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Chiral separation of basic compounds on a cellulose 3,5-dimethylphenylcarbamate-coated zirconia monolithin basic eluents by capillary electrochromatography

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ABSTRACT

Porous zirconia monolith (ZM) modified with cellulose 3,5-dimethylphenylcarbamate (CDMPC) was used as chiral stationary phaseto separate basic chiral compounds in capillary electrochromatography. The electroosmotic flow behavior of bare and CDMPC-modified zirconia monolithic (CDMPC-ZM) column was studied in ACN/phosphate buffer eluents of pH ranging from 2 to 12. The CDMPC-ZM column was evaluated by investigatingthe influences of pH, the type and composition of organic modifier of the eluent on enantioseparation. CEC separations at pH 9 provided the best resolutions for the analytes studied, which are better than those observed on CDMPC-modified silica monolithic columns under similar chromatographic conditions. No appreciable decline in retention and resolution factors after over 200 injections, and run-to-run and day-to-day repeatabilities of the column of less than 3% indicate the stability of the zirconia monolithic column in basic media.

1. Introduction

Enantioseparation is important in pharmaceutical, clinical, food, agricultural and forensic analyses [1,2] as the enantiomers of a chiral compound exhibit different biological activity [3,4]. One enantiomer may produce the desired therapeutic activities while the other may be inactive or produce undesirable effects. Among various methodologies used for chiral separation in both analytical and preparative scales, chromatographic methods have been used most frequently as they are rapid, effective, reproducible and preparative in nature. The chromatographic techniques can be used for the isolation of the enantiomers and for the qualitative and/or quantitative analysis of the enantiomers in bulk substances and pharmaceutical preparations. So, chiral chromatography has become a major method for enantioseparation as well as preparation of enantiopure compounds [5]. Capillary electrochromatography (CEC) is a hybrid technique of HPLC and CE, and is a rapidly developing technique in the area of separation science [6–8] and has been increasingly utilized in studies on the development and evaluation of enantioseparation methods. It shows high efficiency and fast analysis because it uses electroosmotic flow (EOF) to pump the mobile phase and can separate charged as well as uncharged compounds through electrophoresis and chromatographic separation [3].

Three types of columns including, open-tubular (OT-CEC), particulate-packed (P-CEC) and monolithic (M-CEC) capillaries are used for CEC [9]. The major advantage of CEC is the amount of chiral selector required to prepare a chiral column is far less than HPLC. Several reviews have been reported on enantioseparations using CEC as a separation technique [6–8,10]. Monolithic columns are becoming attractive alternative to particle-packed columns in high performance liquid chromatography and electrochromatography [11,12]. A monolithic capillary column is composed of an interconnected skeletal structure that forms a continuous bed with flow-through paths called as through-pores [13]. The preparation of a packed column involves burdensome packing of stationary phase particles in a capillary and preparation of frits by sintering a zone of the packing. Such frits cause formation of air bubbles during the analysis which results in reduction of separation efficiency, and tend to break easily [14–17]. The monolithic capillary columns are devoid of the problems and difficulties associated with packed capillary columns. The monolithic columns allow fast mass transfer at lower pressure drops, enabling much faster separations for large molecules. The continuous monolithic bed in the capillary column also allows high linear velocities that enable high throughput screening and fast separations of enantiomers [11–13,18]. All these qualities have made the monolithic column as an alternative to the packed and open-tubular columns in the field of micro- and nano-scale separation of wide range of compounds.

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Among various classes of chiral selectors [19,20] polysaccharides have occupied a unique place in the chiral separation of a broad range of chiral compounds and been most widely used as the chiral stationary phase. Some 200 kinds of polysaccharide derivatives including cellulose, amylose, chitin, chitosan, galactosamine, curdlan, dextran, xylan, and inulin have been in use for chiral separations [21]. The derivatives of cellulose and amylose usually exhibit higher chiral recognition ability than the other type polysaccharides. A number of studies have been reported for separation of a large number of chiral compounds in different classes by HPLC on polysaccharide-immobilized silica [22–25] and polymer columns [26]. The polysaccharide-based columns can be used in normal phase, polar organic and reversed-phase mode [27].

Silica-based stationary phases have received wide acceptance due to their well-studied surface chemistry. However, the drawbacks of silica-based stationary phases are also well experienced, which includes restricted use in eluents of a limited pH range of 2–8 due to unstable nature of Si–O–Si bond in acidic as well as basic solutions [28,29] and applicability only in limited temperature range [30]. Zirconia is an attractive and suitable alternative to silica as the support, mainly due to its unique and extraordinary chemical, mechanical and thermal stabilities [31–33]. Zirconia particles show no detectable signs of dissolution over the pH range from 1 to 14 and have been used for prolonged periods at temperatures up to 200 °C in chromatographic separations. The specific surface area and pore volume of zirconia are smaller in comparison with silica, but due to its higher density the surface area of zirconia is comparable to that of silica in terms of surface area per unit volume. The unique surface chemistry of zirconia extends different applications for its use in chromatography [31,34]. A number of zirconia-based chiral stationary phases have been evaluated in HPLC [35–39] and capillary electrochromatography (CEC) [40–42]. Recently zirconia-based chiral stationary phases coated with cellulose derivatives have been used in HPLC [35,36,43,44] and CEC in particle-packed [40] and monolithic columns [42]. We recently reported chiral separation of some basic chiral compounds by CEC on a cellulose 3,5-dimethylphenylcarbamate (CDMPC)-coated zirconia monolith (CDMPC-ZM) column in mobile phases of acidic pH [42]. In this work, we investigated the enantiomer separation by CEC of a set of basic chiral compounds on the CDMPC-ZM column in eluent of pH ranging from 2.0 to 12.0 to evaluate the performance and stability of the column in basic media.

2. Experimental

2.1. Materials

Fused silica capillaries (75 μm I.D., 365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Zirconium butoxide, acetic acid, potassium dihydrogen phosphate and sodium hydroxide were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Cellulose (Avicel) was obtained from Merck (Darmstadt, Germany). Triphenylmethyl chloride and 4,4′-diphenylmethane diisocyanate of reagent grade were received from TCI (Tokyo, Japan). Polyethylene glycol (PEG) (MW = 10,000 g mol^{-1}), 3,5-dimethylphenyl isocyanate, tetrahydrofuran (THF) and pyridine were supplied by Aldrich (Milwaukee, WI, USA). All reagents used were reagent grade or better having higher than 99% purity. HPLC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Water was purified with an Elgastat UHQ water purification system (Bucks, UK). Basic chiral compounds including Tröger's base (TB), propranolol (PRO), oxprenolol (OXP), indapamide (IND), atropine (ATR) and homatropine (HATR) were of the highest-purity available from Aldrich (Milwaukee, WI, USA) or TCI (Tokyo, Japan).

2.2. Instrumentation

An Agilent HP ^{3D}CE System (Palo Alto, CA, USA) equipped with a diode-array UV detector, a ±30 kV high voltage power supply and an external nitrogen pressure was used for the CEC separations. An external pressure of 10 bar was applied to both buffer reservoirs. Instrument control and data collection were performed with the ChemStation software. Separations were carried out at 25 °C and monitored at 200, 214, 254 and 280 nm. The morphology of the zirconia monoliths was examined by a field emission scanning electron microscope (FE-SEM S-4100, Hitachi, Japan). A syringe pump from Cole-Parmer (Vernon Hills IL, USA) was used to inject the CDMPC solution into the zirconia monolithic capillaries.

2.3. Column preparation

Zirconia monolithic capillary column was prepared according to the method reported earlier [42]. Briefly, to a homogeneous hydrolysis solution consisted of PEG, water, acetic acid and 1-butanol at suitable concentrations, a required amount of zirconium butoxide was added. The resulting mixture was injected into the activated fused silica capillary up to the required length and allowed to react overnight at 35 °C. Then, the column was heated at 150 °C for 6 h. After completion of heating, the capillaries were cooled to room temperature and then characterized by SEM. CDMPC was prepared as per the reported method [45] and characterized by elemental analyses, IR and NMR spectroscopy. To perform the CDMPC coating on the surface of zirconia monolithic bed, the zirconia monolithic capillaries were initially washed with ethanol and then with THF. Then, a CDMPC solution of 4% by weight in THF was passed through the capillaries at a flow rate of 5 μL min^{-1} using a syringe pump to coat the entire zirconia monolithic bed of the capillary column. Drying and additional coating of the coated monolithic capillary with the CDMPC solution was repeated two more times in order to increase the polymer loading on the surface. The capillary was finally rinsed with methanol and mobile phase, respectively.

2.4. Chromatography

The mobile phases used for the enantioseparation were the mixtures of phosphate buffer of varying pH and ACN or MeOH in different compositions. These mobile phases were filtered through a nylon membrane filter of 0.2-μm pore size and degassed prior to use. The CDMPC-coated zirconia monolithic capillary columns were equilibrated for about 8–10 h in order to reduce baseline noise before CEC runs. Samples dissolved in the mobile phase were injected electro-kinetically at 15 kV for 5 s. Retention times of two consecutive injections were in agreement within 3%. Fresh mobile phase was replenished after each run of sample. The capillaries with total length of 35 cm and monolithic bed length of 25 cm were used for separation. The dead time was measured by injecting acetone.

3. Results and discussion

Zircanol groups on the zirconia monolith (ZM) surface can undergo the following Brönsted acid–base reactions and either positive or negative surface charge can develop depending on the pH of the mobile phase:

$$ZrOH \leftrightarrows ZrO^- + H^+ \quad (1)$$

$$ZrOH_2{}^+ \leftrightarrows ZrOH + H^+ \quad (2)$$

Isoelectric pH of zirconia is reported to be between 5 and 6, which can vary depending on the crystalline form and the material of synthesis. Thus the direction of EOF can be either cathodic above this pH or anodic below this pH [46]. Fig. 1 shows variation

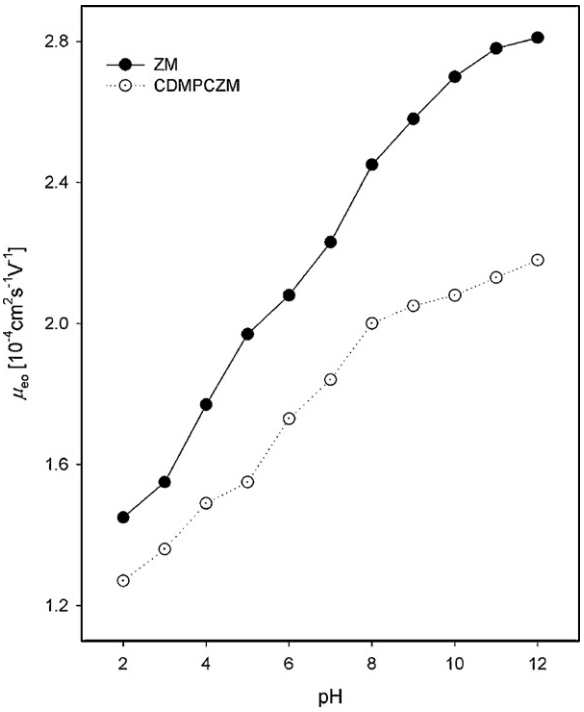


Fig. 1. Effect of pH on electroosmotic mobility, μ_{eo}, on bare ZM and CDMPC-ZM. *Conditions*: column, length 35 cm (monolith bed 25 cm) × i.d. 75 μm; eluent, 50/50 (v/v) ACN/phosphate buffer (50 mM); marker, thiourea; temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV for 5 s; detection, 215 nm.

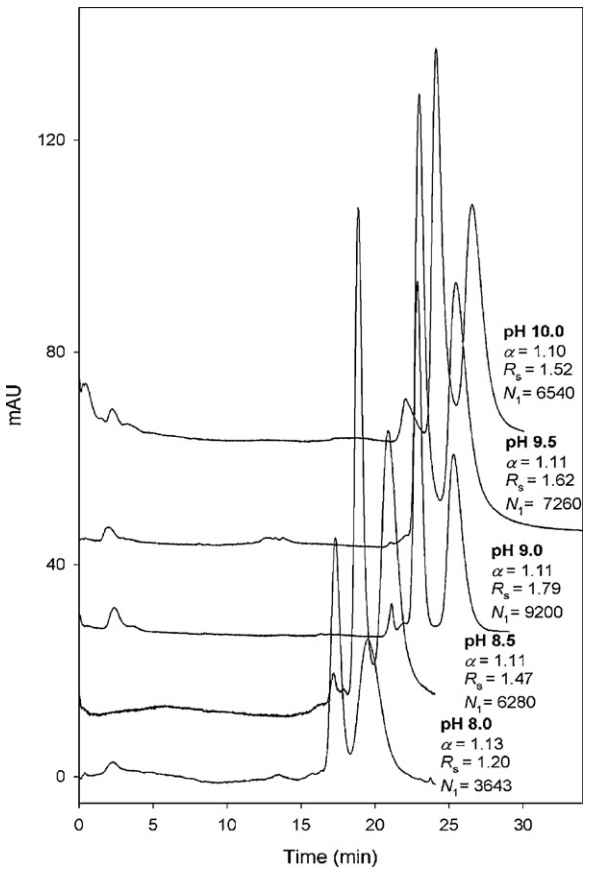


Fig. 2. Effect of pH. *Conditions*: analyte, Tröger's base; eluent, 50/50 ACN/phosphate buffer (50 mM); temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV, 5 s; detection, 214 nm.

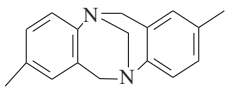
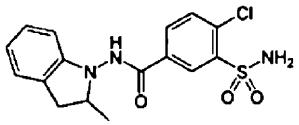
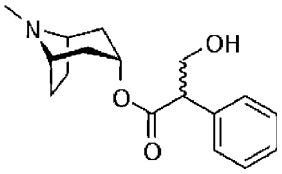
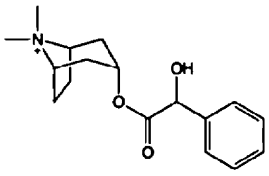
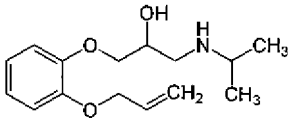
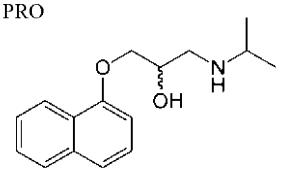
of electroosmotic mobility (μ_{eo}) measured by thiourea on bare ZM and CDMPC-ZM with pH in the 50:50 (v/v) ACN/phosphate buffer. Cathodic EOF was observed for both bare ZM and CDMPC-ZM at all pHs and its magnitude increases with pH, indicating that equilibrium given by Eq. (1) is prevalent, giving negative charge on its surface [42]. Phosphate ions as Lewis bases are able to bind strongly to the Lewis acid sites of zirconia surface [31] to provide extra negative charges and hence cathodic EOF, regardless of the mobile phase pH. The magnitude of EOF increases with pH as more zircanol groups dissociate according to Eq. (1) to increase negative surface charges, resulting in increasing EOF. The magnitude of EOF on CDMPC-ZM is smaller than on bare ZM since the number of exposed dissociable zircanol groups on CDMPC-ZM is decreased as the surface is covered by adsorbed polymer [42].

The pH of the mobile phase will affect the electromigration behavior of a basic analyte as it determines its degree of ionization and hence electromigration behavior. Separation of a representative analyte, Tröger's base, has been examined in eluents of 50:50 (v/v) ACN/phosphate buffer of apparent pH 8.0–10.0 and the results are shown in Fig. 2. With increasing pH migration time of TB increases. With increasing pH the fraction of protonated form of TB molecules decreases, which in turn reduces electrophoretic flow that is co-directional with EOF, resulting increased migration time. Chiral selectivity factor (α) does not change appreciably with pH. Resolution factor (R_s) first increases with pH to give maximum at pH 9.0 and then decreases upon further increase in pH. The relationship, $R_s = 1/4N^{1/2}(\alpha - 1)$ [47] can be used to explain this. As the α values remain essentially unchanged the R_s values change by following the variation of the plate number (N_1) with pH. The mobile phase with pH 9.0 was thus chosen for the further separation of remaining compounds. Table 1 lists chiral separation data for all six compounds in the eluent of 50:50 (v/v) ACN/phosphate buffer at pH 9.0. Better resolution was observed for TB (R_s = 1.79; N_1 = 9200; t_1 = 22.85 min at pH 9.0) on the ZM coated multiply with the CDMPC solution of a low concentration (4 wt%) than on that coated once with a solution of a high concentration (8 wt%) (R_s = 1.35; N_1 = 3970; t_1 = 23.04 at pH 4.4) [42]. It seems that the higher resolution observed with the multiply coated column is due to increased loading with repetitive coating and more homogeneous coating obtained by the use of the coating solution of lower concentration although SEM micrographs of the two monolithic columns do not show noticeable difference in the surface morphology. It is noted that comparison may not be rigorous as the CDMPC-ZM column and pH of the eluent used in this work are not the same as those in the earlier work.

Influence of the ACN content on chiral separation was examined for Tröger's base by varying the ACN content from 40 to 60 v% and resulting chromatograms are shown in Fig. 3. ACN was chosen as the organic modifier as it provides better separation efficiency [40,48]. With increase in the ACN content retention of TB decreases steadily, showing typical RPLC behavior. While selectivity factor decreases only slightly resolution factor for the enantiomers decreases appreciably as increasing amount of ACN causes the solubility in the eluent to increase and hence interactions of the analyte with CDMPC to decrease [49]. It is thought that ACN composition of 50 v% is optimal when retention time and resolution are taken into consideration together.

It is expected that the separation behavior of basic analytes in mobile phase of basic pH is different from that in acidic pH. In the eluent of acid pH the basic molecules are present predominantly in the protonated form while the molecules will less in the protonated form in the eluent of basic pH. The protonated ions migrate electrophoretically in the same direction with cathodic EOF and thus are likely to show shorter retention at acidic pH than basic pH. Fig. 4 shows exemplary chromatograms for the separation of

Table 1
Chiral separation of basic compounds on CDMPC-ZM.[a]

Compound	t_1[b]	t_2[b]	R_s[c]	α[d]	k_{app1}[e]	k_{app2}[e]	N_1[f]	N_2[f]
TB 	22.85	25.31	1.79	1.11	0.29	0.42	9200	3200
IND 	23.89	25.26	1.15	1.06	0.34	0.42	9640	5170
ATR 	25.22	26.59	1.12	1.05	0.42	0.50	10,150	5400
HATR 	21.81	23.36	0.64	1.07	0.23	0.31	1760	1170
OXP 	7.39	7.62	1.76	1.03	−0.58	−0.57	94,280	37,550
PRO 	6.69	6.83	1.52	1.02	−0.62	−0.61	118,110	78,060

[a] *Conditions*: column length, 35 cm (monolith bed, 25 cm) × 75 μm i.d.; mobile phase, 50/50 (v/v) ACN/phosphate buffer (50 mM, pH 9.0); temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV for 5 s; detection, 214 nm; dead time (t_o) marker, acetone. *Abbreviations of compound names*: Tröger's base (TB), propranolol (PRO), oxprenolol (OXP), indapamide (IND), atropine (ATR) and homatropine (HATR).
[b] Migration times of the first and second eluted enantiomer.
[c] Resolution factor.
[d] Apparent enantioselectivity factor ($=t_2/t_1$).
[e] Apparent retention factors of the first and second eluted enantiomer. $k_{app}=(t-t_o)/t_o$.
[f] Plate numbers of the first and second eluted enantiomer.

enantiomers of Tröger's base obtained in eluents of pH 4.4 and 9.0. Retention of TB enantiomers is somewhat shorter at pH 9.0 than pH 4.4. It is likely that overall migration rate determined by the combined flow by electrophoretic and electroosmotic mobility at pH 9.0 is slightly higher than that at pH 4.4. The increased EOF due to much greater negative charge developed by Eq. (1) is likely to compensate the decreased electrophoretic flow of the less protonated form of TB at pH 9.0. At pH 4.4 electrophoretic flow is faster due to increased number of the protonated form but EOF is lower due to smaller negative charge developed by the equilibrium. Much better resolution is observed at basic than acidic pH while no appreciable difference in enantioselectivity is observed at the two pH. TB molecules are less protonated at pH 9.0 than pH 4.4 and hence less hydrophilic than those in the protonated from, giving a lower solubility in the reversed-phase eluent. This will, in turn, increase interaction of the analyte with CDMPC. As more analyte molecules are in neutral form at basic pH, desorption kinetics from the CDMPC polymer will be also faster. It is likely that combination of these two effects results in increased plate number and higher resolution observed at pH 9.0.

It is of interest to compare CEC separation of the analytes on CDMPC-modified zirconia monolith (ZM) with that on CDMPC-modified silica monolith (SM). CEC separation data for Tröger's base (TB) and propranolol (PRO) were available in the literature for comparison although the chromatographic conditions are not exactly the same as those in this work and thus the comparison may not be rigorous. The best resolution factor of 1.33 for TB was observed on CDMPC-SM in 40/60 (v/v) ACN/phosphate buffer (2 mM, pH 7.0) by He et al. [50]. This is compared to the resolution factor of 2.14 observed on CDMPC-ZM in 50/50 (v/v) ACN/phosphate buffer (50 mM, pH 9.0) in this work. Enantiomers of PRO were not resolved on CDMPC-SM in 40/60 (v/v) ACN/phosphate buffer

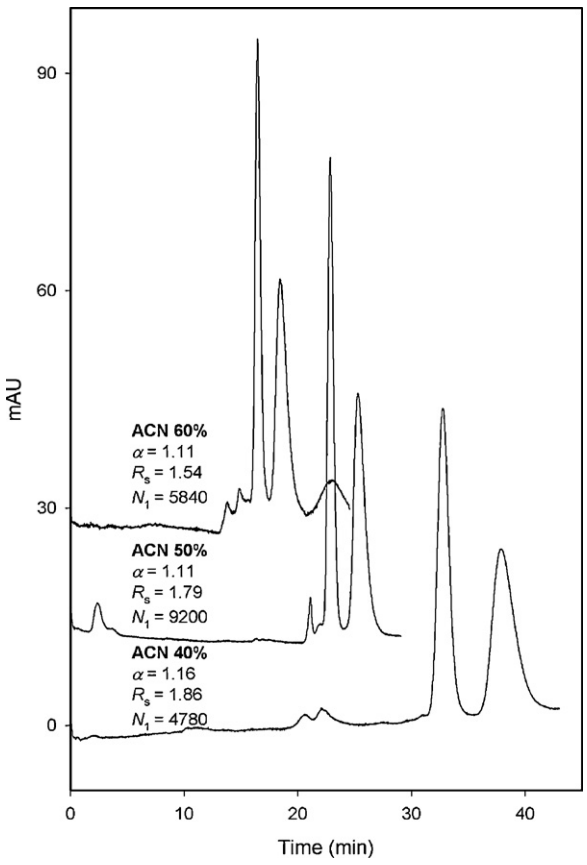


Fig. 3. Effect of ACN content. *Conditions*: eluent, ACN/phosphate buffer (50 mM, pH 9.0). Other conditions are the same as in Fig. 2.

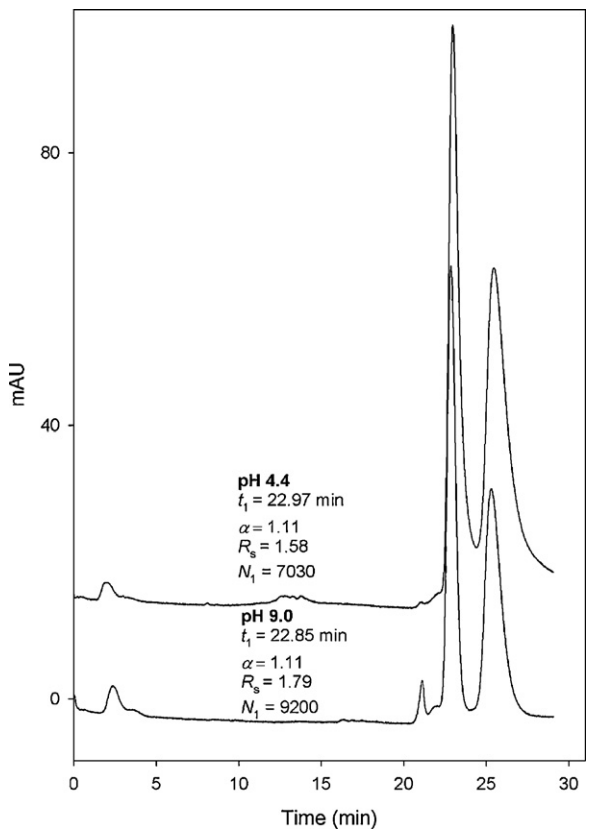


Fig. 4. Comparison of the chiral separations in acidic and basic eluents. *Conditions*: eluent, 50/50 (v/v) ACN/phosphate buffer (50 mM). Other conditions are the same as in Fig. 2.

(2 mM, pH 9.6) while they were resolved with resolution of 1.52 on the CDMPC-ZM in 50/50 (v/v) ACN/phosphate buffer (50 mM, pH 9.0) in this work, which is much better than the best resolution (1.08) obtained by CEC on cellulose tris(3-chloro-4-methylphenylcarbamate)-modified particle-packed column in 50/50 (v/v) ACN/phosphate buffer (50 mM, pH 9.0) by Hendrickx et al. [51].

Column stability and repeatability were checked by determining the RSD values of retention time and resolution for two typical analytes, TB and IND, and the data shown in Table 2. Both run-to-run (RSD < 1.5%) and day-to-day repeatabilities (RSD < 2.5%) of the CEC enantioseparations on the CDMPCZM column in basic eluents are well within the acceptable range. Further, after over 200 injections, no appreciable column deterioration and consequent decay in resolution was observed, indicating the stability of the CDMPC-modified zirconia monolithic column in the basic eluents.

In summary, zirconia monolith modified with cellulose 3,5-dimethylphenylcarbamate was used for separation of a set of basic chiral compounds using CEC. The EOF behavior of bare ZM and CDMPC-ZM column was studied in ACN/phosphate buffer eluents of pH ranging from 2 to 12. Increasing cathodic EOF was observed for both bare ZM and CDMPC-ZM with pH. The CDMPC-ZM column was evaluated by investigating the influences of pH, the type and composition of organic modifier of the eluent on enantiomer separation. CEC separations in the mobile phase of pH 9 provided the best chiral resolutions for the analytes studied, which are better than those observed on CDMPC-modified silica monolithic columns under similar conditions. No appreciable decline in resolution and retention after over 200 injections, and run-to-run and day-to-day repeatabilities of the column of less than 3% indicate the stability of the zirconia monolithic column in basic mobile phases.

Table 2
RSDs for run-to-run and column-to-column repeatabilities.

Compound	% RSD, run to run[a]		% RSD, column to column[b]	
	t_1[c]	R_s[d]	t_1[c]	R_s[d]
TB	0.5	0.8	1.6	1.9
IND	0.7	1.1	2.0	2.3

[a] For five consecutive injections.
[b] For three columns.
[c] Migration time of the first eluting enantiomer.
[d] Resolution factor.

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