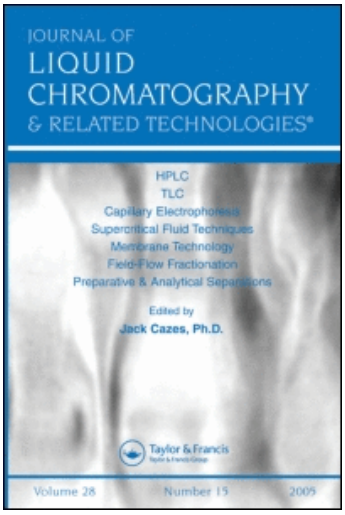

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β-CYCLODEXTRIN-MODIFIED MONOLITHIC STATIONARY PHASES FOR CAPILLARY ELECTROCHROMATOGRAPHY AND NANO-HPLC CHIRAL ANALYSIS OF EPHEDRINE AND IBUPROFEN

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β-CYCLODEXTRIN-MODIFIED MONOLITHIC STATIONARY PHASES FOR CAPILLARY ELECTROCHROMATOGRAPHY AND NANO-HPLC CHIRAL ANALYSIS OF EPHEDRINE AND IBUPROFEN

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ABSTRACT

Chiral monolithic capillary columns for reversed-phase capillary electrochromatography (CEC) and for nano-HPLC were prepared by linking β-cyclodextrin modifier into the acrylic monolithic

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phase. Columns with the physically and chemically bonded β-cyclodextrin derivatives were tested under CEC and nano-HPLC conditions; enatioselective separation of (−)-ephedrine/(+)-pseudoephedrine and (+/−)-ibuprofen was successfully performed. The separation efficiency of CEC and HPLC was examined and compared; resolution of (+/−) ibuprophen was 2.45 and 2.97 for CEC and HPLC respectively, number theoretical plates of thiourea were 41,600 N/m in CEC.

INTRODUCTION

Capillary electrochromatography (CEC) is a very powerful analytical separation technique which combines the efficiency of capillary zone electrophoresis and the selectivity of high performance liquid chromatography. Although, CEC has been applied in many different areas,[1–5] packed-column preparation and low-detection sensitivity remain challenges of this technique. The preparation and the usage of common packed columns is usually connected with the problems having their origin in difficult incorporation of frits into capillaries, the limited stability of packing material, and the formation of bubbles inside the capillary during analysis.

To avoid these problems, the monolithic stationary phases were developed by Svec et al.[6] Monolithic separation medium, prepared by in situ polymerization, is not compressible, does not change its size substantially due to swelling, and does not require frits in the end of capillary. Monolithic columns have also shown a very good performance in micro- and nano-HPLC (see Refs. 7 and 8).

The development and application of monolithic columns in CEC and HPLC has a tremendous growth in the recent years. Surprisingly, enantiomeric monolithic columns have been reported only in a few examples. Nilsson and coworkers used imprinted monolithic capillaries,[9] Koide and Ueno described enantriomeric modifications of monolithic columns, such as physical incorporating of polymeric β-cyclodextrin as a chiral selector,[10] chemical bonding of allyl carbamoylated β-cyclodextrin[11,12] or chemical bonding of chiral crown ether[13] into the monolithic phase. Frechet and co-workers used quinidine-functionalized chiral monomer[14,15] as an enantiomeric selector.

This paper reports the use of novel physically and chemically bonded β-cyclodextrin derivatives for enenatiomeric separations of drugs in the CEC and nano-HPLC, and compares the selectivity and performance of these separation techniques.

EXPERIMENTAL

Materials

3-(Methoxysilyl)propylmethacrylate and resin Vestopal-W were obtained from Fluka (USA). Acetone (ACN), ethylendimethacrylate (EDMA), 2,2′-azobis(2-methylpropionitrile) (AIBN) and butylmethacrylate (BMA) was provided by Merck (Germany). 1-Propanole, 1,4-butandiole, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), acetic acid, sodium acetate, and racemate of ibuprofen were obtained from Aldrich (USA). (−)-Efedrine, (+)-pseudoefedrine and racemate of ketotifene were from Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Peracetyl-2′-O-allyl-β-cyclodextrin[16] and *tert*-butyl-β-cyclodextrin (TB-β-CD)[17] were prepared using described procedures. Fused silica capillary with internal diameter (i.d.) of 100 μm and external diameter (o.d.) 375 μm was obtained from Composite Metal Services (Great Britain).

Instrumentation

CEC experiments were carried out using Crystal CE with UV detector, Unicam 4225 (ATI Unicam, Great Britain). If not otherwise stated, separations were applying potential of +20 kV, with elecrokinetical injection at +3 kV for 2 s. Nano-HPLC was performed using system consisting from: high-pressure pump MHPP 20 (Laboratorni pristroje, Prague, Czech Republic), injection loop, Valko C14W (Valko Europe, Schenkon, Switzerland) with loop volume 60 nL, UV detector, Unicam 4225 (ATI Unicam, Great Britain). Polymerization of pre-polymers in capillaries was performed in a thermostated oven. Mammert 400 (Mammert, Swabach, Germany). Detection of (−)-ephedrine/(+)-pseudoephedrine and ketotifene was carried out at 200 nm,[17] detection of ibuprofen at 254 nm.[22] All experiments were carried out at 25°C.

Microscopy of the monolithic stationary phase was performed using microscope, NFPK (Carl Zeiss Jena, Germany) with camera, Philips CCD–LDH 0670/00 CCIR (Philips, Japan), connected to the computer with software, AnalySis 2.1 (Soft-Imiging Software, Germany).

Preparation of the Monolithic Polymer Capillaries

Monolithic columns were prepared by modifying the procedure described by Peters et al.[18] First, the inner capillary wall was being activated by washing with 1 M NaOH for 24 h. The following step was silanization of the inner

capillary wall, which was done by passing mixture of 10 mL of 6 M acetic acid and 40 μL of 3-(methoxysilyl)propylmethacrylate for 90 h. Then the capillary was rinsed by deionized water (10 min) and dried with nitrogen (10 min). Polymerization was performed for 24 hours at 60°C in a capillary containing pre-polymer, which was plugged using a rubber septum. Pre-polymer contained a polymerization component (A) (40%, w/w) and a porogenic component (B) (60%, w/w). Compositions of components A and B are described in following sections.

Neutral Monolithic Polymer Capillary Column (MCI)

Composition: A-39.5% (w/w) EDMA, 59.5% (w/w) BMA and 1% (w/w) AIBN; B-60% (w/w) 1-propanol, 30% (w/w) 1,4-butandiol and 10% (w/w) deionized water.

Negatively Charged Monolithic Polymer Capillary Column (MCII)

Composition: A-39.4% (w/w) EDMA, 59.3% (w/w) BMA, 1% (w/w) AIBN and 0.3% (w/w) AMPS; B-60% (w/w) 1-propanol, 30% (w/w) 1,4-butandiol and 10% (w/w) deionized water.

Negatively Charged Monolithic Polymer Capillary Column with Bonded Peracetyl-2′-O-β-Cyclodextrin (MCIII)

Composition: A-34.0% (w/w) EDMA, 51.1% (w/w) BMA, 0.85% (w/w) AIBN, 0.25% (w/w) AMPS and 13.8% (w/w) peracetyl-2′-O-allyl-*β*-cyclodextrin; B-60% (w/w) 1-propanol, 30% (w/w) 1,4-butandiol and 10% (w/w) deionized water.

Calculations

In order to evaluate the performance of the uncharged monolithic phase, a suitable equation of effective mobility of molecule in uncharged monolithic stationary phase (μ_{eff}^{mon}) was developed, using the following definitions and presumptions. The separation system consisted of two connected capillaries (see Fig. 1). The first one contained monolithic stationary phase (Fig. 1, part "M") and the second one, a capillary with UV detection window (Fig. 1, part "O"), contained only a separation buffer. Velocity of the molecule in the monolithic

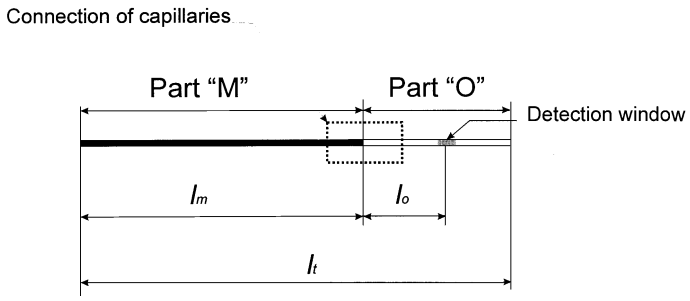

Figure 1. Scheme of the separation system containing two connected capillaries.

capillary "M" is specified as v_m, the one in the capillary "O", v_o. Length of the capillary "M" is specified as l_m, length from the connection of the capillaries to the detection windows in capillary "O" as l_o. Total length of the connected capillaries is specified as l_t. The time within, which the molecule of analyte passes the distance l_m is t_m, the time within, which the molecule of analyte passes the distance l_o is t_o; the total time, which the molecule of analyte needs to reach the detection window is t_d. The time t_d is the only value, which can be directly experimentally measured, and t_d represents the sum of t_m and t_o.

The relation between velocity v_m, length l_m and time t_m can be expressed as:

$$v_m = \frac{l_m}{t_m} \tag{1}$$

Time t_m is not directly measurable. We can measure only the time within, which the molecule of analyte reaches detection window, t_d. For t_m we can write:

$$t_m = t_d - t_o \tag{2}$$

By substituting Eq. (2) for Eq. (1) we obtained:

$$v_m = \frac{l_m}{t_d - (l_o/v_o)} \tag{3}$$

By substituting Eq. (3) for the definition equation of electrophoretic mobility:

$$\mu = \frac{v}{E} \tag{4}$$

we obtain the final equation for calculation of $\mu_{\text{eff}}^{\text{mon}}$:

$$\mu_{\text{eff}}^{\text{mon}} = \frac{l_m}{t_D \times (U/l_t) - (l_o/\mu_{\text{eff}}^{0})} \tag{5}$$

where, μ_{eff}^{0} is the effective mobility of the ion in free-capillary (accessible from tabulated data or easily measurable), U is the applied voltage.

For calculation of retention characteristic of the uncharged monolithic column, the factor k_m is introduced, defined as the ratio of effective mobility of the analyte (μ_{eff}^{0}), and its effective mobility in monolithic column ($\mu_{\text{eff}}^{\text{mon}}$):

$$k_m = \frac{\mu_{\text{eff}}^{0}}{\mu_{\text{eff}}^{\text{mon}}} \tag{6}$$

Connecting Capillaries

To achieve UV detection, an open silica capillary with detection window for UV detection (i.d. 100 μm, o.d. 375 μm) was connected to the capillary containing a monolithic polymer using the method described in Ref. 19. The dimensions of resulting electrophoretic/chromatographic columns were: MCI: $l_m = 43.1$, $l_o = 6.6$, $l_t = 64.7$ cm; MCII: $l_m = 49.5$, $l_o = 8.5$, $l_t = 73.0$ cm; MCIII: $l_m = 50.9$, $l_o = 7.2$, $l_t = 79.5$ cm.

RESULTS AND DISCUSSION

Characterization of Monolithic Stationary Phase

Microscopy of the studied monolithic phases MCI, MCII, and MCIII was performed to obtain the information of the pore distribution. The monolithic stationary phase was fixed in 2% OsO_4, according to the common fixation method[20] and examined under microscope. The microphotography of the structure of the monolithic phase MCI and MCII is shown in Fig. 2. The microphotography of monolithic phase MCIII is not available due to problems with its coating by osmium, possibly caused by the presence of cyclodextrin in polymer.

Capillary Electrochromatography Using Neutral and Negatively Charged Polyacrylamide Columns with Physically Bonded *tert*-Butyl-β-cyclodextrin

There are three prevailing mechanisms causing band broadening in CEC and HPLC:[21] (A) eddy diffusion due to flow in the capillary, (B) longitude diffusion, and (C) resistance to mass-transfer. To examine physical–chemical properties (factors B and C) of the monolithic column separately from the factor A, the monolithic column with uncharged monolytic phase (MCI) was prepared and connected to the piece of empty capillary with UV detection window.

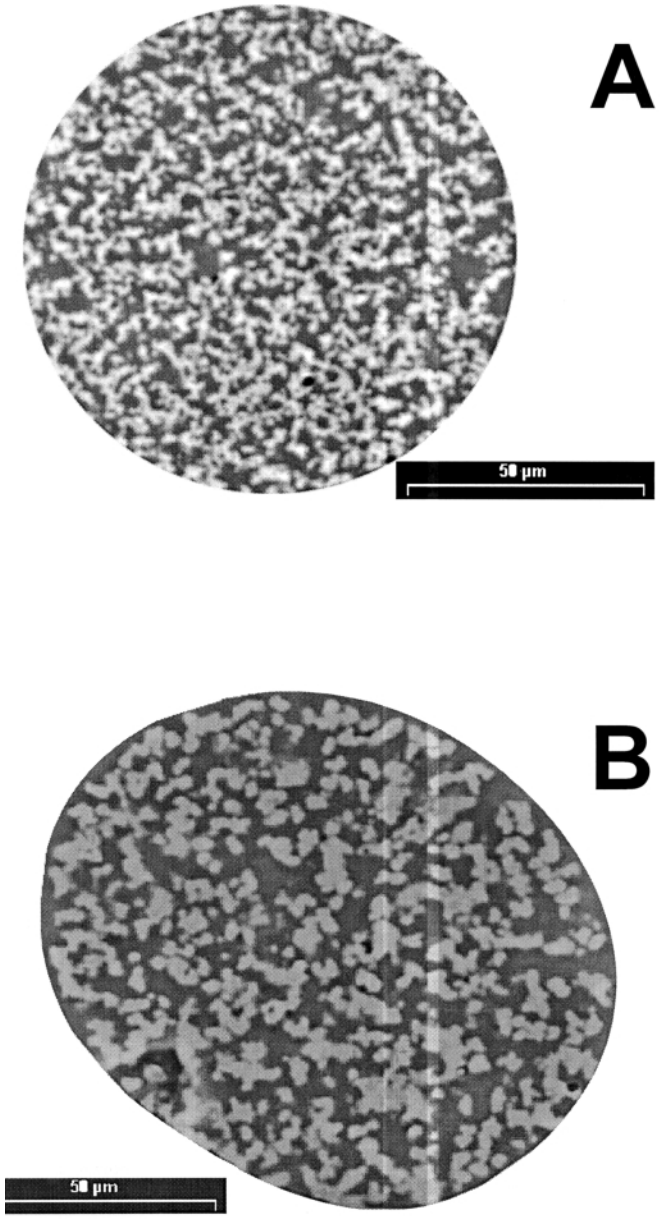

Figure 2. Microphotography of the monolithic phase MCI (A) and MCII (B). Stationary phase is in dark gray; pores are in light gray.

In order to suppress the residual electroosmotic flow, the separations of model compounds, diastereomers (−)-ephedrine and (+)-pseudoephedrine, and enantiomers, (−)-ketotifene and (+)-ketotifene, were performed in 100 mM acetate buffer (pH 4.7). Under these conditions, the model compounds were retained very weakly by the monolithic stationary phase and their mutual separation was not achieved.

The influence of addition of 5 mM *tert*-butyl-β-cyclodextrin (TB-β-CD) into the background electrolyte on the separation of model compounds was examined. The column was washed 60 min with acetate buffer containing 5 mM TB-β-CD prior to the experiments, to achieve the saturation of monolithic stationary phase with TB-β-CD. As shown in Fig. 3, the separation of (−)-ephedrine, (+)-pseudoephedrine in this buffer was achieved; the estimated value of resolution (R_s) of the peaks is 0.82. The enantiomers of ketotifene also interacted with TB-β-CD, but due to low efficiency of the separation, they were not resolved. The retention and peak characteristics of analyzed diastereomers and enantiomers are summarized in Table 1. As is evident from presented data, the enantiomers of ketotifene are able to interact with monolytic stationary phase saturated with TB-β-CD but remain unresolved.

The following set of experiments was carried out with the polyacrylamide monolithic column containing sulfo-groups, supporting stable electroosmotic flow[18] in a wide range of pH (MCII). The efficiency of the column, MCII, was

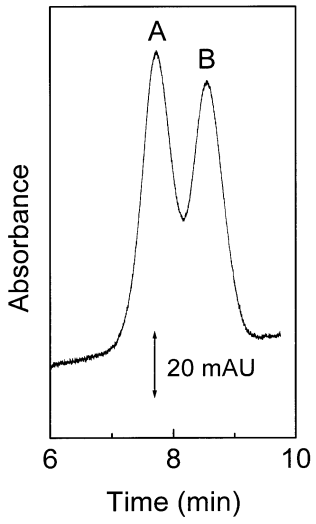

Figure 3. The separation of (+)-pseudoephedrine (A) and (−)-ephedrine (B) using monolithic column MCI with physically bonded *tert*-butyl-β-CD.

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Table 1. Separation of the Enantiomers and Diastereoisomers on the Monolithic Phase MCI Without (A) and With (B) Physically Bonded TB-β-CD

Compound	μ_{eff}^{mon} (A) $cm^2/Vs \times 10^{-4}$	μ_{eff}^{mon} (B) $cm^2/Vs \times 10^{-4}$	k_m (A)	k_m (B)	N/m (A) $\times 10^3$	N/m (B) $\times 10^3$	Peak Asymmetry (A)	Peak Asymmetry (B)
(−)-Ephedrine	2.55	1.84	1.048	1.451	3.45	2.01	2.05	0.98
(+)-Pseudoephedrine	2.53	2.04	1.026	1.274	3.28	1.86	2.31	0.94
(+) and (−)-Ketotifen	1.42	1.29	1.028	1.135	5.83	0.56	20.69	8.08

examined, the number of theoretical plates of thiourea was found at 41,600 N/m. Then, the separation of (−)-ephedrine and (+)-pseudoephedrine diastereomers (concentration 1 mM) was carried out in the column, without the presence of TB-β-CD. As expected, no separation was achieved. Then the column was being washed 60 min with acetate buffer containing 5 mM TB-β-CD to achieve the saturation of monolithic stationary phase with TB-β-CD. The experiment was performed in the acetate buffer without added TB-β-CD. The resolution of the diastereomeric pair of (−)-ephedrine/(+)-pseudoephedrine was similar to the one achieved in neutral polymer, estimated $R_s = 0.76$. The reproducibility of the migration times and the value of resolution was satisfactory, the value of R.S.D <4% for 10 consecutive measurements. Comparative experiments with native β-cyclodextrin as monolithic stationary phase modifier, did not result in effective retention and resolution of studied analytes. Limit of detection based on measurement of 30 μM (−)-ephedrine and (+)-pseudoephedrine was 13 and 11 μM, respectively (S/N = 3).

Capillary Electrochromatography and Nano-HPLC Using Negatively Charged Polyacrylamide Column with Chemically Bonded Peracetyl-2′-*O*-allyl-β-cyclodextrin

The most effective approach on how to avoid the elution of the chiral selector from the column is its chemical incorporation into the monolithic stationary phase. The monolithic column, with chemically bonded cyclodextrin selector (MCIII), was prepared by copolymerization of peracetyl-2′-O-allyl-β-CD with pre-polymer. The presence of strongly acidic sulfo-groups assures constant

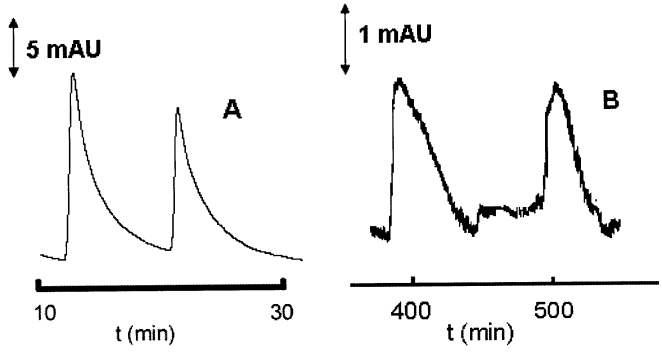

Figure 4. The separation of the enantiomers of ibuprofen using chiral monolithic column with chemically bonded peracetyl-2′-*O*-allyl-β-cyclodextrin by nano-HPLC (A) and CEC (B).

Table 2. Enantioseparation of (+/−)-Ibuprofen Using Peracetyl-2′-*O*-allyl-*β*-cyclodextrin Modified Monolithic Phase by Nano-HPLC and CEC

	t_p (min)	R_s	N/m × 10^3
CEC	391.2 (516.5)[a]	2.45	1.81 (2.34)
Nano-HPLC	13.2 (23.5)	2.97	0.38 (1.10)

t_p retention time.
[a]Values in parenthesis represents values of the second peak of the enantiomeric pair.

magnitude of EOF in a wide range of pH. The separation of (+/−)-ibuprofen was performed using this column in CEC and nano-HPLC mode. The efficiency of the column MCIII was examined, the number of theoretical plates of thiourea was found to be 37,800 N/m.

Figure 4 shows the separation of 1 mM (+/−)-ibuprofen using CEC and nano-HPLC. Retention and peak characteristics of both izomers are summarized in Table 2. As is evident from the presented data, achieved resolution of enantiomers is higher in nano-HPLC (resolution 2.97 in nano-HPLC versus 2.45 in CEC). Time of the analysis is shorter in nano-HPLC mode, due to higher flow rate. The efficiency of the separation, based on number of theoretical plates, is higher in the CEC mode (1.81–2.34 N/m) than in the nano-HPLC (380–1100 N/m), due to the "plug" flow profile of mobile phase. Limit of detection of (+/−)-ibuprofene was 3 μM in nano-HPLC (based on measurement of 10 μM of compound; S/N = 3) and 120 μM in CEC (based on measurement of 300 μM of compound; S/N = 3).

CONCLUSION

We have demonstrated that the monolithic columns with physically and chemically bonded *β*-cyclodextrin derivatives can be used for chiral CEC and nano-HPLC separations. Prepared monolithic columns showed the ability to separate the model mixtures of important drug enantiomers and proved to be sufficiently stable within three months of testing.

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