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Optimized preparation of poly(styrene-*co*-divinylbenzene-*co*-methacrylic acid) monolithic capillary column for capillary electrochromatography

Preparation of a poly(styrene-*co*-divinylbenzene-*co*-methacrylic acid) monolithic stationary phase for the use in capillary electrochromatography (CEC) has been improved by optimizing the polymerization conditions. It is observed that the reaction time strongly affects column efficiency, while the proportion of isooctane in porogen influences peak symmetry of some solutes seriously. The lifetime of the monolithic columns prepared mainly depends on the pH of buffers used. Reproducibility of electroosmotic flow (EOF) from batch to batch columns are lower than 2.8% relative standard deviation. Unlike other types of capillary electrochromatographic monoliths, a pH-dependent EOF was observed on this type of column. Separation of various types of compounds including aromatic hydrocarbons, hormones, anilines, basic pharmaceuticals, and peptides was achieved. The facile preparation and wide application of this monolithic column may make styrene-based polymer a potential stationary phase in CEC.

Keywords: Basic pharmaceuticals / Capillary electrochromatography / Hormones / Peptides / Stationary phase / Styrene-based monolith DOI 10.1002/elps.200305579

1 Introduction

Capillary electrochromatography (CEC) is a microseparation technique that combines high efficiency of capillary electrophoresis (CE) and high selectivity of high-performance liquid chromatography (HPLC) [1–4]. In contrast to HPLC, the flat flow profile generated by electroosmotic flow (EOF) in CEC is expected to be more advantageous to separation process. Recently, monolithic columns containing a wall-supported continuous porous bed have shown a great potential for CEC due to simple procedures of their preparation [5–9]. Up to now, CEC with monolithic columns has been successful in the separation of neutrally low-molecular-weight compounds, such as aromatic compounds, herbicides, aldehydes and ketones, retinyl esters, and steroids [10–15]. However, separation of ionic compounds, especially ionic biomolecules with CEC, still poses difficulties due to the involvement of complicated electrophoresis mechanism [9, 16–19].

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Abbreviations: MAA, methacrylic acid; **γ-MAPS**, γ-methacryloxypropyltrimethoxysilane

The monolithic columns in CEC can be classified into two classes: particle-fixed columns and porous polymer monolithic columns [20]. The particle-fixed columns are unique but inconvenient to be prepared, due to the fact that they are always fabricated with multistep procedures. Therefore, the polymer monolithic columns in CEC have extensively been studied, which include inorganic and organic polymer monoliths. Inorganic porous polymer columns based on silica gel are typically prepared using a sol-gel process [21–23]. The organic porous polymer columns can be prepared by polymerization of organic monomers in the presence of a porogen. The preparation of organic monoliths is quite similar to that in HPLC, whereas charged groups should be concomitantly coupled to the surface of polymer monoliths to generate EOF. Generally, three types of organic monoliths based on polyacrylamides [12, 14, 15, 24, 25], polymethacrylate esters [7–9, 26–28] and polystyrenes [29–32], have been prepared as the stationary phases for CEC. Despite their wide use in HPLC, polystyrene-based stationary phases with outstanding chemical stability in a broad pH range have not attained much attention in CEC. Zhang *et al.* [29] reported the synthesis and applications of poly(styrene-divinylbenzene) encapsulated silica (PS-DES) as the stationary phase for CEC, but the preparation process was much more complicated due to the involvement of packing silica beads into capillaries. The porous-layer open-tubular (PLOT) columns by polymerization of vinyl-

benzyl chloride and divinylbenzene on the surface of a capillary wall were prepared by Gusev *et al.* [30]. Recently, C_{12} alkyl chains were introduced into the polymer as hydrophobic groups for retention of compounds by Huang *et al.* [31]. The separation of tripeptides and proteins in those columns has been achieved. However, multistep procedures are required in for preparation of those columns. In addition, the amount of charged groups incorporated in the polymer is not easily controlled, which would result in a relatively low reproducibility of the EOF. A negatively charged porous monolith with methacrylate acid, styrene, and divinylbenzene as the monomers has been prepared in a single step by Xiong *et al.* [32]. Several groups of aromatic compounds were well separated on the prepared columns. However, polymerization reaction with about 20 h makes preparing the monolith rather slow.

In this work, the previous method developed by Xiong *et al.* [32] has been improved to prepare a poly(styrene-*co*-divinylbenzene-*co*-methacrylic acid) monolith within 4 h on the condition that the pretreatment process has been completed. This ready preparation method exhibited good reproducibility, and diverse series of neutral and ionic samples, such as aromatic hydrocarbons, hormones, anilines, basic pharmaceuticals, and peptides, could be well separated on the prepared CEC columns.

2 Materials and methods

2.1 Materials and instruments

Styrene, methacrylic acid (MAA), toluene and isooctane, purchased from Shenyang Xinxi Chemical Plant (Shenyang, China), were distilled under a vacuum before use. 80% divinylbenzene, purchased from Aldrich (Milwaukee, WI, USA), was extracted with 10% aqueous sodium hydroxide and water, and subsequently dried over anhydrous magnesium sulfate. γ-Methacryloxypropyltrimethoxysilane (γ-MAPS) was obtained from Sigma (St. Louis, MO, USA). Formamide and all aromatic compounds were of analytical grade and were purchased from Tianjin Chemical Plant (Tianjin, China). Pharmaceuticals and corticosteroids were obtained from Sigma and peptides from Serva (Heidelberg, Germany). Double-distilled water purified by Milli-Q (Millipore, Milford, MA, USA) was utilized throughout the experiments. The sample solution of aromatic hydrocarbons was prepared by dissolving them in ACN with volume ratio at 1:10, and then further diluted to the appropriate concentration ranging from 0.01–0.03 μg/μL with the mobile phase before injection. Anilines, hormones, pharmaceuticals, and peptides were dissolved directly in the mobile phase with the concentration ranging from 0.1–2 μg/μL. Capillaries of 100 μm inner diameter and 375 μm outer diameter were purchased from the Yongnian Optic Fiber Plant (Hebei, China). All experiments were carried out on a Beckman P/ACE 2200 and 5500 instruments (Beckman, Fullerton, CA, USA) at room temperature. A Waters 510 HPLC pump (Waters, Milford, MA, USA) was utilized to flush the columns.

2.2 Preparation of monolithic columns

Prior to the polymerization, the capillary was pretreated with the following procedure. Firstly, the capillary column with a length of 35 cm was rinsed with 0.1 M NaOH for 1 h and then with water until the outflow reached pH 7.0. After subsequent flushing with methanol for about 10 min, it was dried by passage of nitrogen gas. γ-MAPS solution by its dilution with methanol at a volume ratio of 1:1 was injected into the capillary with a syringe. It was sealed with rubber at both ends and then submerged in a bath of water at 50°C for overnight. Finally, the capillary was rinsed with methanol and water to flush out the residual reagent. Thereby, Si-O-Si-C bonds were formed between the capillary wall and the reactive methacryloyl groups, which are available for subsequent attachment of monolith to the wall during the polymerization reaction. The monolithic column was prepared from polymerization reaction of mixtures, consisting of styrene, divinylbenzene, MAA, toluene and isooctane using 2,2'-azo-bis(isobutyronitrile) (AIBN; 1 wt% with respect to the monomers) as an initiator. The polymerization mixture was sonicated for 20 min to obtain a homogeneous solution, and then purged with nitrogen for 10 min. After the pretreated capillary was completely filled with the mixture, the capillary was sealed at both ends with rubber stoppers. The sealed capillary was submerged into a bath of water and allowed to react for 0.5–24 h at 70°C. The resultant monolithic capillary column was washed with methanol about 2 h using an HPLC pump to remove unreacted monomers and porogens. At the end of this period, the detection window was made by burning off 1–2 mm of both the coated polymer outside and the monolith inside of the capillary using flames [6]. The ashes of the organic monolith inside the capillary were flushed out by methanol for about 30 min with the HPLC pump under the applied pressure at about 80 bar. Capillaries, without visible compression of the monolith, were cut at both ends to a total length of 27 cm. Finally, the column was equilibrated at 5 kV for 30 min before running. Macroscopic materials prepared in larger amounts of corresponding mixtures in empty HPLC columns were washed with methanol using an HPLC pump for about 12 h, after that, the polymer was flushed out from the column, cut into small pieces, and

dried under vacuum at 25°C for 24 h. Mercury intrusion porosimetry was used to characterize the pore size and surface area of monolith.

3 Results and discussion

3.1 Evaluation of column performance

It was reported that the preparation of macroporous stationary phases in CEC usually lasted about 8–24 h for polymerization reaction alone. In our experiments, by optimizing the composition of the polymerization mixture based on Xiong *et al.* [32], a monolithic capillary column could be prepared within 4 h starting from the pretreated capillary, involving 1 h for the polymerization, 2.5 h for flushing out the porogen and making the detection window, and 30 min for equilibration. To our knowledge, such rapid and single-step preparation of monolithic columns has not been reported as of yet. No derivatizations on the monolith surface are needed. The comparison of the composition of the polymerization mixture, together with the properties of the monolithic polymers prepared by Xiong *et al.* [32] and us are listed in Table 1. It can be seen that although the reaction time decreases from 24 h to only 1 h, the highest efficiency of the column still remains good. In our experiment, it was also observed that the reaction time affects the column efficiency and migration time of void marker strongly, as shown in Table 2.

According to the theory of CEC, it is known that the EOF velocity depends not only on the level of charged surface functionalities but also on the size of the transport channels [7]. It is well known that pore and channel size of monolith decreases, but specific area of monolith increases generally with increasing polymerization reaction time according to the theory of nucleation and phase separation [33]. It was observed by Peters *et al.* [8] that the EOF increases with an increase in pore size of monolith, which means that the reaction time should have negative contribution to the EOF velocity. However, the reaction time will lead to increase in the specific area of monolith, which results in a positive contribution of reaction time to the EOF. In our case, the migration time of thiourea increases with reaction time, which can be explained by the fact that the negative contribution of pore size on EOF is stronger than the positive contribution of the specific area. In addition, the slow exchange of the solutes across the stationary phase boundary caused by the decrease of the pore diameter may also decrease column efficiency with the increase of polymerization time. As reported by Peters *et al.* [7], excessively small transport channels in monolith will lead to deviations from a plug-like flow profile, which also results in the low column efficiencies. It was observed that the column efficiency decreased rapidly with the reaction time in our experiment, except for the 0.75 h polymerization involved columns, in which the frame network of polymer may not be formatted due to the excessively short reaction time.

Table 1. Composition of polymerization mixture and the porous and chromatographic properties of different monolithic CEC columns

	Composition of polmerization mixture				
	Styrene (μL)	Divinyl-benzene (μL)	MAA (μL)	Toluene (μL)	Isooctane (μL)
Xiong [32][a)]	50	100	50	800	0
This work[b)]	50	100	50	300	300

	Pore structure and chromatographic properties			
	Mean diameter (nm)	Pore volume (mL/g)	Average surface area (m^2/g)	Highest column efficiency (*N*/m)
Xiong [32][a)]	40[c)]	0.09872[c)]	82.418[c)]	140 000
This work[b)]	1095[d)]	3.41[d)]	12.46[d)]	>200 000[e)]

a) Polymerization time, overnight
b) Polymerization time, 1 h
c) Specific surface area was calculated from the experimental data of nitrogen adsorption/desorption according to the Brunauer-Emmett-Teller (BET) equation.
d) Mercury intrusion porosimetry was used for determination of the average surface area.
e) The column efficiency is 243 000 *N*/m for caffeine and 210 000 *N*/m for barbital under the experimental conditions (Fig. 6).

Table 2. Column efficiency of thiourea on the monolithic columns prepared under different reaction times

Reaction time (h)	0.75	1	2	4	12	24
Column efficiency (*N*/m)	63 000	124 000	117 000	48 000	26 000	19 000
Retention time (min)	3.89	3.92	4.11	4.36	4.85	5.12

Experimental conditions: column, effective length 20.5 cm (total length 27 cm) × 100 μm ID × 375 μm OD; mobile phase, 10 mM phosphate buffer containing 60% acetonitrile, pH 7.0; temperature, 25°C; applied voltage, 10 kV; injection, 5 kV for 2 s; detection wavelength, 254 nm

In addition to what was mentioned above, the isooctane proportions in the porogenic mixture strongly affected the peak symmetry of the late eluting solutes, such as isopropyl toluene and butyl benzene. The less the proportion of isooctane in the porogen, the more serious the tailing of the peaks was. It was observed that with the increase of the ratio of isooctane, from 16% to 50%, the efficiency of the column and symmetry of the peaks were improved. The best peak symmetry and column efficiency was obtained when the proportion of isooctane in porogen is 50%. In fact, the addition of a poorer solvent to the polymerization reaction mixture results in an earlier phase separation of the polymer [7], which not only accelerates the polymerization process, but also increases the mean diameter of the pores [33–35]. Since the size of pores within the monolithic material is expected to affect the chromatographic efficiency, the very large pores and voids within the monolith enable the rapid exchange of solvents near the precipitated polymer layer. The fast release of the redissolved species in the flow of the solvent allowed them to form sharp zones thereby leading to good separation and high column efficiency [36]. It could be speculated that with the increase of isooctane, the pore structure of the monolith tends to be more adapted for convection mass transfer, which further improves the symmetry of the peaks.

The lifetime of the column mainly depends on the pH of the electrolyte solutions. The columns exhibited good stability in neutral and acidic mobile phase, as they could be used for one month without apparent loss of column efficiency. Under strong alkaline conditions (pH 11), the column could also be used for about one week, after that, an unstable current was often observed during the experiments. This might be due to the gradual break off of the stationary phase caused by the alkaline erosion of the capillary inner wall, as there still remain residual silanol groups on the surface of the capillary inner wall even after the pretreatment process. In addition, the Si-O-Si-C bonds between capillary wall and monolithic rod could also be damaged. However, it should be noticed that other types of monolithic capillary columns, especially silica-based columns, can not be used at all under such strong basic conditions.

Run-to-run, column-to-column, as well as batch-to-batch reproducibility was investigated in terms of t_0 and the retention factor (k') of aromatic compounds, including benzene, toluene, ethyl benzene, and propyl benzene. It was observed that reproducibility of EOF for the batch-to-batch preparation of columns was RSD smaller than 2.8% (n = 3), and the reproducibility of the k' values for different solutes based on the run-to-run injections, column-to-column preparation, and batch-to-batch preparation of columns was smaller than 1.6% RSD (n = 6), 3.5% (n = 5), 7.3% (n = 3), respectively. These results indicated that the reproducibility of the prepared monolithic columns is acceptable.

The EOF on this prepared monolithic column is mainly generated by the dissociated functionalities of the MAA, incorporated in the polymer. This leads to pH-dependent EOF, due to the relatively high pK_a of MAA. It is known that the pK_a of MAA is between 6 and 7. Therefore, under pH lower than 7, the ionization content of MAA is increased with increasing pH value of the buffer. Until the pH value exceeds 7, MAA on the surface will be completely ionized, and the EOF on the column reaches a maximum. The obtained result is illustrated in Fig. 1. It was observed that the EOF of the column reached 1.36×10^{-8} $m^2v^{-1}s^{-1}$ at pH about 7.0, and stayed nearly constant above this value. In CZE, it has been reported that EOF decreased with increasing acetonitrile content in the mobile phase [37], which was attributed to a concomitant decrease in zeta potential on the inner capillary wall. Although the reversed trend in CEC has been reported by some authors [38–40], we have found the same trend for changes in EOF as that in CZE, that is, the EOF decreased with increasing acetonitrile content, as shown in Table 3. Similar results weres also reported by Yamamoto *et al.* in 1992 [41]. In their work, the decrease in EOF was even more evident, and the phenomenon was considered to be caused from the decrease in dielectric constant and the magnitude of the zeta potential. The results

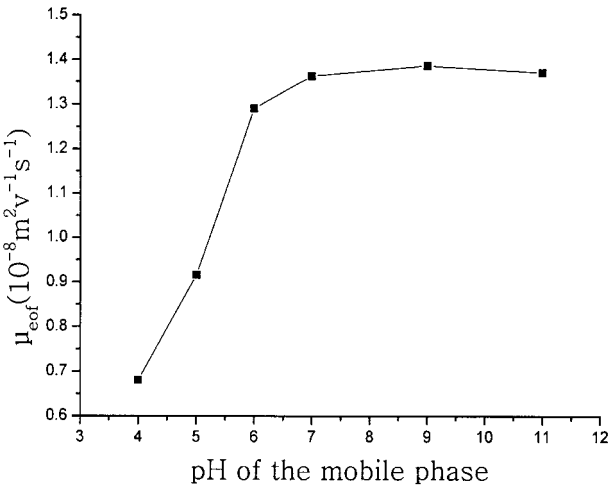

Figure 1. Influence of the pH on electroosmotic mobility (μ_{eof}). Experimental conditions: column, effective length 6.5 cm (total length 27 cm) × 100 μm ID × 375 μm OD; mobile phase, 60% acetonitrile in 5 mM phosphate buffer containing 50 mM KCl as neutral salt, which is mainly responsible for the ionic strength. EOF marker, thiourea; applied voltage, 5 kV; injection, 5 kV for 4 s; temperature, 25°C; detection wavelength, 214 nm.

Table 3. Influence of the acetonitrile concentration on electroosmotic mobility (μ_{e0f})

Acetonitrile concentration (v%)	40	50	60	70
Electroosmotic mobility (μ_{eof}, $10^{-8}m^2v^{-1}s^{-1}$)	2.60	2.35	2.30	2.05

Experimental conditions: mobile phase, 10 mM phosphate buffer containing various concentration of acetonitrile, pH 7.0. Other conditions are the same as in Fig. 2

acquired in our experiments are in contradiction with previous results [32], which may have been caused from the dramatic difference in average pore diameter between two cases, leading to the opposite effect of ACN concentration on zeta potential in our work from Xiong's results. Judging from these contradictory experimental phenomena in literature, the fundamental effect of acetonitrile content on EOF in CEC still needs to be elucidated in more detail. It is possible that there are others factors, such as the pore structure to affect the zeta potential. The relationship between the theoretical plate height (H) and the velocity of mobile phase for ethyl benzene and butyl benzene has also been studied. It was found that even when the velocity increased to 2.0 mm/s, no apparent loss of column efficiency was observed (Fig. 2), which would be one of the typical behaviors of the monolithic stationary phases, and holds great promise for the use of such a monolithic column in fast analysis of samples. Throughout our experiments, column efficiencies in the range of 120 000–186 500 theoretical plates per meter were observed for different aromatic hydrocarbons.

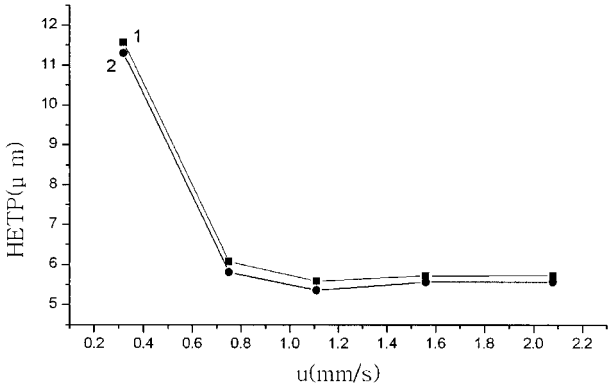

Figure 2. Plot of plate height (HETP) of (1) ethyl benzene and (2) butyl benzene *versus* the linear velocity. Experimental conditions: column, effective length 20.5 cm (total length 27 cm) × 100 μm ID × 375 μm OD; mobile phase, 10 mM phosphate buffer containing 60% acetonitrile, pH 7.0; applied voltage, from 5 to 25 kV; injection, 5 kV for 1 s; detection wavelength, 254 nm.

Furthermore, even higher column efficiency could be obtained for the separation of basic compounds. For example, the column efficiency for the selected basic pharmaceuticals is even higher than 200 000 theoretical plates per meter.

3.2 Separation of different types of samples on the monolithic column

Separation of neutral compounds can be used for evaluation of CEC-columns. For this purpose, the prepared column was applied for separation of neutral compounds at pH 7. Firstly, nine aromatic hydrocarbons were separated on the monolithic column within 5 min and the highest column efficiency was 186 500 N/m. A typical chromatogram is shown in Fig. 3. From the elution order of benzene, toluene, ethyl benzene, propyl benzene, and butyl benzene, it can be deduced that the separation of neutral solutes is based on the reversed-phase mechanism. A mixture of hormones as the second family of neutral compounds was separated on this column. Conventional methods for separation of hormones were performed on HPLC, but some related separations have been reported in CEC [42, 43]. Presently, four corticosteroid compounds, together with deoxycortone, have been baseline-separated within 7 min on the monolithic column (Fig. 4). Unlike early attempts in the separation of hormones in CEC, no gradient elution was needed, thus the separation procedure was simplified.

As mentioned above, CEC has been a useful tool for separation of neutral organic molecules. However, the separation of ionic compounds is still a challenge for this

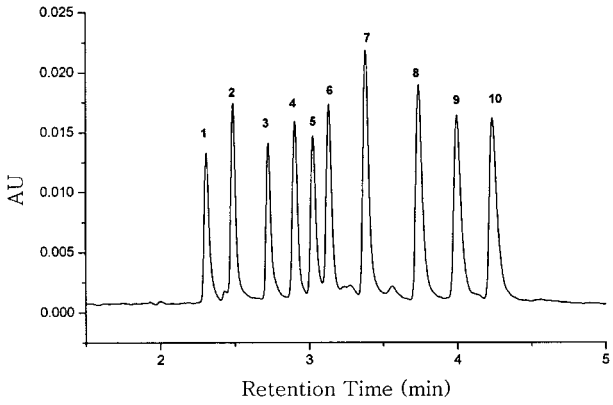

Figure 3. Separation of aromatic compounds on the monolithic column. Experimental conditions: 10 mM phosphate buffer containing 50% acetonitrile; applied voltage, 15 kV; injection, 5 kV for 1 s. Other conditions are the same as in Fig. 2. Peaks: (1) thiourea; (2) 2,4-dinitrotoluene; (3) 1,2-dinitrobenzene; (4) benzene; (5) 2-nitrotoluene; (6) toluene; (7) ethyl benzene; (8) propylbenzene; (9) 1-methyl-4-isopropyl-benzene; (10) butyl benzene.

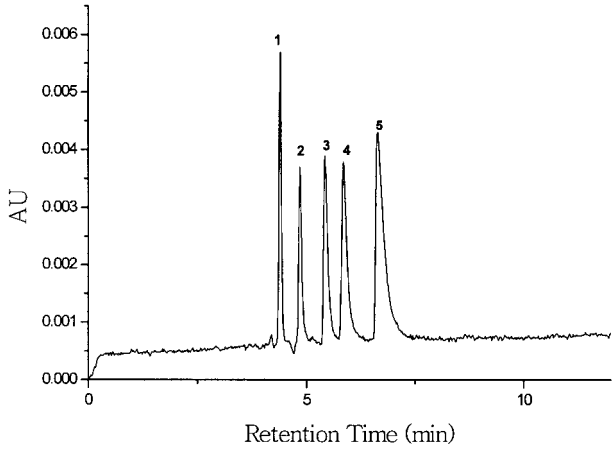

Figure 4. Separation of hormones on the monolithic column. Experimental conditions: 10 mM phosphate buffer containing 60% acetonitrile; applied voltage, 10 kV; injection, 5 kV for 2 s; detection wavelength, 214 nm. Other conditions are the same as in Fig. 2. Peaks: (1) estriol; (2) diethylstilbestrol; (3) cortisone; (4) estradiol; (5) deoxycortone.

technique. For example, during the separation process of basic compounds on silica-based stationary phases, severe peak tailing often occurs in typical RP-CEC. This is due to the interaction of these compounds with residual silanol groups on silica gel. While long analysis times are encountered in the case of acidic compounds in silica-based CEC columns, due to the different direction of their electrophoretic mobility from EOF. Some approaches have now been undertaken to overcome these problems [29, 44–47]. In order to improve the peak symmetry of basic compounds, a conventional method would be to add a competing base to the mobile phase to compete with the analytes [44–46]. This was expected to result in efficient separations with improved peak shapes. But the influence of the competing base on baseline was obvious, as they always involve more complicated mechanisms, which even lead to the change of elution order of some solutes [46]. Recently, Wu *et al.* [47] reported a mode for separation of ionic compounds driven only by electrophoretic mobility on a neutrally hydrophobic monolithic column. All the ionic peptides, including basic peptides, showed good peak symmetry. Svec *et al.* [13] adopted the ion-suppressed mode to separate a group of anilines at pH 12. Under these conditions, the basic analytes were in their neutral form, but the sulfonic groups on the monolithic polymer remained dissociated, ensuring a flow velocity sufficient to achieve the separations in a short period of time. Thereby, symmetric peaks were obtained due to suppressed electrostatic interaction between the neutral solutes and the negatively charged monolithic surface.

Similar attempts as the ion-suppressed mode of CEC were carried out to separate anilines and basic pharmaceuticals since the styrene-based monolith showed good stability with a strong basic pH. The ionization of selected basic compounds can be suppressed at high pH value. Electrochromatograms for separations of anilines and basic pharmaceuticals are shown in Figs. 5 and 6, respectively. It can be observed that the peak symmetry of the anilines is good, indicating that the ionization of the anilines had been mostly suppressed at high pH. The peak of basic pharmaceuticals is even more symmetric

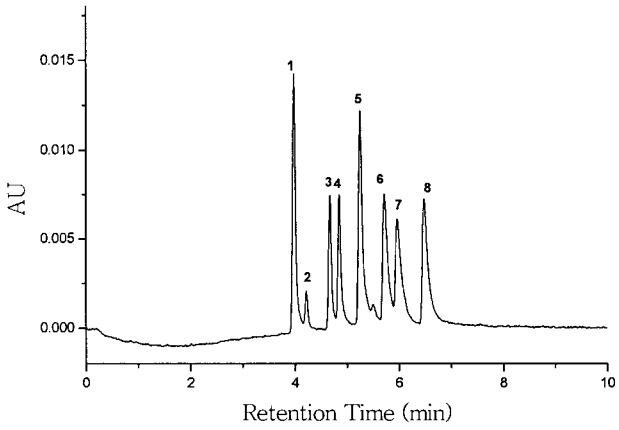

Figure 5. Separation of anilines on the monolithic column. Experimental conditions: 10 mM phosphate buffer containing 70% acetonitrile, pH 10.5; voltage, 10 kV; injection, 5 kV for 2 s; detection wavelength, 214 nm. Other conditions are the same as in Fig. 2. Peaks: (1) thiourea; (2) 1,4-phenylenediamine; (3) *o*-phenylenediamine; (4) 3-nitroaniline; (5) 2,4-dinitroaniline; (6) 3,3-dimethoxybenzdine; (7) *o*-tolidine; (8) 4-aminobiphenyl.

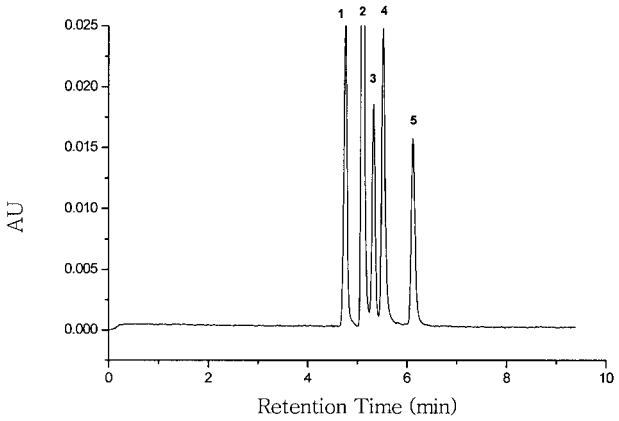

Figure 6. Separation of basic pharmaceuticals on the monolithic column. Experimental conditions: 10 mM phosphate buffer containing 50% acetonitrile, pH 10.5; injection, 5 kV for 2 s; detection wavelength, 214 nm. Other conditions are the same as in Fig. 2. Peaks: (1) cinchonine; (2) caffeine; (3) barbital; (4) isoamyl barbital; (5) phenyl barbital.

than that of the anilines. It was further proved that the ion-suppressed mode is an efficient method to separate basic compounds. The elution mechanism of the basic compounds under the suppressed mode is mainly based on the hydrophobic interaction between solutes and polymer rod, because the elution order of basic pharmaceuticals in CEC is the same with that in RP-HPLC. We have investigated the relationship between log *P* and log *k′* of anilines. The former and latter parameters illustrate the relative hydrophobicity and the retention factor of anilines on the stationary phase, respectively. It was observed that these two factors were basically in agreement with each other. The Log *P* values of solutes increase with the log *k′* values correspondingly with the exception of the lastly eluted compound 4-aminobiphenyl. This result may further support that the separation of basic solutes under this condition is mainly based on the mechanism of reversed-phase mode.

Peptides and proteins are usually separated at acidic pH under reversed-phase HPLC. However, in CEC most peptides are positively charged and will migrate electrophoretically to the cathode under acidic buffer. If the surface of the stationary phase in CEC is negatively charged, strong electrostatic interaction between the positively charged analytes and the negatively charged stationary phase occurs and results in long elution times or elution is even impossible. In order to suppress the strong electrostatic interaction, higher amounts of salt are often added into the mobile phase [20] which will result in serious Joule heating and bubble formation. In fact, in order to eliminate the strong electrostatic interaction of the charged groups between the peptides and the organic polymer monoliths, a so-called "counterdirectional mode" has been reported [18, 19], under which the peptides migrate electrophoretically in a direction opposite to that of the EOF. For example, an anodic EOF will be generated on the stationary phase carrying positive charges, and positively charged peptides will migrate electrophoretically to the cathode. The disadvantage of this mode may be that the separation time could take much longer, at least in principle, than that of separation of neutral compounds as the direction of EOF on the column and the electrophoretic mobility of solutes is opposite. Recently, Wu *et al.* [48] reported the separation of peptides under mixed mode of reversed-phase and ion-exchange on a monolithic column with column efficiency higher than 280 000 N/m.

However, in the case of MAA as the EOF-generating group, if the peptides are separated in acidic pH, the interaction between the analytes and the stationary phase can be successfully controlled due to the dependence of the ionization of the MAA on the pH. Furthermore, the elution times can be expected to be shorter than that in counterdirectional mode. In our experiment, separation of peptides was performed at pH 4.0. Under this condition, the EOF and the electrophoretic migration of most peptides are of the same direction, from anode to cathode, as most peptides carry positive charges, while the EOF is still large enough to serve as the driven force to generate velocity of mobile phase. At the same time, electrostatic interaction is largely suppressed, because the MAA incorporated in the monolith is not completely dissociated at pH 4. A mixture of six peptides was baseline-separated on the monolithic column due to their differences in the electrophoretic mobility and chromatographic interaction with the stationary phase (Fig. 7). It can be seen that the first three peptides, Arg-Gly, Met-His, and Ala-Ala-Ala, are eluted before formamide, which means that the electrophoresis mechanisms play a predominant role in their separation, because the p*I* of those peptides is higher than the pH of the buffers. The other three peptides are eluted after formamide, which indicates that chromatographic interaction is the main contribution to their retention behavior. In addition, separation of peptide isomers was performed on the prepared column (Fig. 8). It can be seen that isomers of both basic and hydrophobic peptides can be well resolved. Compared to other conventional CEC columns, this stationary phase seems to be an alternative for the separation of peptides, as not only a wider pH range could be adopted, but also the EOF and the retention of peptides on the stationary phase could be adjusted by adjusting the pH of buffer. It may be deduced that peptide separations can in principle even be performed under strong basic conditions, as in this case electrostatic interaction between the peptides and the stationary phase is also largely absent.

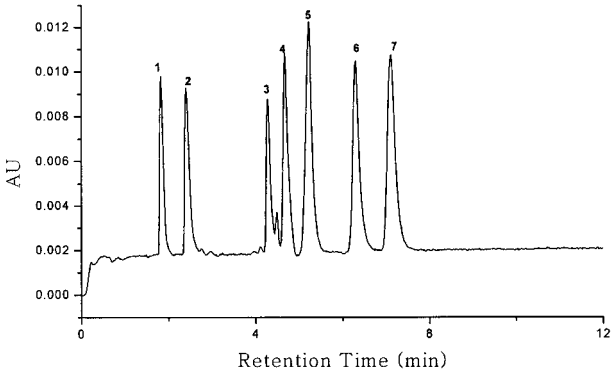

Figure 7. Separation of peptides on the monolithic column. Experimental conditions: 60% ACN in 10 mM citrate, pH 4.0; applied voltage, 20 kV; injection, 5 kV for 2 s; detection wavelength, 200 nm. Other conditions are the same as in Fig. 2. Peaks: (1) Arg-Gly; (2) Met-His; (3) Ala-Ala-Ala; (4) formamide; (5) Phe-Gly; (6) Phe-Tyr; (7) Phe-Phe.

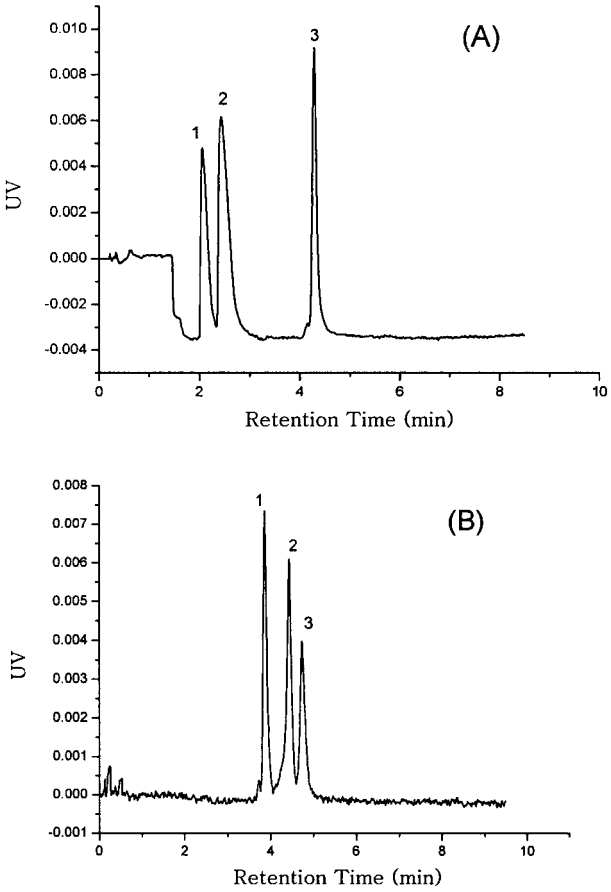

Figure 8. Separation of peptide isomers on the monolithic column. Experimental conditions: column, effective length 20.5 cm (total length 27 cm) × 100 μm ID × 375 μm OD; mobile phase, 60% ACN in 10 mM citrate; pH 4.0; applied voltage, 25 kV; injection, 5 kV for 2 s; detection wavelength, 200 nm; temperature, 25°C. (A) Peaks: (1) Met-His; (2) His-Met; (3) formamide. (B) Peaks: (1) thiourea; (2) Tyr-Phe; (3) Phe-Tyr.

4 Concluding remarks

A method for the preparation of a poly(styrene-*co*-divinylbenzene-*co*-methacrylic acid) monolithic stationary phase in CEC has been improved by optimizing the composition of mixture and polymerization conditions. The monolith rods synthesized in this way exhibited good performance in CEC separations. Improved peak shape was obtained by adjusting the amount of isooctane in the monomer mixture, proving that the proportion between the good solvents and poor solvents has an important effect on the structure of the monolithic polymer in capillary. As the EOF of this column is pH-dependent, the pH and thus the separation mechanism can be adapted for different families of compounds. Neutral compounds such as aromatic hydrocarbons and corticosteroids were well separated under reversed-phase mode of CEC, while basic compounds such as anilines and basic pharmaceuticals were separated under ion-suppressed mode. Peptides, including basic and neutral samples, were well separated in a combination of chromatographic and electrophoretic mechanisms. The facile preparation and wide application of this monolithic column may make styrene-based polymer a potential matrix as the stationary phase in CEC.

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