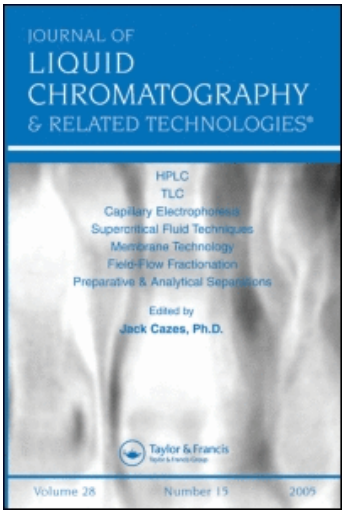

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Mixed Mode of Hydrophilic and Ionic Interaction Pressurized Capillary Electrochromatography for Separation of Basic Compounds

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ABSTRACT

The separation of six basic compounds was obtained using a mixed mode of hydrophilic interaction and strong cation exchange (SCX), pressurized capillary electrochromatography (pCEC) on a novel pCEC instrument. The effects of organic modifier concentration, ionic strength, and pH value in the buffer on retention behaviors of solutes were investigated. The effects of pressure and voltage on separation also were evaluated. Moreover, the separation result was compared with that in reversed phase pCEC, and the separation mechanism was discussed. A satisfactory reproducibility and stability in the separation system was also achieved

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in our study. This paper demonstrates the mixed mode pCEC is potentially applicable for separation of basic compounds.

Key Words: Pressurized capillary electrochromatography; Mixed mode; Hydrophilic interaction; Ionic interaction; Basic compounds.

INTRODUCTION

Capillary electrochromatography (CEC), which combines the high selectivity of high performance liquid chromatography (HPLC) with high efficiency of capillary electrophoresis (CE), has received considerable interest during the last decade. With rapid development of CEC in theory, instrument, and column fabrication, it has developed into a powerful separation technique.[1–4] Capillary electrochromatography is very attractive for pharmaceutical analysis owing to high separation power, rapid speed, and low consumption in samples and solvent.

Compared with neutral compounds, separation of basic compounds is very difficult. In order to become neutral, basic compounds would require a high pH mobile phase, while the stationary phase is detrimental at too a high pH. Although Smith and Evans[5] showed the basic compounds can be separated by reversed phase CEC with C18 column, the peak was bad tailing. To improve the separation and peak shape, amines were added as a modifier,[6,7] and separation of neutral, basic, and acidic compounds was reported with Hypersil C8 and C18 column in CEC.[7,8]

Another effective method separating basic compounds is using ion-exchange chromatography. Smith[5] suggested the use of strong cation exchange because the sulphonet group of stationary phase is negatively charged and can promote EOF even in strongly acidic solution due to their low pK_a value. Cikalo et al.[9] demonstrated the potential of strong cation exchange (SCX) materials in CEC. For data, some groups studied the separation of basic compounds and peptides by ion-exchange CEC.[10–14] These separations are mainly based on ion-exchange or hydrophobic interaction.

Wei et al.[15] reported the feasibility of separation of basic compounds by CEC with bare silica as a stationary phase, and observed that the retention mechanism involved multiple interaction, including reversed phase, ion-exchange, and normal phase chromatography.

Hydrophilic and ionic interaction chromatography is a novel and effective tool for separation of polar compounds including protein, peptides, and amine acid. Alpert[16–18] observed that the separation mechanism of this chromatography in HPLC was mainly hydrophilic interactions between the solutes and stationary phase, similar to normal phase chromatography. Up to now, little

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was reported on the separation of basic compounds in pressurized capillary electrochromatography (pCEC) according to mixed mode of ion exchange and hydrophilic interaction.

For results, CEC experiments were mainly performed on commercially available CE equipment or "home-built" system, but there existed some difficulties such as bubble formation, column dry-out, and gradient elution in CEC.[19,20] In our laboratory, a novel special CEC instrument, Trisep™ 2000 GV CEC system, has been developed, in which the mobile phase is driven by electroosmotic (EOF) and pressurized flow. Bubble is suppressed and CEC operation is easy in this system. In pCEC, the electrophorestic migration rate and flow rate of the mobile phase can be operated independently, since the retention factor, in theory, can be tuned well, adjusting pressure and electrical field[21,22] and thereby, selectivity in the separation was increased.

In this paper, the separation of six basic compounds was carried out with poly(2-sulfoethyl aspartamide)-silica (SCX) column by pCEC. The influences of operational parameters such as organic modifier, ionic strength, and pH value in the mobile phase, as well as applied voltage and supplementary retention behaviors of basic solutes were studied. The separation mechanism was also discussed. Again, the reproducibility and stability of the separation system is good, which shows our pCEC system is satisfactory.

EXPERIMENTAL

Apparatus

In the study, all experiments were carried out with a CEC separation system, which was comprised of a solvent gradient delivery module, a high voltage power supply, a variable wavelength UV/Vis on-column detector, a micro fluid manipulation module (including a 20 nL four ports injector), and a data acquisition module. The mobile phase was driven by two micro-HPLC pumps through a filter to the splitting cross. The back pressure regulator made sure the pressure in the cross was maintained at a constant value. The grounded electrode was set to the splitting cross and the other electrode was set to the tee in connection with the outlet end of the column. Samples were injected by a 20 nL volume four ports injector.

Materials

An SCX column [Poly(2-sulfoethyl aspartamide)-silica capillary columns] [250 mm × 100 (150) μm i.d.], packed with 5 μm particles and ODS capillary columns (250 mm × 100 μm i.d.), packed with 3 μm particles were

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supplied by Unimicro Technologies. Inc. (Pleasanon, CA). Potassium dihydrogen phosphate (KH_2PO_4), phosphoric acid, potassium hydroxide, sodium chloride, and toluene were all of analytical grade (obtained from Tianjin Chemical Company, Tianjin, China). Acetonitrile was chromatographic grade (obtained from Xinke Chemical Inc., Heibei, China). Double deionized water was used. Samples: indapamide, methatone, celiprolol, atenolol, timolol, and isoprenaline (Fig. 1) were obtained from Tianjin Institute of Pharmaceutical Control, (Tianjin, China).

Procedures

Samples were first dissolved in water to obtain a solution containing 1 mg mL^{-1} each of compounds, then were further diluted with mobile phase to

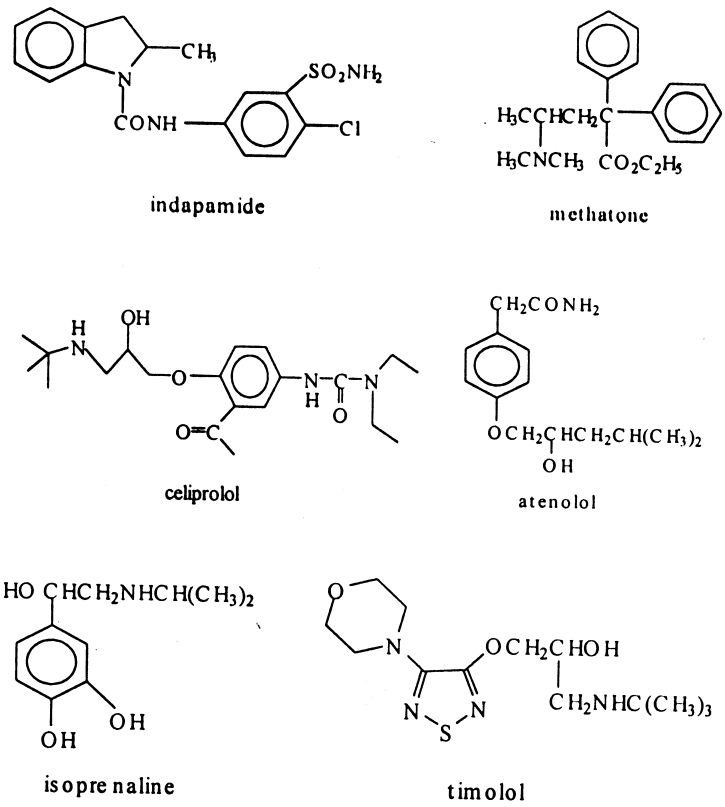

Figure 1. Structure of basic compounds.

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give the compounds an approximate concentration of 0.1 mg mL^{-1}. All these solutions were filtered with 0.22 μm micro filter. Mobile phase solutions were first prepared adjusting the KH_2PO_4 buffer to the desired pH value and then mixing with the appropriate amount of acetonitrile and sodium chloride buffer. Mobile phase solution was degassed in an ultrasonic bath for 10 min before using. A negative voltage was added on the column outlet and the column inlet was grounded. Pressure was applied to the column inlet during the separation. Total flow rate of the two pumps was 30 μL min^{-1}. The wavelength of the UV/Vis detector was set at 214 nm. The injector had an internal loop of 20 nL.

RESULTS AND DISCUSSION

Selectivity and Retention

In this study, the separation of six basic compounds was studied in RP-pCEC with a C18 column and in SCX-pCEC with a poly(2-sulfoethyl aspartamide)-silica (SCX) column. As shown in Fig. 2, in RP-pCEC, six compounds are not completely resolved and tailing. In contrast, in SCX-pCEC, all compounds are resolved to baseline with good peak shape and no tailing. As expected, the elution orders of solutes in SCX-pCEC are approximately the opposite of that in RP-pCEC. This suggests the separation on the SCX column is due to hydrophilic interaction. It is believed that the retention mechanism in HPLC on poly(2-sulfoethyl aspartamide)-silica columns is multifunctional, mainly involving a hydrophilic interaction and a cation-exchange mechanism.[16,17] In pCEC, the retention becomes even more complicated owing to additional electrophoresis effect. In order to investigate the retention behavior in pCEC, the effects of organic modifier, ionic strength, and pH value on retention factor k^* were studied. The capacity factor of pCEC k^* was defined with the following equation:[21,22]

$$k^* = \frac{t_r - t_0}{t_0} \tag{1}$$

where t_r and t_0 is the retention time for the retained charged solute and unretained neutral solute. Toluene was selected as the neutral marked in this separation system.

To study the relationship between retention and operational parameters, k^* can be also expressed as Eq. (2):[21,22]

$$k^* = \frac{\lambda k' p + (\mu_{eo} k' - \mu_{em})E}{\lambda p + (\mu_{eo} + \mu_{em})E} \tag{2}$$

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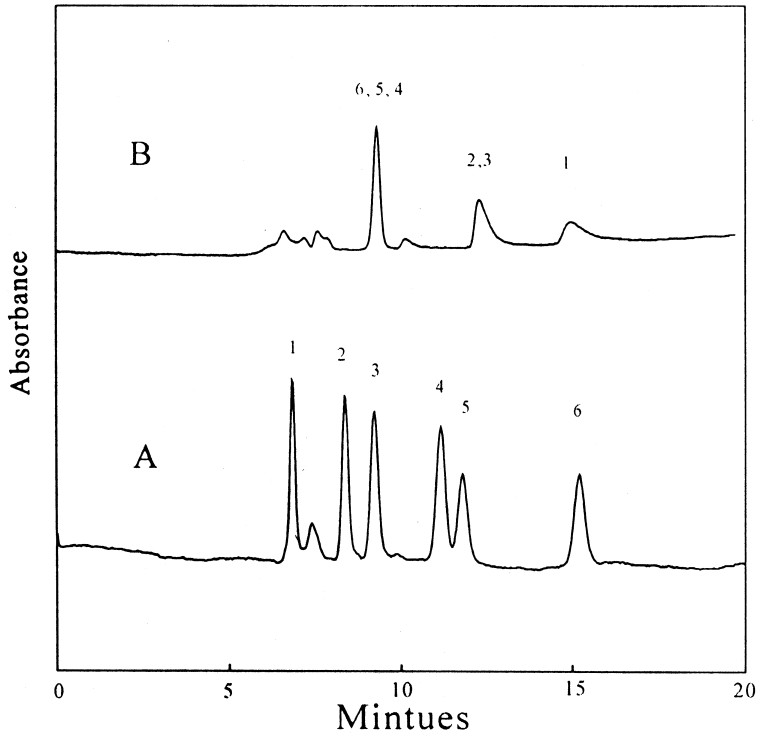

Figure 2. Electrochromatograms showing the comparison of SCX-pCEC (A) and reversed phase pCEC (B) for the separation of six basic compounds. Column: A: EP-100-25-5-SCX; B: EP-100-25-3-C18; mobile phase: A: 70% (v/v) acetonitrile in 5 mM KH_2PO_4 (pH = 4.5); B: 70% methanol in 5 mM KH_2PO_4 (pH = 4.5) buffer; flow rate: 0.03 mL min^{-1}; voltage: A: 5 kV; B: 3 kV; pressure: A: 500 psi; B: 1000 psi; injection volume: A and B: 20 nL; detection wavelength: 214 nm; samples: 1 indapamide, 2 methatone, 3 celiprolol, 4 atenolol, 5 timolol, and 6 isoprenaline.

where μ_{eo} and μ_{em} are the electoosmotic and electrophoretic mobilities, respectively, k' is the capillary factor of the solute in the same column operated in HPLC mode, λ is a constant for the same column and solvent.

Effects of Acetonitrile, Ionic Strength, and pH

The effect of organic modifier is shown in Fig. 3. As the acetonitrile concentration in the mobile phase was raised from 50% to 60%, k^* for all solutes increased little. A further increase in the acetonitrile concentration

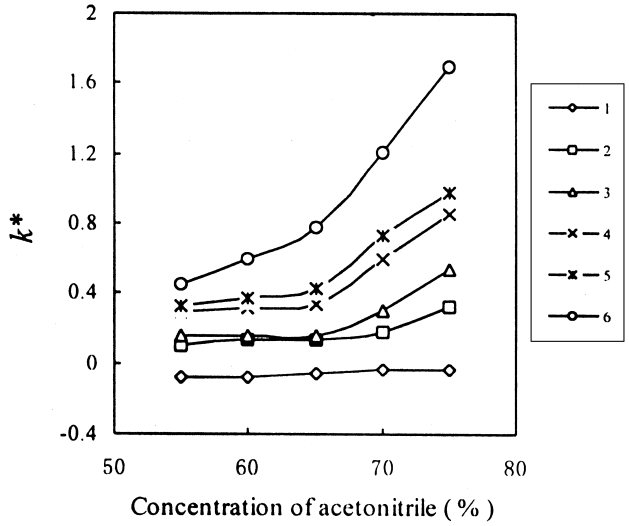

Figure 3. Effect of concentration of acetonitrile on retention. Column: EP-100-25-5-SCX mobile phase vary concentration acetonitrile in 5 mM KH_2PO_4 buffer pH 3.5; pressure: 500 psi; voltage: 5 kV; samples: as in Fig. 2.

up to 75%, a marked increase in k^* (expect for 6), was observed. As shown in Fig. 3, at a high level of acetonitrile, k^* are governed primarily by hydrophilic interaction between the solutes and the stationary phase. The separation mechanism is similar with a normal phase separation model. In six solutes, the separation of 2 and 3, as well as 4 and 5, is difficult, so the two selector factors α are selected as an optimal criterion [Eq. (3)]. Table 1 shows the effects of acetonitrile concentration on $\alpha_{2,3}$ and $\alpha_{4,5}$. The mobile phase consisting of 70% acetonitrile is an optimal condition, as shown in Table 1.

$$\alpha = \frac{k_2^*}{k_1^*} \tag{3}$$

In order to examine the effect of ionic strength, the experiments were performed at fixed organic concentrations of 70% and pH 4.5. The retention of all solutes decreased quickly with increasing sodium chloride concentration from 0 mM to 100 mM, as shown in Fig. 4. This indicates that electrostatic interaction between solutes and stationary phase was reduced at high ionic strength. The tendency is similar with that in ion-exchange CEC.[10] However, the decrease of electrostatic interaction may not be the only reason for the decreased k^* of solute. From Eq. (2), k^* in pCEC can be controlled by other

Table 1. Effect of acetonitrile concentration on selector factor.

	55%	60%	65%	70%	75%
$\alpha_{2,3}$	1.58	1.20	1.18	1.67	1.64
$\alpha_{4,5}$	1.18	1.21	1.25	1.24	1.14

Note: The experiment conditions were same as Fig. 3.

factors such as μ_{eo}. With ionic strength increased, the decreased electrostatic interaction causes decrease of k', it will lead to decrease of k^*; at the same time, decreased μ_{eo} can also influence the k^* of solute. Although, pressure can suppress the bubble formation in the column, Joule heating becomes very strong in high ionic strength mobile phase, and it might be another reason for decreased k^* of solute. From Fig. 4, it can be seen that addition of sodium chloride is not satisfactory for separation of the six solutes.

Figure 5 shows the migration times of six basic solutes in buffers of different pH in the mobile phase. From Fig. 5, the migration times increases

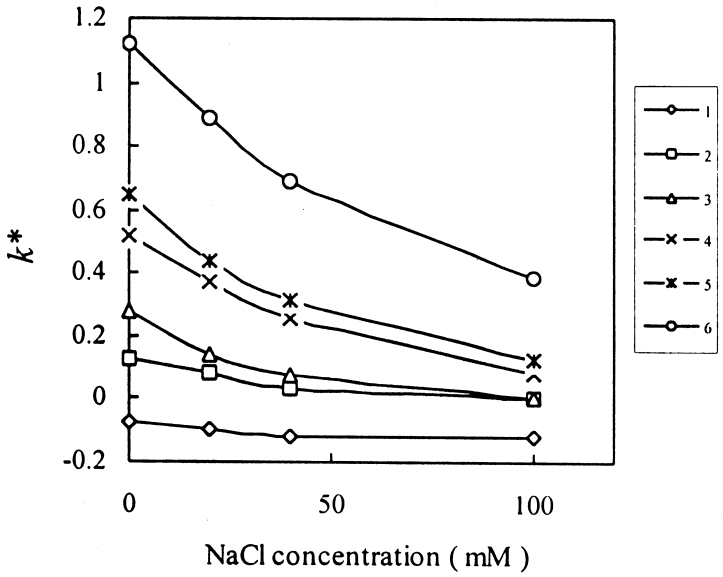

Figure 4. Effect of ionic strength on pCEC separation. Column: EP-100-25-5-SCX mobile phase vary concentration NaCl in 5 mM KH_2PO_4 buffer pH 4.5 which consists of 70% acetonitrile; pressure: 500 psi; voltage: 5 kV; samples: as in Fig. 2.

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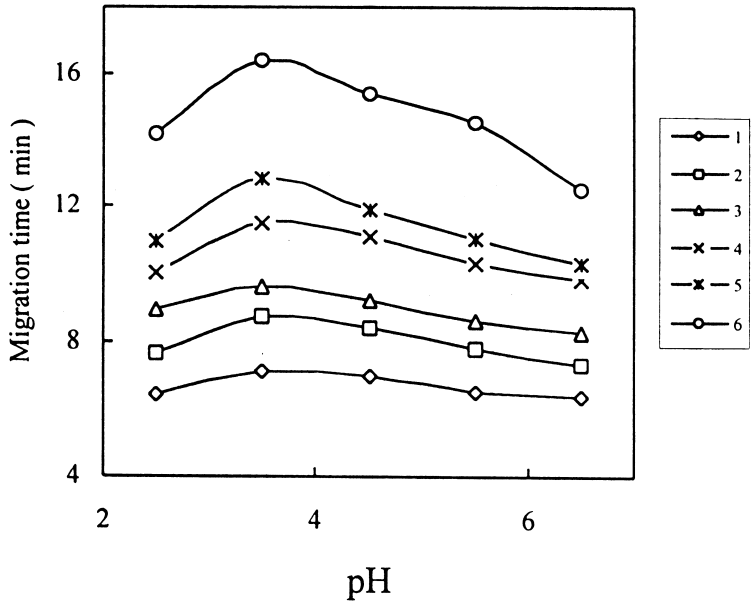

Figure 5. Effect of pH on retention of pCEC separation. Column: EP-100-25-5-SCX mobile phase 70% acetonitrile in 5 mM KH_2PO_4 vary concentration pH value buffer; pressure: 500 psi; voltage: 5 kV; samples: as in Fig. 2.

with increasing pH value from 2.5 to 3.5, while they decrease with further increasing pH value from 3.5 to 6.5. The results are similar with that in SCX-CEC. It may be the reason that electrostatic interaction between solutes and stationary phase can be effected by pH in buffer. At low pH, ionization of the sulphonic group on the stationary phase was partly suppressed and the level of its ionization increased with increasing pH, so electrostatic interaction also got stronger. At high pH, the sulphonic group was ionized completely, and the positively charged density of solutes decreased with increasing pH from 3.5 to 6.5, so retention time of solutes decreased. Again, in different pH buffers, μ_{em} and μ_{eof} also had some effects on retention of solutes.

Effect of Applied Voltage and Pressure

In pCEC, the effect of pressure or flow rate on the column on k^* was seldom reported.[22] But, different from that in HPLC, pressure in pCEC

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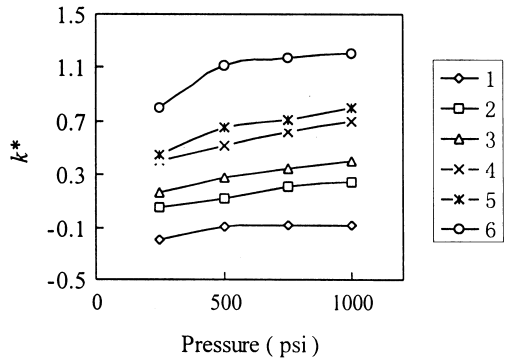

Figure 6. Effect of pressure on retention. Column: EP-100-25-5-SCX mobile phase 70% acetonitrile in 5 mM KH_2PO_4 buffer (pH 4.5); voltage: 5 kV; samples: as in Fig. 2.

becomes a factor tuning retention behavior of solute. Figure 6 shows the effect of pressure on the k^* for solutes. From Fig. 6, it can be seen that k^* for solutes increased with increasing pressure on the column. In the buffer, basic compounds are apparently positively charged and the direction of μ_{em}, μ_{eo} and μ_{ep} are the same. As the pressure is raised, with μ_{ep} increasing, k^* tends to k'. The results can be interpreted from the theory aspect.[23] Selector factor α for 2 and 3, 4, and 5 can be also optimized by tuning pressure on the column, as shown in Table 2.

In CEC, the effect of voltage on retention is different in a reversed phase column and ion exchange column.[24,25] In pCEC, the effect of applied voltage on separation might be more complicated. Figure 7 shows separation of a neutral solute and three basic solutes under different applied voltage. From Fig. 7, both the retention orders and migration rates of solutes could be tuned by changing voltage. Moreover, the direction and magnitude of voltage can be tuned more freely in pCEC than in pure CEC. It can be expected that rapid and highly efficient separations in complicated separation

Table 2. Effect of supplementary pressure on selector factor.

	250 psi	500 psi	750 psi	1000 psi
$\alpha_{2,3}$	3.4	2.33	1.67	1.6
$\alpha_{4,5}$	1.12	1.26	1.15	1.14

Note: The experimental conditions were same as Fig. 6.

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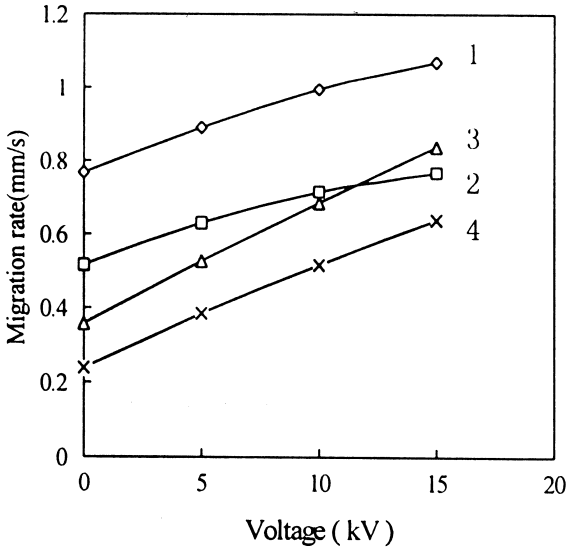

Figure 7. Effect of voltage on retention. Column: EP-150-25-5-SCX mobile phase 70% acetonitrile in 5 mM KH_2PO_4 buffer pH 4.5; pressure: 500 psi; voltage: 0, 5, 10, 15 kV; sample: 1 indapamide, 2 thiourea, 3 atenolol, 4 isoprenaline.

systems can be obtained easily with pCEC through adjusting the ratio of the pressure and voltage.

Reproducibility and Stability

In pCEC, the mobile phase is driven by both EOF and pressure flow. The application of pressure can avoid the formation of bubbles and reduce the Joule heating in the column, so that the repeatability is increased. In our separation system, samples were injected through a rotary type HPLC injection valve. Compared with other injection methods such as voltage injection and pressure injection, the valve injection is more accurate in injection volume and injection time, since the repeatability can be also improved. Table 3 lists the RSD values of the retention time for six solutes obtained from five runs in pCEC. Prior to use, the column was rinsed with mobile phase for 90 min to equilibrate the column. At moderate pH, the reproducibility of less than 2% of RSD in retention time was achieved. From Table 3, the reproducibility at pH 4.5 is better than that at pH 2.5. The reason might be that ionization of stationary phase is not completely at low pH, and equilibrium of separation systems is not satisfactory.

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Table 3. Reproducibility (5 times) of pCEC.

	pH 2.5		pH 4.5	
	Mean RT (min)	RSD (%)	Mean RT (min)	RSD (%)
1	6.35	2.25	6.99	0.97
2	7.45	0.84	8.41	0.65
3	8.79	2.45	9.36	1.08
4	9.87	1.03	10.27	0.75
5	10.77	2.53	12.03	0.72
6	13.91	3.24	15.24	1.28

Note: The experimental conditions were same as Fig. 2.

CONCLUSION

In the study, separation of six basic compounds was achieved in pCEC with poly(2-sulfoethyl aspartamide)-silica capillary columns. Compared with reversed phase C18 columns for basic compounds, the peak shape can be improved and retention order can be reversed using this column. The separation mechanism is similar with normal phase chromatography, while it is more suitable in CEC owing to the aqueous mobile phase used. The results show that separation of basic compounds are based on the mixed mode of hydrophilic interaction, ionic exchange, and electrophoresis, and this mixed mode may be very useful for separation of polar compounds such as basic compounds and peptides. In pCEC, the retention behaviors of compounds can be well tuned by changing applied voltage and supplementary pressure, and it is attractive for separation of complicated samples. Under control of pressure, some problems encountered on pure CEC such as bubble formation and Joule heating, were resolved, therefore, good reproducibility in retention time was achieved.

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