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Short communication

Fast separations of chiral β-blockers on a cellulose *tris*(3,5-dimethylphenylcarbamate)-coated zirconia monolithic column by capillary electrochromatography

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ABSTRACT

Cellulose *tris*(3,5-dimethylphenylcarbamate) (CDMPC) is an excellent chiral selector for enantioseparation of a wide variety of chiral compounds. The monolithic chiral columns are becoming popular in liquid chromatography and capillary electrochromatography. In this work, we present the fast separation of chiral β-blockers on a CDMPC-modified zirconia monolithic column by capillary electrochromatography (CEC). The porous zirconia monolithic capillary column was prepared by using the sol–gel technology and then zirconia surface modified with CDMPC. The enantioseparations were performed in reversed-phase (RP) eluents of a phosphate solution (pH 4.4) modified with acetonitrile or alcohol. The enantioseparations of a set of eight chiral β-blockers were achieved in less than one minute. Influences of the applied voltage, column temperature, concentration of acetonitrile and the type of alcohol as the organic modifier in the mobile phase, and sample injection time on enantioseparation were investigated. CEC separations at the applied voltage of 10 kV and 15 °C in the ACN-modified mobile phase provided the best resolutions for the analytes studied. Run-to-run and day-to-day repeatabilities of the column in the RP-CEC separation were less than 1 and 2%, respectively.

1. Introduction

Capillary electrochromatography (CEC) is considered as a relatively novel separation technique that is a combination of high performance liquid chromatography and capillary electrophoresis [1]. In CEC, the electroosmotic flow (EOF) is used to drive the mobile phase through the capillary. The flat profile of the EOF makes the electrochromatographic separations more efficient compared to pressure-driven ones [2]. CEC has been used for separation of both charged and neutral compounds with high efficiency. In recent years, CEC is also a rapidly developing and popular technique for enantioseparation [3–5], due to its advantages such as high efficiency associated with electrically driven separation, high selectivity of chromatographic stationary phases, low solvent and selector consumption [3].

Beta blockers (β-blockers) or β-antagonists are a class of adrenergic drugs that block the action of endogenous catecholamines (adrenaline and noradrenaline) on β-adrenergic receptors [6,7]. The majority of these β-blocker drugs are chiral. The enantiomers of a chiral compound often exhibit different biological activities [8]. Usually the pharmacologically inactive enantiomer shows unwanted effects, antagonistic function and even toxic effects. Thus, the biological and toxicological tests of each new chiral drug entity and its individual enantiomers are highly significant and become essential in the pharmaceutical field [9]. According to the guidelines of the U.S. Food and Drug Administration, pharmaceutical companies have to separate and study each enantiomer of therapeutically active chiral drugs for their pharmacological and metabolic pathway [10]. Therefore, synthesis and enantioseparation of chiral drugs carry equal significance in pharmaceutical investigations such as pharmacological and toxicological studies.

Among a number of chiral stationary phases (CSPs), polysaccharides have occupied a unique place in the chiral discrimination for a broad range of chiral compounds and have been the most widely used chiral selector for enantioseparation. These polysaccharide-based CSPs have shown very good resolutions for different classes of analytes in normal phase (NP), reversed-phase (RP) and polar organic (PO) phase conditions [11]. Krause et al. reported enantioseparations of β-blockers in capillaries packed with silica gel modified with cellulose *tris*(3,5-dimethylphenylcarbamate) (CDMPC) [12]. In their work, different separation modes were utilized including, NP and RP nano-HPLC and CEC with and without pressure-assistance. Chankvetadze et al. also reported enantioseparation by HPLC of chiral β-blockers on monolithic silica modified

Abbreviations: CDMPC, cellulose *tris*(3,5-dimethylphenylcarbamate); CDMPCZM, CDMPC-modified zirconia monolith.

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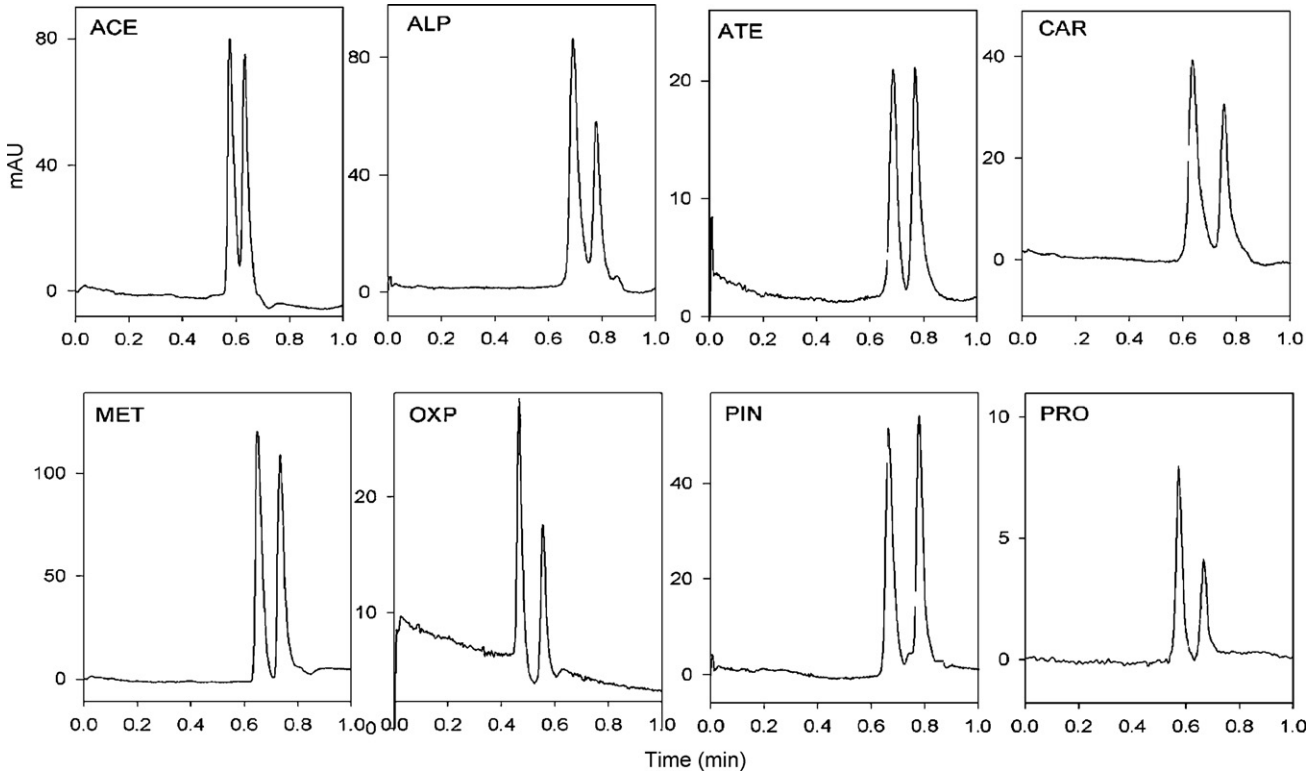


Fig. 1. Chromatograms for the chiral separation of β-blockers. Conditions: column, 35 cm length (monolith bed, 25 cm) × 75 μm I.D.; run buffer, 50/50 (v/v) ACN/KH_2PO_4 (50 mM, pH 4.4); sample concentration, 0.2–0.5 mg/mL; temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV, 3 s; detection wavelength, 214 nm.

with CDMPC [13]. A very fast enantioseparation of 2,2,2-trifluoro-1-(9-anthryl)ethanol in normal phase LC has been reported on a commercial 5-cm monolithic silica column modified with CDMPC [14]. Zirconia columns coated with cellulose derivatives as the CSP have been used in HPLC [15–18] and recently in CEC in the form of particle packed [19] and monolithic columns [20]. In this work we report fast enantioseparations of chiral β-blockers in less than one minute on a CDMPC-modified zirconia monolithic (CDMPCZM) column in reversed-phase (RP) mobile phases composed of aqueous phosphate and acetonitrile or alcohol as the organic modifier.

2. Experimental

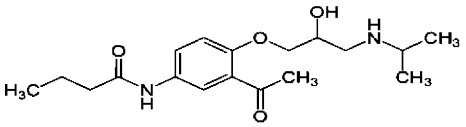
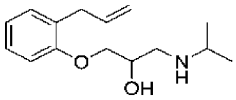
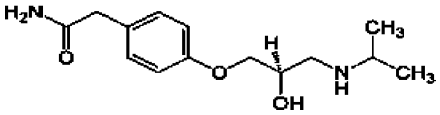
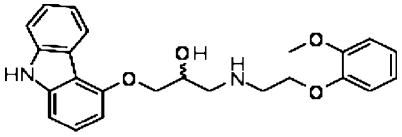
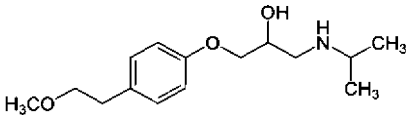
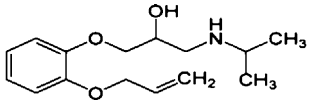
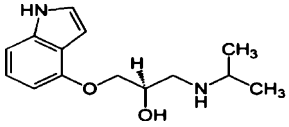
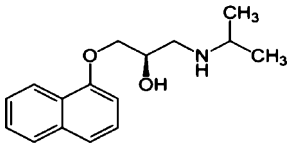
Fused silica capillaries (75 μm I.D., 365 μm O.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). Zirconium butoxide, acetic acid, potassium dihydrogen phosphate and sodium hydroxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cellulose (Avicel) was obtained from Merck (Darmstadt, 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 better than 99% purity. HPLC-grade acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), 1-propanol (1-PrOH), 2-propanol (2-PrOH), 1-butanol, acetone and diethyl ether were obtained from J.T. Baker (Phillipsburg, NJ, USA). Water was purified with an Elgastat UHQ water purification system (Bucks, UK). All racemic β-blocker compounds used in the study were commercially available and were of the highest-purity available from Aldrich (Milwaukee, WI, USA) or TCI (Tokyo, Japan).

An Agilent HP^{3D} CE System (Palo Alto, 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 unless stated otherwise and monitored at 200, 214, 254 and 280 nm. The morphology of the zirconia monolith was examined by a field emission scanning electron microscope (FE-SEM S-4100, Hitachi, Japan). A syringe pump used to inject the CDMPC solution into the zirconia monolithic capillary was from Cole-Parmer (Vernon Hills, IL, USA).

Preparation of the CDMPC-modified zirconia monolith (CDMPCZM) in a capillary involves two steps. The fused silica capillary was washed with 1.0 M NaOH for 2 h and flushed with water, acetone and diethyl ether successively for 30 min each. The capillary was then dried using a nitrogen gas flow for about 1 h and then kept in GC oven at 150 °C for 1 h. Zirconia monolith was synthesized using the procedure reported earlier [20] and characterized by SEM. CDMPC was prepared as per the reported method [21] and characterized by elemental analyses, IR and NMR spectroscopy. To perform the CDMPC coating on the surface of zirconia monolithic bed, the zirconia monolithic (ZM) capillary was initially washed with ethanol and then with THF. Then, a CDMPC solution in THF was passed through the capillary at a flow rate of 5 μL min^{-1} using a syringe pump to coat the entire ZM bed of the capillary column. The capillary (length, 35 cm; monolithic bed, 25 cm) was dried and rinsed with methanol and mobile phase, respectively.

The mobile phases used for the enantioseparation were mixtures of aqueous KH_2PO_4 solution and organic modifiers such as ACN, MeOH, EtOH, 1-PrOH and 2-PrOH in different compositions. The mobile phases were filtered through a nylon membrane filter of 0.2-μm pore size and degassed prior to use. The CDMPCZM columns were equilibrated for about 8–10 h in order to reduce baseline noise before analyzing the analytes. Sample solutions were prepared by dissolving the chiral β-blocker drugs in the mobile

Table 1
Separation data of chiral β-blockers.*

β-Blocker[a]		t_1[b]	t_2[b]	R_s[c]	α[d]	N_1[e]	N_2[e]
ACE		0.58	0.63	1.53	1.09	4643	4538
ALP		0.69	0.78	1.70	1.13	4081	3914
ATE		0.68	0.77	1.67	1.13	5068	4999
CAR		0.64	0.75	1.82	1.17	5043	4419
MET		0.65	0.73	2.02	1.13	5357	4774
OXP		0.47	0.56	2.79	1.19	5271	4926
PIN		0.66	0.78	2.62	1.18	5626	5017
PRO		0.57	0.66	2.48	1.16	5511	5026

* Conditions: column length, 35 cm (monolith bed 25 cm) × 75 μm I.D.; mobile phase, 50/50 (v/v) ACN/KH_2PO_4 buffer (50 mM, pH 4.4); temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV, 3 s; detection wavelength, 214 nm.
[a] Abbreviations for β-blockers: acebutolol (ACE), alprenolol (ALP), atenolol (ATE), carvedilol (CAR), metoprolol (MET), oxprenolol (OXP), pindolol (PIN) and propranolol (PRO).
[b] Migration times of the first and second eluted enantiomers.
[c] Resolution factor.
[d] Apparent enantioselectivity factor given by t_2/t_1.
[e] Number of theoretical plates for the first and second eluted enantiomers.

phase and injected electrokinetically at 15 kV for 3 s unless mentioned otherwise. Subsequent injections were preceded by flushing of the column with the mobile phase for 15 min.

3. Results and discussion

Zirconia is an amphoteric oxide, acting as both Brönsted acid and base. There also exist Lewis acid sites on zirconia, which are capable of binding strongly to Lewis bases such as phosphate ions present in the mobile phase. The adsorbed phosphate ions on the surface also provide additional negative charges, thereby yielding fast cathodic EOF irrespective of the eluent pH [19]. For CDMPC-coated particle-packed capillaries, zirconia showed greater EOF than silica as the concentration of surface hydroxyl groups is somewhat higher (~9.8 and ~8 μmol/m^2 for fully hydroxylated zirconia [22] and silica [23], respectively) and more adsorbed phosphate ions on zirconia than silica provide additional negative charges for increased zeta potential [19]. It is very likely that the same is true for monolithic columns as well.

The CDMPC-coated zirconia monolithic column was utilized to explore the fast separations of a group of ten chiral β-blockers including atenolol (ATE), acebutolol (ACE), alprenolol (ALP), carvedilol (CAR), metoprolol (MET), nadolol (NAD), oxprenolol (OXP), pindolol (PIN), propranolol (PRO) and labetalol (LAB) by CEC. Chromatograms for enantioseparation of eight β-blockers are shown in Fig. 1 along with chromatographic data in Table 1. For LAB there are two chiral centers giving four possible stereoisomers (*R*,*R*), (*R*,*S*), (*S*,*S*) and (*S*,*R*). Nadolol has three stereogenic centers and is expected to have eight stereoisomers. As the two hydroxyl groups on its cyclohexane ring are conformationally locked in the *cis*-form [24], only four stereoisomers to be formed (*RSR*, *SRS*, *RRS*, and *SSR*). For both LAB and NAD partial separations with only two peaks were obtained. Chiral β-blockers were resolved within one minute in the RP eluent composed of 50/50 (v/v) ACN/KH_2PO_4 (pH 4.4). In particular MET, OXP, PIN and PRO were resolved with R_s of greater than 2.0. All the β-blockers are basic compounds and thus are present in the fully protonated form in the mobile phase of pH 4.4. The electrophoretic movement of the cationic analytes is cathodic and co-directional with EOF, resulting in very fast separations.

Table 2
Enantioseparation results of chiral β-blockers under different conditions.*

β-Blocker[a]	t_1[b]	t_2[b]	R_s[c]	α[d]	N_1[e]	N_2[e]
Applied voltage (kV)						
OXP						
5.0	1.09	1.32	2.21	1.21	3404	3059
7.5	0.90	1.05	2.43	1.17	4507	3949
10.0	0.47	0.55	2.78	1.17	5271	4926
PRO						
5.0	1.74	2.11	1.94	1.21	3213	3319
7.5	0.92	1.08	2.05	1.17	4214	4011
10.0	0.57	0.66	2.28	1.16	5511	5026
Temp. (°C)						
MET						
15	0.97	1.16	2.27	1.19	4574	4431
20	0.79	0.91	2.16	1.15	5121	4890
25	0.65	0.73	2.02	1.13	5357	4774
30	0.52	0.59	1.89	1.13	5850	5520
ACN (v%)						
ALP						
45	0.56	0.63	1.50	1.12	3675	3460
50	0.69	0.78	1.70	1.13	4081	3914
55	1.14	1.32	1.93	1.16	4435	4186
ATE						
45	0.57	0.64	1.34	1.12	4755	4512
50	0.68	0.77	1.67	1.13	5068	4999
55	0.96	1.14	2.01	1.18	5234	5045
Type of added alcohol[f]						
PIN						
MeOH	0.68	0.78	1.79	1.15	5290	5160
EtOH	0.72	0.81	1.66	1.15	4883	4584
1-PrOH	0.93	1.08	2.05	1.16	4523	4214
2-PrOH	1.01	1.17	2.44	1.17	5646	5319
PRO						
MeOH	0.70	0.78	1.52	1.11	5640	5420
EtOH	0.75	0.84	1.39	1.12	4985	4732
1-PrOH	0.88	1.05	1.74	1.19	4215	4150
2-PrOH	0.97	1.16	1.86	1.19	5114	5070

Repeatability

	Run to run,[g] % RSD					Day to day,[h] % RSD				
	t_1[b]	t_2[b]	R_s[c]	N_1[e]	N_2[e]	t_1[b]	t_2[b]	R_s[c]	N_1[e]	N_2[e]
MET	0.8	0.9	1.3	3.8	4.7	1.2	1.5	1.6	5.4	5.8
OXO	0.9	1.1	1.5	4.2	3.9	1.5	1.6	2.0	7.8	6.5
PIN	0.5	0.7	1.0	2.8	3.4	1.0	1.4	1.8	4.9	5.7
PRO	0.7	0.8	1.1	3.2	3.5	1.7	1.2	1.5	6.5	6.9

* Conditions: column length, 35 cm (monolith bed 25 cm) × 75 μm I.D.; mobile phase, 50/50 (v/v) ACN/KH_2PO_4 buffer (50 mM, pH 4.4); temperature, 25 °C; applied voltage, 10 kV; injection, 15 kV, 3 s; detection wavelength, 214 nm.
[a] Abbreviations for β-blockers: acebutolol (ACE), alprenolol (ALP), atenolol (ATE), carvedilol (CAR), metoprolol (MET), oxprenolol (OXP), pindolol (PIN) and propranolol (PRO).
[b] Migration times of the first and second eluted enantiomers.
[c] Resolution factor.
[d] Apparent enantioselectivity factor given by t_2/t_1.
[e] Number of theoretical plates for the first and second eluted enantiomers.
[f] 50 v% alcohol in the mobile phase.
[g] For five consecutive injections.
[h] For five consecutive days.

Influences of the applied voltage, capillary temperature, sample injection time and composition of ACN on enantioresolution of representative β-blockers on the CDMPCZM column were investigated to find the optimum settings, and the results summarized in Table 2. The increasing voltage gives decreasing migration times due to increasing EOF. Enantioselectivity decreases slightly while resolution and plate numbers increase with voltage. Taking into account of resolution and analysis time together, applied voltage of 10 kV was chosen. With increasing temperature migration time, enantioselectivity and resolution decrease while the plate number increases due to faster mass transfer. Decrease in migration time is due to increased dielectric constant to viscosity ratio (ε/η) and hence EOF as the mobile phase viscosity (η) is decreased with temperature. The decreases in enantioselectivity and resolution with temperature are due to weakening of the interactions between the enantiomer and the chiral selector. As the injection time increases resolution and plate numbers deteriorate rapidly as a consequence of increased sample loading [12,25]. Sample injection time of 3 s was thus selected to obtain good separation efficiency and proper detection sensitivity. The change in the organic modifier composition in the RP eluent can alter partition of the analyte between the mobile and stationary phase, affecting retention and selectivity of the analytes. Migration times increase with increasing amount of ACN. The ε/η ratio decreases as the ACN content increases [26], which gives lowered EOF and hence increased migration time over the composition range studied. With increasing ACN content resolution, enantioselectivity and efficiency improve at the expense of increased migration time. Improved enantioresolution with increased ACN content is likely due to increased interactions of the analyte enantiomers with CDMPC since the solubility of the

protonated analyte becomes lower in the less dipolar eluent as the amount of ACN, which is less dipolar than water [27], increases and in turn causes the analyte to spend more time in the stationary phase to undergo interactions with the chiral selector.

The effect of the type of alcohol as the organic modifier on the enantioseparation of the β-blockers was investigated (Table 2). Upon changing the alcohol in the order of MeOH, EtOH, 1-PrOH and 2-PrOH, retention times of the β-blockers increase in the same order as the ε/η ratios decrease in the same order [28] to generate lowered EOF, which results in increasing retention of the analytes. From going MeOH to EtOH enantioselectivities for the β-blockers increase while resolution and plate numbers decrease in general. However, from going EtOH to 1-PrOH both resolution and enantioselectivity increase while the plate numbers continue to decrease as the alkyl chain length of the alcohol increases. From going 1-PrOH to 2-PrOH resolutions still improve with negligible change in enantioselectivity while plate numbers improve. Among the four alcohols 2-PrOH gives the best enantioresolution with slightly longer retention time than the other alcohols. Performance of the 2-PrOH modified eluent is somewhat lower but close to that of the ACN-modified eluent. It is not clear at present how the 2-PrOH modified eluent gives comparable enantioresolutions to those with the ACN-modified eluent. The characteristic and the extent of interactions between the chiral analyte and CDMPC are likely to be very similar in the 2-PrOH and ACN-modified eluents since such mobile phase properties as the HB donating acidity [27] and HB accepting basicity [29] of 50/50 (v/v) 2-PrOH/water are very similar to those of 50/50 (v/v) ACN/water (HB acidity, 0.89 vs. 0.90; HB basicity, 0.66 vs. 0.68) even if the corresponding properties of the two pure solvents are quite dissimilar [27]. Although 2-PrOH-modified mobile phase gives somewhat longer retention and lower resolution than ACN-modified mobile phase, 2-PrOH is deemed useful as the organic modifier in RP-CEC enantioseparation of the β-blockers on CDMPCZM.

Stability and repeatability were checked by determining the RSD values of migration times, resolutions and plate numbers for four typical analytes, MET, OXP, PIN and PRO, and the data shown in Table 2. Both run-to-run (RSD < 1%) and day-to-day repeatabilities (RSD < 2%) of the CEC enantioseparations on the CDMPCZM column are well within the acceptable range. Further, after more than 200 injections using different mobile phases, no appreciable column deterioration and consequent decline in resolution was observed, indicating the stability of the CDMPC-modified zirconia monolithic column in the RP-CEC operations.

Fast enantioseparation methods are becoming more prevalent in the last few years due to the rapid developments in chemical, pharmaceutical, clinical, agriculture, genomics and proteomics where a large number of analytes are generated. This work described fast enantioseparations of eight chiral β-blockers on the CDMPC-modified zirconia monolithic column by RP-CEC within one minute with good resolutions and selectivities. Influences of the applied voltage, temperature, concentration of acetonitrile and alcohol modifier in the eluent on enantioseparation were investigated. Among the conditions investigated the separation at applied voltage of 10 kV and 15 °C in the ACN-modified eluent provided the best resolutions for the analytes studied. Run-to-run and day-to-day repeatabilities of the columns were less than 1 and 2%, respectively, indicating the reliability of the zirconia monolithic column.

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