4, 4 and 1 mg/ml solutions in water for ribonuclease A, myoglobin, carbonic anhydrase II and β-lactoglobulin A, respectively. The test mixture was prepared by mixing 10 μl of all stock solutions with 20 μl of the selected background buffer. Samples were introduced hydrodynamically at an injection time of 5 s. Separations were routinely run at 20 kV at 13°C and monitored for absorbance at 214 nm. Methanol was used as electroosmotic flow (EOF) marker and Sudan III was used to determine and characterize the elution window. In preparation for separation, the capillary was consecutively rinsed with 0.1 *M* NaOH for 2 min, water for 2 min and running buffer for 5 min.

Microemulsions were prepared according to the methods reported in the literature [1–5]. Microemulsion-forming organic solutes, SDS, butanol were mixed with borate buffer that was optimized with respect to buffer concentration (1–20 m*M*) and pH (7–11).

MEKC separations were performed in 10 m*M* borate with SDS, which were optimized with respect to pH (8–9.5), and SDS concentration (50–120 m*M*). One hundred m*M* phosphate, 100 m*M* borate and 50 m*M* phosphate–50 m*M* borate were employed as CZE buffers. The exact compositions are given in the figures.

3. Results and discussion

3.1. MEEKC separation

The optimum conditions for separation using the microemulsion system were 2.5 m*M* borate buffer, pH 8.5–9.5, 80 m*M* octane or heptane, 900 m*M* butanol and 120 m*M* SDS (Fig. 1). The highest number of theoretical plates was $7.43 \cdot 10^3$ (myoglobin).

Under alkaline conditions, the electroosmotic mobility of the bulk solutions was nearly identical. Separations at pH values of 7, 8 and 11 were not satisfactory. In the range of pH 8.5–9.5, good separations were obtained as shown in Fig. 2.

In contrast to neutral solutes, the charged proteins possess their own electrophoretic mobility in the bulk aqueous phase, as well as partitioning into microemulsion phase. Therefore, in MEEKC the mobility of a protein would be the weighed sum of the mobility of the microemulsion phase and its own mobility in the aqueous phase. The migration velocity of the solute (U_x) in MEEKC was as follows:

$$U_x = \frac{1}{1+k'} \cdot (\mu_{eo} + \mu_{ep}) \cdot \frac{V}{L_t} + \frac{k'}{1+k'} \cdot \mu_{me} \cdot \frac{V}{L_t} \quad (1)$$

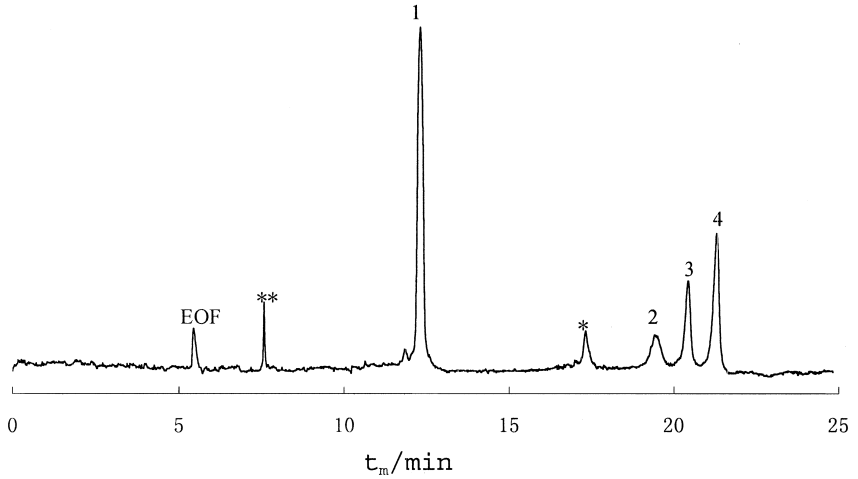

Fig. 1. Separation of the test mixture of proteins by MEEKC. Separation conditions: 120 m*M* SDS, 80 m*M* heptane, 900 m*M* butanol in 2.5 m*M* borate, pH 8.5, 20 kV, 13°C. Peaks: 1 = ribonuclease A, 2 = carbonic anhydrase II, 3 = β-lactoglobulin A and 4 = myoglobin, * = impurity in ribonuclease A, ** = impurity in carbonic anhydrase II.

As $t_m = L_s/U_x$, Eq. (1) can be given by

$$\frac{1}{t_m} = \frac{1}{1+k'} \cdot (\mu_{eo} + \mu_{ep}) \cdot \frac{V}{L_t L_s} + \frac{k'}{1+k'} \cdot \mu_{me} \cdot \frac{V}{L_t L_s} \quad (2)$$

where k' is the retention factor of the solute in MEEKC; μ_{me} and μ_{ep} are the electrophoretic mobilities of microemulsion and solute, respectively; μ_{eo} is the electroosmotic mobility; V is running voltage; L_t and L_s are the total and separation lengths of the capillary, respectively; and t_m is the migration time.

The linear relationships between $1/t_m$ and V have

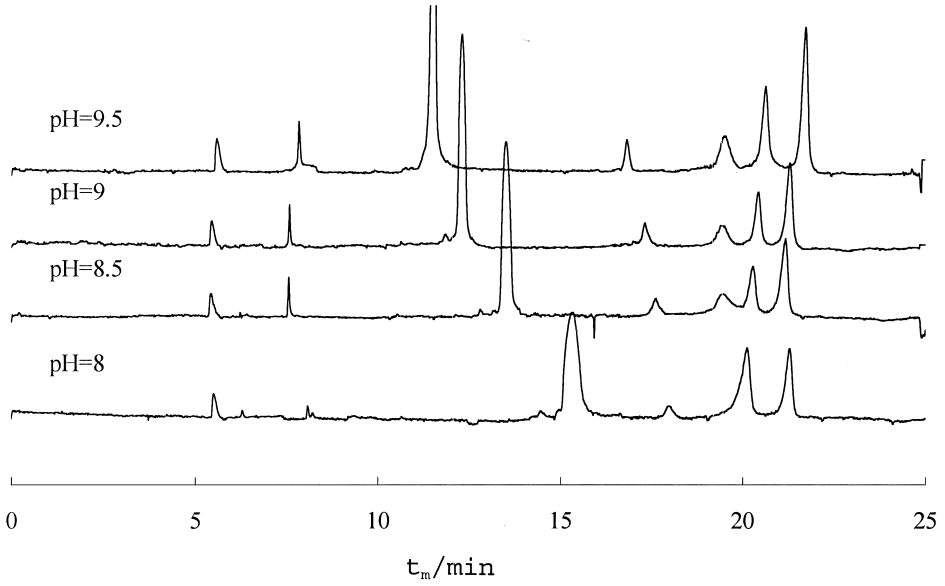

Fig. 2. Influence of pH on the separation of protein mixture by MEEKC. Separation conditions as for Fig. 1 except for the pH of the buffer.

been observed only when the voltage is less than 25 kV. This is the result of the high Joule heat in the capillary under high running voltage.

Fig. 3 shows the influence of the running voltage on the plate numbers of ribonuclease A, carbonic anhydrase II and β-lactoglobulin A. As can be seen in Fig. 3, the optimum separation voltage is 20 kV where the theoretical plate number is up to 80 000. Furthermore, the curves are very similar to the relationship between the theoretical plate number and velocity of mobile phase in chromatography. However, it does not mean the loss of efficiency at higher voltage was caused by the slow mass transfer involving the pseudo-stationary phase, because the plate number of each protein was decreased significantly when the temperature increased from 13 to 40°C in MEEKC. This appears to an experimental phenomenon.

The influence of the temperature of capillary on the separation was also investigated. The efficiency was improved significantly when the separation temperature decreased. EOF increased when the temperature increased. The relationship between the t_m and temperature can be deduced as follows.

The mobility of EOF is defined as

$$\mu_{eo} = \frac{\epsilon_0 \epsilon \zeta}{\eta} \tag{3}$$

where ζ is the zeta potential of the capillary wall; ϵ_0 and ϵ are the dielectric constants of vacuum and electrolyte, respectively; and η is the viscosity of electrolyte in the capillary, which is given by

$$\eta \approx nhe^{\ni/kT} \tag{4}$$

where $\ni$ is the energy required for the molecule moving in the solution, and h, k and n are the Planck constant, Boltzmann constant and molecular density, respectively. Thus, μ_{eo} is given by

$$\mu_{eo} = \frac{\epsilon_0 \epsilon \zeta}{nhe^{\ni/kT}} \tag{5}$$

$$\ln \mu_{eo} = -\frac{\ni}{kT} + \ln\left(\frac{\epsilon_0 \epsilon \zeta}{nh}\right) = \frac{C_1}{T} + C_2 \tag{6}$$

where C_1 and C_2 are nearly constant within the test temperature. As μ_{eo} is proportional to $1/t_m$, Eq. (6) can be given by

$$\ln t_m = C_3/T + C_4 \tag{7}$$

where C_3 and C_4 are constant in the range of test temperature. The linear relationship between ln t_m and $1/T$ (unit of T: Kelvin) is very good (see Fig. 4). Therefore EOF can be changed by varying the temperature.

The influence of the microemulsion was difficult

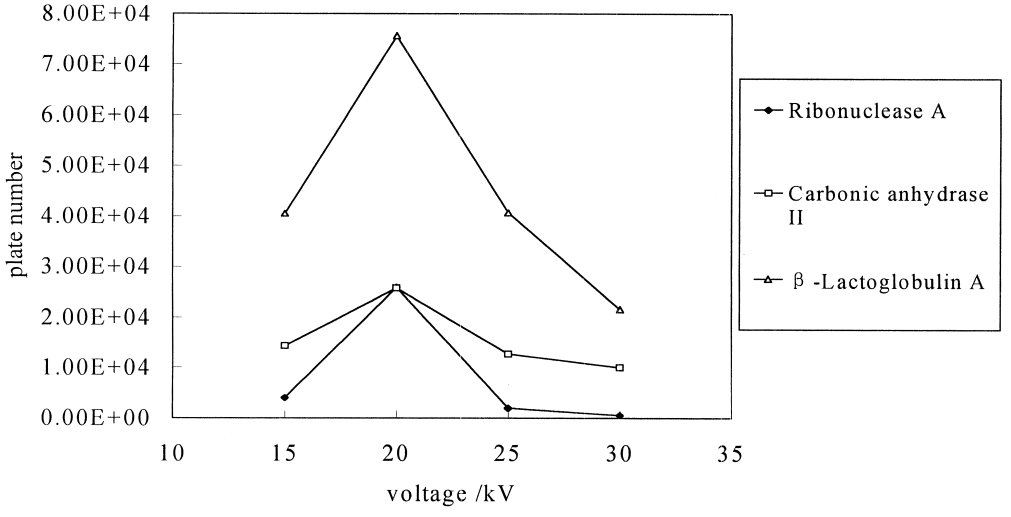

Fig. 3. Relationship between running voltage and theoretical plate number. Separation conditions as for Fig. 1 except for the running voltage.

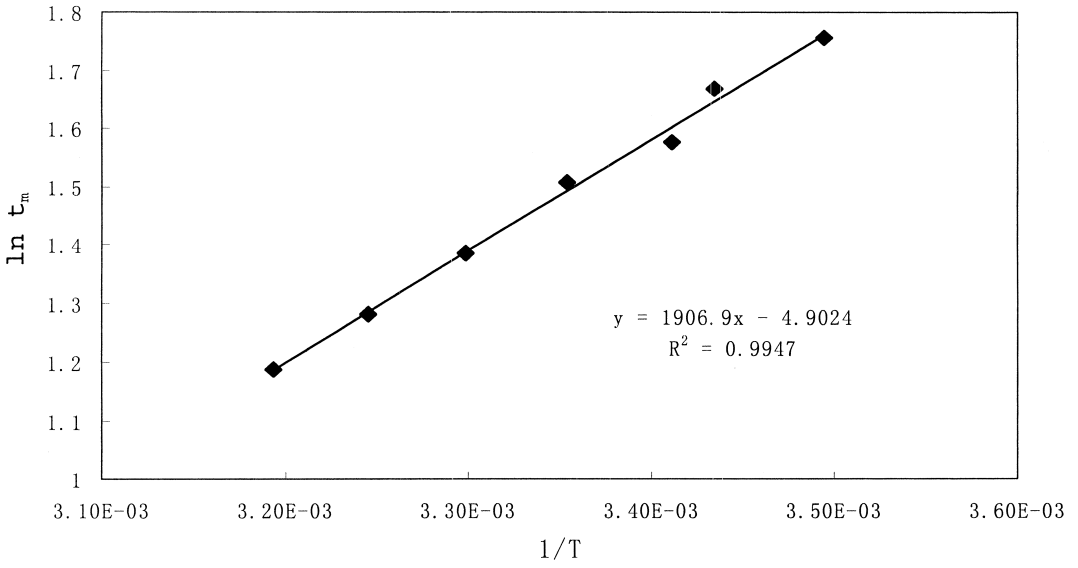

Fig. 4. Relationship between ln t_m and $1/T$. Separation conditions: applied voltage, 20 kV; current, 32.0–82.7 μA from 13 to 40°C; other parameters as for Fig. 1.

to determine since the components of the microemulsion needed to be balanced within a limited range.

Variation of borate concentration in the range of 1–15 m*M* affected the mobilities of EOF and solutes very little, implying that the partition procedure of protein between aqueous phases and microemulsion phases were not affected by borate concentration. Moreover, the current only increased by 31.3% when the borate concentration ranged from 1 m*M* to 15 m*M*, indicating that microemulsion is the main cause of current.

The mobility of EOF was not affected by the variation of SDS concentration ranging from 60 to 120 m*M* as can be seen in Fig. 5. The electrophoretic mobilities of proteins in the test mixture decreased as the SDS concentration was increased. The mobility of β-lactoglobulin A decreased more slowly than that of carbonic anhydrase II and myoglobin. The optimum SDS concentration was 120 m*M*. This phenomenon illustrates that the influence of SDS concentration on the partition procedure of protein is varied with the hydrophobicity of protein.

The mobility of EOF and the mobilities of proteins were nearly constant when the carbon numbers in the oils ranging from 5 to 10 were used. Partitioning between aqueous phases and the emulsion phases did not depend on carbon numbers in oils, but on the stable microemulsion system.

3.2. Comparison with CZE and MEKC

Fig. 6 shows representative electropherograms of a mixture containing four model proteins by CZE separation, which was carried out in the buffers of borate, phosphate and phosphate–borate mixture, respectively. The pH was 8.0 for all three buffers. Optimization of buffer concentration and applied voltage was made in the same CE column and identical buffer pH. The theoretical plate numbers for the four proteins ranged from 27 146 to 48 000 in phosphate, from 15 400 to 58 400 in borate and from 14 600 to 89 500 in phosphate–borate, with averages of 41 882, 29 121 and 40 249 respectively. As shown in Fig. 6, the four proteins, as well as several impurities, were well resolved in all three buffers. When the concentration of phosphate or borate was reduced to 20 m*M*, protein adsorption on capillary surface increased significantly. This process led to band broadening and resulted in far lower actual plate numbers than would be expected in the buffers of high concentration.

The electropherograms of the protein mixture by

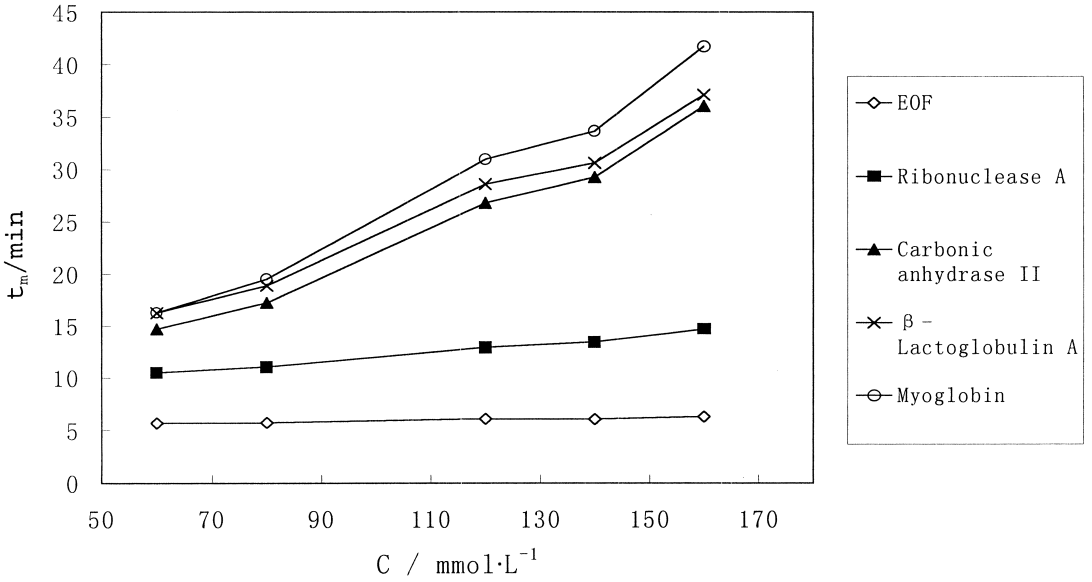

Fig. 5. Influence of SDS concentration on the protein separation in MEEKC. Separation conditions as for Fig. 1.

MEKC in different SDS concentrations are shown in Fig. 7, and the buffer pH ranged from 8 to 9.5. When the SDS concentration was 50 m*M*, only ribonuclease A was separated. Improved separation was obtained when the SDS concentration was increased to 120 m*M* at pH 8.5. The plate number ranged from

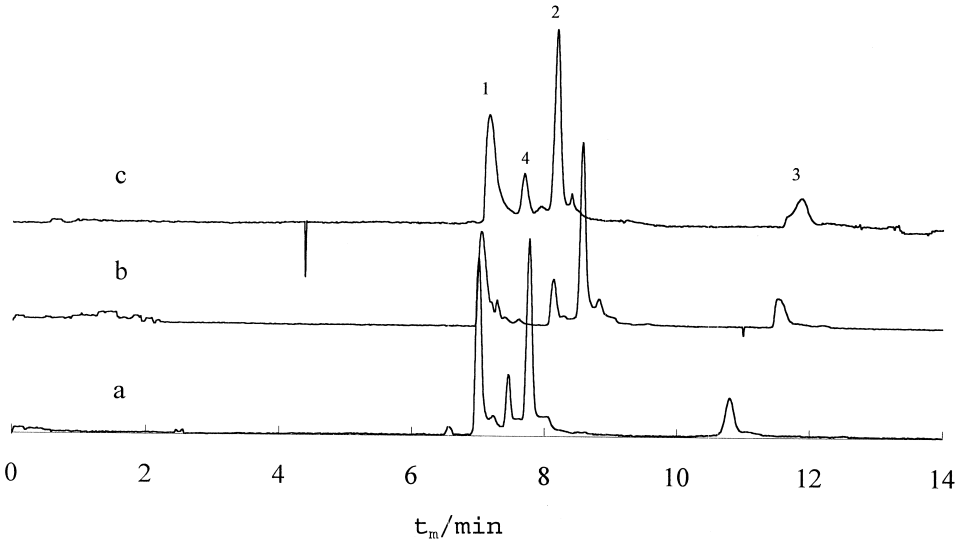

Fig. 6. CZE separations of protein mixture. Buffer composition: 100 m*M* phosphate (a), 100 m*M* borate (b) and 50 m*M* phosphate–50 m*M* borate (c). Other parameters: pH 8.0; voltage, 12 kV; temperature, 20°C. Identification of peaks as in Fig. 1.

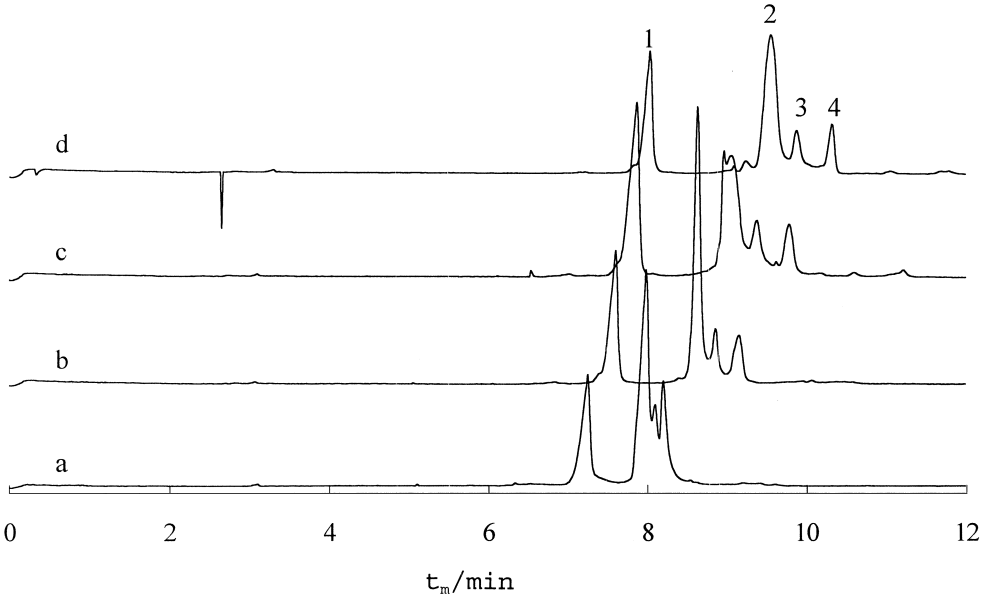

Fig. 7. Separation of the protein mixture by MEKC. SDS concentration: 50 m*M* (a), 80 m*M* (b), 100 m*M* (c) and 120 m*M* (d). Other parameters: 10 m*M* borate, pH 8.5; voltage, 12 kV; temperature, 13°C. Identification of peaks as in Fig. 1.

14 462 (carbonic anhydrase II) to 56 704 (myoglobin) with an average of 35 982. The resolution and efficiency were slightly less than those obtained with CZE, and the migration order of the proteins in CZE was different from that of MEKC.

Compared with CZE and MEKC, the effect of pH on the mobilities of acidic and basic proteins was different in MEEKC (Fig. 2). The mobilities of all the proteins in MEEKC were decreased significantly. The migration order of the proteins in MEEKC was the same as MEKC, but different from CZE.

As a general rule, proteins are too large to partition into a true micellar system [13], Unlike MEKC, proteins can partition into microemulsions in MEEKC. So there are three possible mechanisms for protein separation in MEEKC: electrophoretic mobility, partition procedure due to protein hydrophobicity, and binding action by SDS. Although the protein migration order in MEEKC and MEKC is identical, we cannot deduce that MEEKC is based on the same separation mechanism as MEKC due to the better resolution and the higher retention factor in MEEKC. Protein hydrophobicity may play an important role in protein separation with MEEKC.

3.3. Application

Three genetically engineering pharmaceuticals, rhGM-CSF, glycosylated rhG-CSF and non-glycosylated rhG-CSF preparations, were analyzed, all of which contain high amounts of human serum albumin as stabilizer. The concentrations were 300, 150 and 50 μg/ml for non-glycosylated rhG-CSF, glycosylated rhG-CSF and rhGM-CSF injections, respectively, and all the samples were desalted before injection.

The microamount of bioactive ingredients in the complex preparations could be resolved from the matrix effectively using MEEKC, and the representative electropherograms were shown in Fig. 8.

4. Conclusion

MEEKC resolved the protein mixture with high efficiency and could be used to separate both basic and acidic proteins simultaneously. If a stable microemulsion formed, the efficiency for protein separation was significantly affected by the running

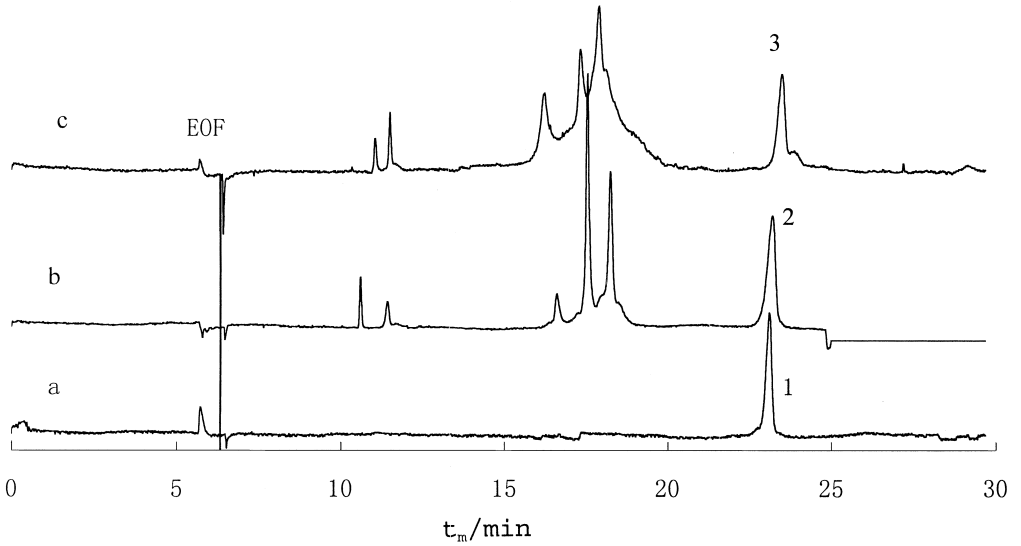

Fig. 8. MEEKC analyses of non-glycosylated rhG-CSF injection (a), glycosylated rhG-CSF injection (b) and rhGM-CSF injection (c). Peaks: 1 = non-glycosylated rhG-CSF, 2 = glycosylated rhG-CSF and 3 = rhGM-CSF. Separation conditions as in Fig. 1 except for the buffer (pH 9.0).

voltage and temperature, but only slightly affected by the pH, concentration of the background electrolyte and the composition of microemulsion.

Acknowledgements

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