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Separation of acidic and basic compounds in capillary electrochromatography with polymethacrylate-based monolithic columns

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Abstract

Methacrylate-based monolithic columns with electroosmotic flow (EOF) or very weak EOF are prepared by in situ copolymerization in the presence of a porogen in fused-silica capillaries pretreated with a bifunctional reagent. Satisfactory separations of acidic and basic compounds on the column with EOF at either low or high pH are achieved, respectively. With sulfonic groups as dissociation functionalities, sufficient EOF mobility still remains as high as 1.74×10^{-4} cm^2 s^{-1} V^{-1} at low pH. Under this condition, seven acidic compounds are readily separated within 5.7 min. Moreover, at high pH, the peak shape of basic compounds is satisfactory without addition of any masking amines into running mobile phase since the secondary interaction between the basic compounds and the monolithic stationary phase are minimized at high pH. Reversed-phase mechanism for both acidic and basic compounds is observed under investigated separation conditions. In addition, possibilities of acidic and basic compound separations on a monolithic column with extremely low EOF are discussed.
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1. Introduction

Capillary electrochromatography (CEC), as a hybrid of high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), features the high selectivity of HPLC and the high efficiency of CE [1]. It is rapidly gaining popularity owing to its powerful separation ability and compatibility with mass spectrometry. However, some inherent problems such as tedious packing procedure and poorly constructed frits have been the barrier to this separation technique in the early stage of its development. As an alternative to the conventional packed columns, monolithic columns have attracted great attention in recent years due to their simple preparation procedure and no need for frits. So far, silica rod [2–4], polymethacrylate-based monolith [5–7], polyacrylamide monolithic column [8,9] and polystyrene continuous bed [10–12] have been fabricated and applied to the separations of peptides [13], proteins [14], steroids [15], polymers [6] and amino acids [16] and so on. Several reviews on in situ polymerization of synthetic organic polymers as monolithic columns for CEC have been published recently [17–19].

Octadecyl silica (ODS) columns are frequently used in reversed-phase CEC (RP-CEC), since most of the well-established knowledge of ODS for HPLC can be transferred to CEC directly. However, it still remains problematic for the separations of acidic and basic compounds in CEC. Although both neutral and charged species can be resolved by CEC, the analysis of acidic compounds is relatively difficult. As we know, electroosmotic flow (EOF) originates from the negatively charged packing surface in RP-CEC due to the dissociation of silanol groups. Its direction is therefore from anode to cathode. Meanwhile, the electrophoretic flow of acidic compounds goes against EOF, resulting in rather long analysis time or even no peaks when the electrophoretic flow velocity is higher than EOF velocity. The retention behavior of acidic compounds at high pH has been investigated in the literature [20,21]. However, some acidic species with higher electrophoretic mobility than that of EOF cannot be electrokinetically injected into CEC columns. Therefore it is

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recommended to utilize ion-suppressed mode where a low pH electrolyte is used as an eluent [22], and EOF is rather low since EOF is a function of pH value. Additionally the stationary phase might degrade if the pH of mobile phase is lower than 2.5, which leads to the gradual deterioration of column performance.

The analysis of basic compounds on ODS columns often suffers from broadening peaks and serious tailing which is caused by the secondary interaction between basic solutes and residual silanol groups. To overcome the problem of peak tailing, Smith [23] reported the separation of basic compounds on a cationic-exchange column with extremely high column efficiency over 1 000 000 theoretical plates/meter. However splitting peaks occurred and such high column efficiency was neither fully explained nor reproducible. Zhang and El Rassi [24] used a laboratory-made octadecyl-sulfonated silica phase to separate nucleosides and bases. CEC performance of basic compounds on conventional reversed-phase materials can be improved by adding competing amines into running mobile phase [25,26]. However, the use of amines slows down or even reverses EOF, and also affects ionic strength in CEC.

In this paper, the polymethacrylate-based monolithic columns are utilized to separate acidic and basic compounds at extreme pH values. Due to the high stability of polymethacrylate-based monolithic column over pH range from 2 to 12, the separations of above compounds are achieved at either low or high pH. The retention mechanism on such columns is investigated. Moreover, in order to separate them under a mild condition, an attempt is made to prepare the polymethacrylate-based monolithic column with very weak EOF. The potential application and limitation of this column are discussed.

2. Experimental

2.1. Instrumentation and material

All the experiments were performed on a P/ACE 5010 (Beckman, Fullerton, CA, USA) equipped with Gold software (version 8.10) for data acquisition. An HPLC pump (Elite Analytical Instrument, Dalian, China) was used to flush monolithic columns with mobile phase for conditioning. A manual syringe pump was obtained from Unimicro Technologies (Pleasanton, CA, USA) to chase bubbles out of capillaries. Fused silica capillaries (100 μm i.d. × 365 μm o.d.) were purchased from Yongnian Optic Fiber Plant (Hebei, China).

2.2. Chemicals and buffers

Acetonitrile was of chromatographic grade, and other chemicals were of analytical grade. Chemicals for column preparation such as ethylene dimethacrylate (EDMA) and butyl methacrylate (BMA) were distilled in vacuo to remove the inhibitor prior to use. Azobisisobutyrinitrile (AIBN) was purified by recrystallization. Ultra pure water was produced by a CLEAR SG water purifier (SG Wasseraufbereitung und Regenerierstation, Barsbüttel, Germany). Mobile phase was prepared by adjusting buffers to desired pH value, then mixing with the appropriate amount of organic modifier. The mobile phase was degassed in an ultrasonic bath for 15 min. The pH value mentioned here was that of stock buffer solution.

2.3. Column preparation

2.3.1. Pretreatment of the inner wall of capillaries

In order to improve the stability of the monolithic columns, the capillaries were treated with 3-(trimethoxysilyl) propyl methacrylate, a bifunctional reagent, prior to polymerization [26–28]. Briefly, the capillaries were washed with 1 mol l^{-1} NaOH for 2 h so that siloxane groups at inner surface of raw fused silica capillary could hydrolyze to increase the density of silanol groups serving as anchors for subsequent silanization. Thereafter, the capillaries were washed with ca. 50 column volumes of deionized water and acetone, respectively. Subsequently, capillaries were purged with nitrogen gas at ambient temperature for 5 h. After that, a solution of 30% (v/v) 3-(trimethoxysilyl) propyl methacrylate in acetone was injected into the capillaries by means of a syringe. With both ends sealed with silicone septums, the capillaries were left at ambient temperature overnight, then washed by ca. 50 column volumes of acetone, and finally purged with nitrogen gas for 5 h. By this means, methoxy groups of this compound react readily with silanol groups at the surface of capillary wall, leaving methacryloyl end to react later with the methacrylic groups present in the monomers, so that continuous bed became covalently bound to capillary wall.

2.3.2. Formation of the stationary phase with EOF

Monolithic columns used in this experiment were fabricated as reported in the literature [5,6]. The reaction solution consisting of 400 mg of the mixture of 40% (w/w) EDMA, 59.4% (w/w) of BMA and 0.6% (w/w) 2-acryloylamido-2-methylpropanesulfonic acid (AMPS), 600 mg of ternary porogen composed of 10% (w/w) water, 64% (w/w) 1-propanol and 26% (w/w) 1,4-butanediol, and 6 mg of AIBN [1.5% (w/w) with respect to the monomers] was injected into the capillaries partially. Thereafter columns were put into a gas chromatography oven and kept at 60 °C for 20 h with both ends sealed with silicone septums. In this paper, this kind of column with EOF was named as column A.

2.3.3. Formation of the stationary phase with extremely low EOF

In order to obtain the monolithic columns with very low EOF, AMPS monomer was omitted during the polymerization. The reaction solution consisting of 400 mg of

the mixture of 40% (w/w) EDMA and 60% (w/w) BMA, 600 mg of ternary porogen composed of 10% (w/w) water, 62% (w/w) 1-propanol and 28% (w/w) 1,4-butanediol, as well as 6 mg of AIBN, was injected into the capillaries. The rest procedures were the same as above. This kind of column was named as column B.

Any movements of both kinds of the stationary phases were not observed in the experiment, which indicated that the stationary phases had been covalently bound to the inner wall of the capillaries through the bifunctional reagent indirectly. Detection window was made with a razor manually right after the stationary phase.

2.4. Separation conditions

Before electrochromatographic experiments, the monolithic columns were flushed with the mobile phase for 1 h with the HPLC pump, and then the columns were conditioned on the instrument with the mobile phase for another 1 h. Applied voltage was first ramped from 0 to 10 kV. Any abrupt change of applied voltage was carefully avoided to minimize the likelihood of bubble formation. During the experiments, the columns were equilibrated for about 30 min after the mobile phase was changed. Column temperature was kept at 25 °C and detection wavelength was set at 214 nm. The effective and total length of the capillaries were 20 and 27 cm, respectively.

3. Results and discussion

3.1. Separation of acidic and basic compounds on the polymethacrylate-based monolithic column with EOF

Column-to-column reproducibility is a very important parameter for column performance. Since the preparation of these monolithic capillaries is a real single-step process, the number of effects that have a deleterious influence on column-to-column reproducibility is dramatically reduced. In our experiments, the relative standard deviation (R.S.D.) for t_0 in five different columns prepared from the identical polymerization mixture is found to be 3.1%, which proves the good column-to-column reproducibility of these columns.

Different from the stationary phases for HPLC, CEC stationary phases not only affect separations but also enable EOF. The generation of EOF is due to the dissociation of ionizable moieties on the surface of monolithic columns. Therefore, during designing a monolithic column for CEC, an ionizable monomer is prerequisite to support EOF. Here AMPS was selected as the charged monomer. The pK_a of sulfonic acid on the surface of the stationary phase is much smaller than that of silanol. Hence the stationary phase could remain high EOF velocity even in ion-suppressed mode. Fig. 1 shows the separation of seven acidic compounds including *p*-hydroxybenzoic acid, *p*-methoxybenzoic acid,

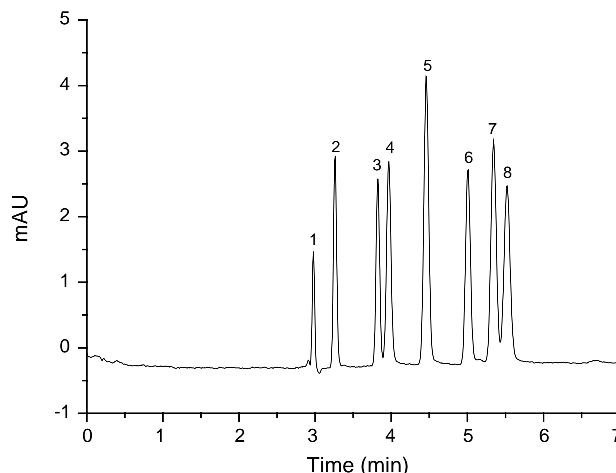

Fig. 1. Electrochromatogram of acid compounds. Stationary phase, column A. Mobile phase, acetonitrile–5 mmol l^{-1} phosphate (pH 2.32) (55:45, v/v). Electrokinetic injection, 3 kV, 3 s. Applied voltage, 16 kV. Peaks: 1, thiourea; 2, *p*-hydroxybenzoic acid; 3, *p*-methoxybenzoic acid; 4, *o*-aminobenzoic acid; 5, *o*-toluic acid; 6, *p*-chlorobenzoic acid; 7, *p*-bromobenzoic acid; 8, *m*-bromobenzoic acid. Other conditions as in Section 2.

o-aminobenzoic acid, *o*-toluic acid, *p*-chlorobenzoic acid, *p*-bromobenzoic acid and *m*-bromobenzoic acid on the polymethacrylate-based monolithic column with EOF at pH 2.32. Under this condition, EOF mobility is as high as $1.74 \times 10^{-4}\,cm^2\,s^{-1}\,V^{-1}$, which is sufficient to achieve the fast separation of acidic compounds. Moreover, the uncharged solutes yield symmetrical peaks with high efficiency up to 177 000 plates m^{-1} and tailing factors within the range from 0.96 to 1.0. The R.S.D. for t_0 and retention times of the aromatic acids in eight consecutive runs are 0.64% and less than 0.78%, respectively, which indicates the high stability of the monolithic stationary phase even at extremely low pH. Reverse-phase mechanism for the separation of acidic compounds is observed with linear correlation coefficient of the regression between $\log k$ and acetonitrile content better than 0.99. In addition, buffer concentration is also an important parameter for method optimization. With the increase of phosphate concentration from 3 to 7 mmol l^{-1}, EOF mobility decreases from 1.80×10^{-4} to $1.72 \times 10^{-4}\,cm^2\,s^{-1}\,V^{-1}$ due to the suppression of double layer. In our experiment, the variation of buffer concentration in the range of 3–7 mmol l^{-1} has no significant effect on column efficiency and resolution. Comprising buffer capacity and separation speed, 5 mmol l^{-1} phosphate is selected as the final condition. Furthermore, as shown in Fig. 1, the fast baseline separation of seven acidic compounds within 5.7 min could be obtained in ion-suppressed mode, which indicates that such a monolithic column is suitable for the separation of acidic compounds.

In RP-CEC, the analysis of basic compounds usually suffers from peak broadening and serious tailing owing to the secondary interaction between the stationary phase and basic solutes. Due to high stability of the polymer monolithic

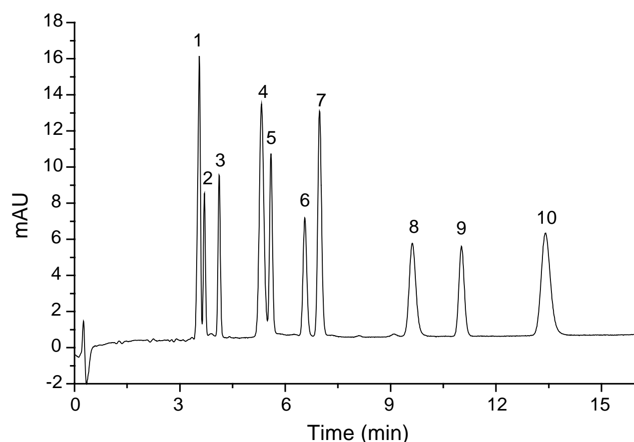

Fig. 2. Electrochromatogram of basic compounds. Mobile phase, acetonitrile–10 mmol l^{-1} sodium hydroxide (pH 12) (55:45, v/v). Electrokinetic injection, 3 kV, 3 s. Applied voltage, 12 kV. Peaks: 1, thiourea; 2, *o*-phenylenediamine; 3, *p*-phenylenediamine; 4, 4,4′-methylenedianiline; 5, aniline; 6, 3,4-dimethylaniline; 7, *m*-nitroaniline; 8, naphthylamine; 9, dimethylaniline; 10, 2,6-dichloro-4-nitroaniline. Other conditions as in Section 2.

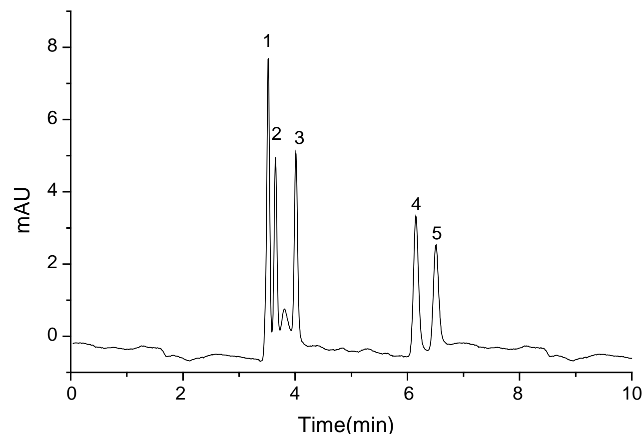

Fig. 3. Separation of positional isomers. Stationary phase, column A. Mobile phase, 60% acetonitrile in 10 mmol l^{-1} sodium hydroxide (pH 12). Injection, 3 kV, 3 s. Applied voltage, 12 kV. Peaks: 1, thiourea; 2, *o*-phenylenediamine; 3, *p*-phenylenediamine; 4, *m*-nitroaniline; 5, *o*-nitroaniline. Other conditions as in Section 2.

column over the range of pH 2–12, nine basic compounds including *o*-phenylenediamine, *p*-phenylenediamine, 4,4′-methylenedianiline, aniline, 3,4-dimethylaniline, *m*-nitroaniline, naphthylamine dimethylaniline and 2,6-dichloro-4-nitroaniline are tested at pH 12. Under this condition all the basic compounds are neutral, and the inherent reason for tailing is, therefore, eliminated. As shown in Fig. 2, satisfactory separation of the basic compounds was obtained with symmetric peaks and column efficiency up to 114 000 plates/m without addition of any competing amines in the mobile phase. Tailing factors for the basic compounds are in the range from 0.92 to 1.2. EOF mobility is $2.1 \times 10^{-4}\,cm^2\,s^{-1}\,V^{-1}$, increasing by 20%, compared with that at pH 2.32, which results from the fully ionized sulfonic groups. Lower column efficiency for the basic compounds might be due to the slight hydrogen bonding between the carbonyl groups of the stationary phase and the amino group of the analytes. Retention mechanism for most of basic compounds is reversed-phase except *o*-phenylenediamine (data not shown). Since there are two amino groups in this molecule, its hydrogen bonding interaction with the stationary phase might be stronger than others. Therefore the retention mechanism for *o*-phenylenediamine might be based on not only hydrophobic interaction but also hydrogen bonding. It can be seen from Fig. 3, the positional isomers of phenylenediamine and nitroaniline can be readily separated under the identical condition. It should be mentioned that such extreme pH conditions cannot be tailored with typical silica-based packing. Otherwise, the hydrolysis of silica-based support, leading to the loss in column efficiency gradually, will be very serious. Therefore high pH stability of monolithic polymer provides great versatility in method optimization. The columns could be used continuously for half a month and there is no significant loss of column efficiency at either high or low pH.

3.2. Possibilities of the separation of acidic and basic compounds on the polymethacrylate-based monolithic column with low EOF

Difficulties in the separations of acidic and basic compounds mainly originate from the charged moieties on the surface of stationary phase in CEC. Therefore an attempt to prepare monolithic column with very low EOF is made so that acidic and basic compounds could be separated under a mild condition. In CEC, the migration of neutral species is driven by EOF. While for a charged species, it can be driven by both electrophoretic flow and EOF. EOF is not, consequently, prerequisite for all separations, especially when the direction of EOF is opposite to that of electrophoretic flow of the charged species. Moreover, the charged moieties on the surface of stationary phase are usually the source of tailing and peak broadening for basic compounds due to the secondary interaction between the stationary phases and the analytes. The ease of the preparation of monolithic columns makes us have more ability to tailor the final property of stationary phases.

The monolithic column with very weak EOF is prepared by omitting AMPS monomer during the polymerization. One of the differences in column A (with EOF) and column B (without EOF) is the surface property of the monolithic columns. EOF mobility on column B is about $9 \times 10^{-6}\,cm^2\,s^{-1}\,V^{-1}$ even at pH 9.8, which is much lower than that on the monolithic column A. Hence, electroosmotic mobilities can be neglected so that the migration of charged acidic compounds is only driven by their electrophoretic mobilities. Consequently, separation mechanism in this case may be electrophoretic migration and chromatographic partition between the stationary phase and

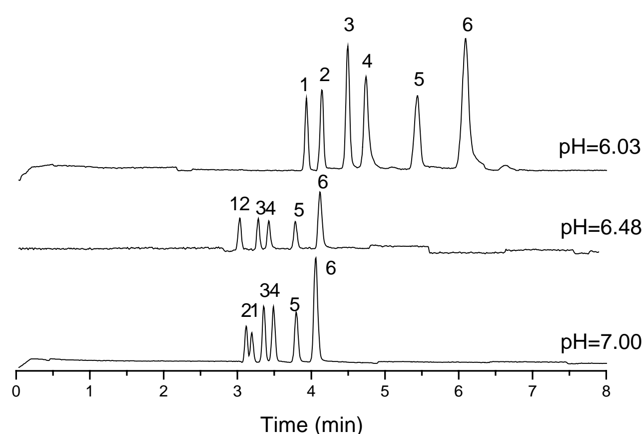

Fig. 4. Influence of pH value on acidic compound separation. Stationary phase, column B. Mobile phase, acetonitrile–5 mmol l^{-1} phosphate (70:30, v/v). Applied voltage, 10 kV. Peaks: 1, *p*-amino phenylsulfonic acid; 2, *p*-methylphenylsulfonic acid; 3, *m*-nitrophenylsulfonic acid; 4, *p*-bromobenzoic acid; 5, α-naphthylacetic acid; 6, *p*-hydrohoxybenzoic acid. Other conditions as in Section 2.

the mobile phase, if the charged solutes still have enough hydrophobicity for chromatographic partition with the stationary phase. Six acidic compounds, *p*-amino phenylsulfonic acid, *m*-nitrophenylsulfonic acid, *p*-bromobenzoic acid, α-naphthylacetic acid, *p*-methylphenylbenzoic acid and *p*-hydrohoxybenzoic acid were selected as test solutes. Column-to-column reproducibility of this kind of column is investigated. The R.S.D. of the migration time of *p*-amino phenylsulfonic acid is 3.5% at pH 7.0 ($n = 3$), which is nearly the same as that obtained on column A. Fig. 4 demonstrates the effect of the pH value on the acidic compound separation on the monolithic column B. The pH value of mobile phase has a significant effect on selectivity and migration velocity. With the increase of pH of mobile phase from 6.03 to 7.00, *p*-amino phenylsulfonic acid and *p*-methylphenylsulfonic acid are coeluted or even the elution order is reversed. Migration velocities of the acidic compounds become faster gradually, which is caused by the changes of the ionization extent of solutes. Although the migration of solutes is driven by their electrophoretic mobilities, the baseline separation of the six test acidic compounds can be achieved within reasonable time. Compared to the conditions in ion-suppressed mode, separation conditions adopted here are much milder to the stationary phase. The R.S.D. of migration time of the acidic solutes is less than 1.40%. To investigate whether chromatographic partition contributes to the acidic compound separation, capillary zone electrophoresis experiment is performed under the identical condition using a fused silica capillary coated with polyacrylamide. However, little difference of separation results is found, which demonstrates that the chromatographic partition between the stationary phase and ionized analytes is very weak. The ionized acidic compounds in this experiment are relatively hydrophilic resulting in the weak chromatographic partition with the stationary phase. This column is, hence, suitable for the separation of acidic compounds that still have enough hydrophobicity for chromatographic partition with the stationary phase. Another limitation of this column is that the organic modifier content cannot be too low in order to increase the retention of charged species, otherwise, bubble formation will become significant due to the poor wettability between the stationary phase and the solutes. The use of this stationary phase in pressure-assisted CEC and μHPLC may be feasible. Since there is no charged moiety on column B for the secondary interaction with basic analytes, the basic compounds could be expected to be separated with good peak shape without addition of competing amines. Further experiments are under way in our group.

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