Synthesis and Characterization of β-CD Derivatized Bovine Serum Albumin Protein as Chiral Selector in Pressurized Capillary Electrochromatography

Rongji Dai,[1] Li Tang,[1,2] Hong Li,[1] Yulin Deng,[1] Ruonong Fu,[3] Zahida Parveen[1]

[1]*School of Life Science and Technology, Beijing Institute of Technology, Beijing 100081, People's Republic of China*
[2]*Department of Environment Science, Zhejiang Wanli University, Ningbo, Zhejiang 315100, People's Republic of China*
[3]*Department of Chemistry, School of Science, Beijing Institute of Technology, Beijing 100081, People's Republic of China*

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ABSTRACT: The synthesis and characterization of β-cyclodextrin derivatized bovine serum albumin (β-CD-BSA) as chiral selector in pressurized capillary electrochromatography were investigated in this article. β-CD-BSA was synthesized by crosslinking β-cyclodextrin and BSA using ethylene glycol diglycidyl ether in boric acid buffer at pH 8.7. The internal surface of the fused silica capillary was coated with amino groups using triethoxylmethylsiloxane and (3-aminopropyl)trimethoxysiloxane by sol–gel technology. Then the inner surface of the capillary was further changed to aldehyde groups surface after being treated using glutaraldehyde. β-CD-BSA aqueous solution was pumped in to the capillary, and β-CD-BSA was successfully covalently bonded to the inner surface of the capillary. Chlorpheniramine, phenylalanine, tryptophan, and ibuprofen were separated by β-CD-BSA modified capillary. The operation voltage has certain effect on the separation of the enantiomers. The data showed that higher operation voltage resulted in good separation of tryptophane. However, when the other chiral selectors, β-CD and BSA were employed, the racemic tryptophan was not separated. This indicated that a good chiral separation was obtained when β-CD-BSA was used as chiral selector. J Appl Polym Sci 106: 2041–2046, 2007

Key words: chiral; protein; separation techniques; chiral selector; pressurized CEC

INTRODUCTION

Cyclodextrins (CDs), their derivatives and proteins play very important role in the separation of enantiomeric isomers, and thousands of enantiomeric isomers were separated on CDs, their derivatives and proteins by gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), and supercritical fluid chromatography (SFC).[1–7]

CDs and their derivatives are the most popular and most widely used chiral selectors up till now.[8–10] A large amount of CDs and their derivatives have been developed for chiral separation. Low price, stable chemical properties, and low UV absorbance make them as useful chiral selector in CE and CEC. CDs consist of 6,7 and 8 D-(+)glucopyranose units, corresponding to α-CD, β-CD, and γ-CD, respectively. Enantioselection is based on the formation of inclusion complexes between the CD host and a chiral solute. The type of CDs is of primary importance for achieving successful enantiomeric separation. CDs, β-CD, and their derivatives are the most popular chiral selectors because of their lowest price, unique structure, and size. However, owing to the existence of intramolecular hydrogen bonding, the solubility of β-CD in aqueous solution is less than 0.020 mol. But the solubility can be improved by modification.

In 1973, for the first time, it was demonstrated that the enantiomers of D,L-tryptophan could be resolved by liquid chromatography using bovine serum albumin (BSA) on agarose supports. At present, owing to their high enantioselectivity, a number of proteins have been employed as immobilized chiral selectors in HPLC. A large variety of proteins, such as BSA, human serum albumin, acid glycoprotein, avidin, conalbumin, cellulose, ovomucoid, cellobiohydrolase, and casein were used as chiral selector. By changing pH, not only the structure of protein could be changed, but also the charged state of protein or a solute could be changed. The pH would be a very important parameter in the chiral separation in CEC.[11–13]

Correspondence to: R. Dai (dairongji@bit.edu.cn).
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A better chiral selectivity was obtained when two kinds of chiral selectors were used in the separation of enantiomers by chromatography.[14,15] Previously, several kinds of CDs derivatives were synthesized and used in the separation of enantiomers and chiral drugs in our lab. To find good chiral selectors for enantiomeric separation, three β-CD derivatized proteins, β-CD BSA, β-CD ovalbumin, and β-CD pancreatin were synthesized by crosslinking β-CD and protein using ethylene glycol diglycidyl ether (EGDE) in our former short communication,[16] which was a very simple study of β-CD pancreatin used as chiral selector. To investigate the chiral separation properties of β-CD BSA in pressurized CEC, the main purpose of this article was to make a further study of β-CD BSA used as chiral selector.

MATERIALS AND METHODS

Reagents and chemicals

β-cyclodextrin (β-CD) was obtained from Yunan Cyclodextrins Factory (Guangdong, China). Bovine serum albumin (BSA), ethylene glycol diglycidyl ether (EGDE), triethoxymethylsilane, and 3-aminopropyl-trimethoxysilane (3-APS) were purchased from Fluka. All chemicals were of analytical grade.

Instrumentation

Bruker Equinox 55 FTIR spectrometer was used for the determination of functional groups in molecules. The separation of chiral compounds was conducted on pressurized capillary electrochromatographic system TriSep^{TM}-2010GV, Unimicro (Shanghai) Technologies. The fused silica capillary with an internal diameter of 50 μm was bought from Hebei Yongnian Optical Fiber, Hebei Province, P. R. China. The dialysis bag (2 kDa) was purchased from Fluka.

Synthesis of β-CD-BSA

BSA (1 g) and β-cyclodextrin (1 g) were dissolved in a buffer solution (50 mL) of 0.05 mol/L sodium borate adjusted to pH 8.7 with hydrochloric acid, then EGDE (0.25 mL) was added drop wise to the reaction solution. The reaction mixture was stirred at 37°C for 4 h and then cooled to room temperature. The salts and other small molecules were removed from solution by dialysis in water using dialysis tubing with 2K molecular weight cut-off. The solution of β-CD derivatized BSA was dried under lyophilization, and 1.1 g of white plate crystals were obtained.

^{1}H NMR ($CDCl_3$): 0.85–3.25(m, H of BSA), 3.28–3.38(m, 2H, 6-H), 3.55–3.57(m, 1H, 4-H), 3.63–3.66(m, 3H, H of β-CD hydroxyl), 4.44–4.45(m, 1H, 2-H), 4.82–4.83(m, 1H, 3-H), 5.69–5.71(m, 1H, 5-H), 5.72–5.74(m, 1H ,1-H). FTIR(KBr, cm^{-1}): 3304, 1657, 1541, 1036. MS(MALDI-TOF, *m/z*): 67758.

The treatment of capillary inner surface

The inner surface of the fused silica capillary was treated by the following steps. Firstly, it was washed with 1.0 mol/L NaOH for 2 h, and then with distilled water for 10 min, 1.0 mol/L HCl for 10 min, distilled water for 10 min, and with methanol for 10 min. Finally, the capillary was dried under N_2 for 3 h.

The modification of capillary inner surface with amino group and aldehyde group

A mixture of toluene (5 mL), triethylamine (0.06 mL), and 3-APS (0.1 mL) was stirred for 10 min, and then pumped into the capillary. The sol–gel mixture was blow-out with N_2 in order to form a thin film on the inner surface of the capillary. The capillary was heated at 100°C for 12 h, then washed with methanol and dried under N_2.

Glutaraldehyde (wt 5%) was dissolved in 0.1 mol/L sodium phosphate buffer at pH 7.0. The glutaraldehyde aqueous solution was continuously injected into the capillary for 5 h. The capillary was washed with 0.1 mol/L sodium phosphate buffer at pH 7.0 for 10 min.

The preparation of β-CD-BSA capillary column

The saturated aqueous solution of β-CD-BSA was continuously pumped into the aldehyde groups modified capillary for 30 min. The reaction was carried out at ambient temperature overnight. The unreacted β-CD-BSA aqueous solution was washed out with sodium phosphate buffer at pH 7.0.

The preparation of β-CD capillary column

A mixture of pyridine (5 mL), triethoxymethylsilane(0.06 mL), and β-CD (0.12 g) was stirred for 20 min until the solution became a sticky liquid (The target sol–gel mixture) and then was pumped into the capillary. The sol–gel mixture was blow-out with N_2 in order to form a thin film on the internal surface of the capillary. The capillary was heated at 80°C for 12 h, then washed with methanol and dried under N_2 for 3 h.

The preparation of BSA capillary column

The saturated aqueous solution of BSA was continuously pumped into the aldehyde groups modified capillary for 30 min. The reaction was carried out at

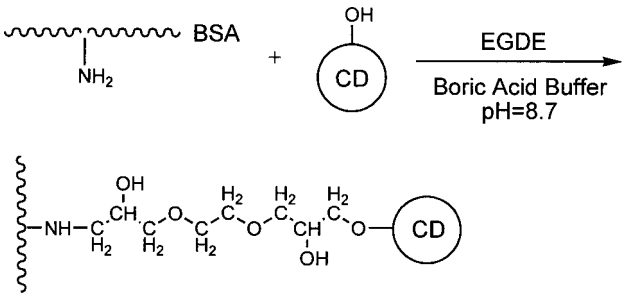

Figure 1 The synthesis of β-CD-BSA.

ambient temperature overnight. The unreacted BSA aqueous solution was washed out with sodium phosphate buffer at pH 7.0.

Electrochromatography

The separation of chiral isomers was carried out in 0.02 mol/L sodium phosphate buffer at pH 4.0. The operation voltage was −15 kV and the detecting UV wavelength is 214 nm. After two rounds of operation, the capillary was rinsed with sodium phosphate buffer at pH 4.0 for 3 min.

RESULTS AND DISCUSSION

The synthesis of β-CD-BSA

The synthesis of β-CD-BSA was shown in Figure 1. The target compound was synthesized by crosslinking BSA and β-cyclodextrin using EGDE in boric acid buffer at pH 8.7. Namely, the two glycidyl groups of EGDE simultaneously reacted respectively with the amino groups of protein and 2-hydroxyl group or 6-hydroxyl group of β-cyclodextrin respectively to produce β-CD-BSA. The synthesized β-CD-BSA maybe a mixture of the resulting compounds that 2-hydroxyl and 6-hydroxyl group of β-CD were reacted with BSA because of their competition reaction. The crude β-CD-BSA product was purified by dialysis and dried by lyophilization.

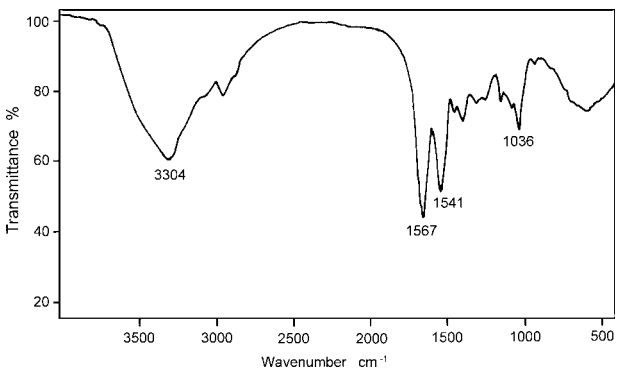

Figure 2 The FTIR spectra of β-CD-BSA.

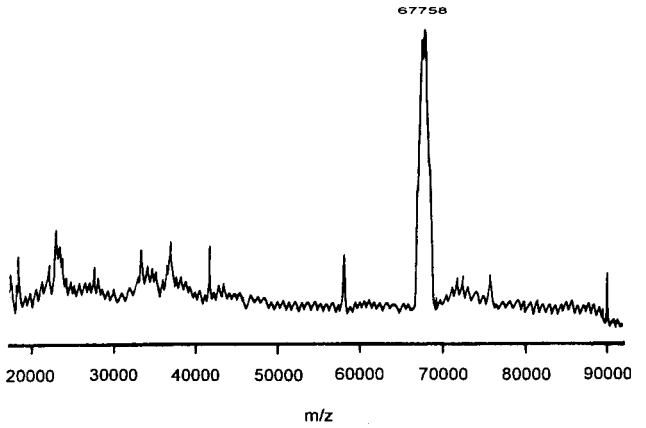

Figure 3 The MALDI-TOF mass spectra of β-CD-BSA.

The structure of β-CD-BSA was characterized by FTIR (as shown in Fig. 2), MALDI-TOF mass spectrum (as shown in Fig. 3), and ^{1}H NMR (as shown in Fig. 4). The characteristics peaks of hydroxyl groups of β-CD and amino group of protein were observed at 3304, 1657, 1541, and 1036 cm^{-1} respectively in the FTIR spectra of the purified β-CD-BSA. In addition, the absorption peak at1036 cm^{-1} was assigned to the C—O—C of β-CD characteristic absorption.

The molar mass of β-CD-BSA was determined to be 67,758 g/mol, while BSA was 66,375. The difference of 1383 g/mol confirmed that only one molecular of β-CD was reacted with BSA.

The ^{1}H NMR spectra of β-CD-BSA was somewhat complex. There is no obvious characteristics peak to be observed.

Column preparation

The chemical covalent bonding of β-CD-BSA on the capillary internal surface was shown in Figure 5. First, the internal surface of the capillary was modified with amino groups by sol–gel technology using

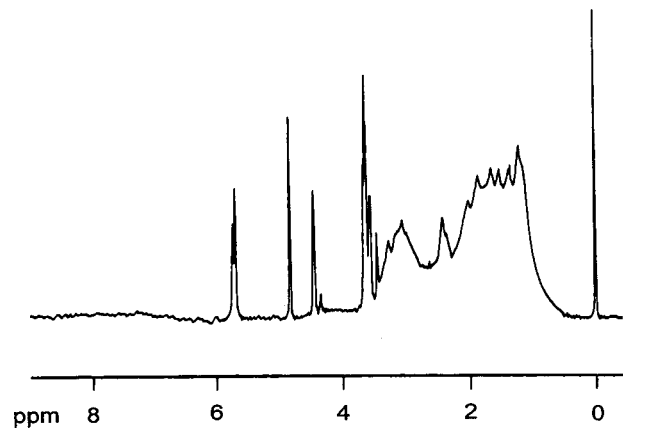

Figure 4 The ^{1}H NMR spectra of β-CD-BSA.

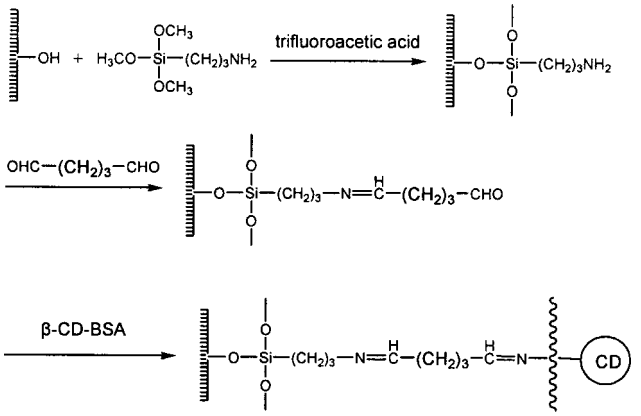

Figure 5 The covalent binding of β-CD-BSA the capillary internal surface.

3-APS in toluene in the presence of triethylamine. Second, the amino modified capillary was treated with glutaraldehyde to produce the aldehyde surface-functionalized capillary. One side of the glutaraldehyde was reacted with the amino groups on the inner surface of the capillary, and the other side remained unreacted. Finally, the saturated aqueous solution of β-CD-BSA was filled into the capillary, and left for overnight. The amino groups of protein were reacted with the aldehyde on the inner surface of the capillary.

BSA capillary column was similarly prepared. The saturated BSA aqueous solution was pumped into the capillary to fabricate the BSA capillary column. The preparation of β-CD was completed using sol–gel technology in pyridine in the presence of triethoxymethylsilane and β-CD.

Separation of enantiomeric isomers

The chiral compounds used here were ibuprofen, tryptophan, phenylalanine, and chlorpheniramine. Their chemical structures were shown in Figure 6. The effects of pH, operation voltage as well as the comparisons of chiral separation results on three kinds of capillary columns were discussed below. All the chiral separations were performed in the pressurized CEC system which only had one pressure of 100,000 Pa. Retention time was not more than 2 min for all chiral compounds.

The resolution of chiral separation was calculated according to eq. (1):

$$Rs = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

The related retention time was the ratio of two retention time, as shown in eq. (2):

$$\alpha = \frac{t_2}{t_1} \quad (2)$$

where t_1 and t_2 were the retention times of two chiral compounds, w_1 and w_2 were the bottom widths of two peaks.

Effect of buffer pH

Sodium phosphate buffer, 0.020 mol/L, was used in the chiral separation. The pH value was adjusted in the range from 3.5 to 7.0 using hydrochloride acid or sodium hydroxide. The different pH values resulted in different chiral separation. The chiral separation results were listed in Table I. The best separation of tryptophan was observed at pH 4.0, but not good separation at pH 5.0. Phenylalanine was separated only at pH = 3.5. The best separation of chlorpheniramine was obtained at pH 3.5, and no separation was observed at pH > 5.0. The best separation of ibuprofen was obtained at pH 5.0, and no separation was observed when pH < 4.0 and pH > 6.0. The capillary electrochromatograms were given in Figure 7.

Effect of operation voltage

The four compounds were analyzed under different operation voltages and the data indicated that the resolution of the enantiomers increased as we increased voltage up to 20 kV. At higher voltage, the Joule heating effect began to dominate, leading to unstable baselines and possible damage to stationary phase in the capillary. The operation voltage range in this work was from −10 to −18 kV, and the highest voltage for the analysis was −18 kV.

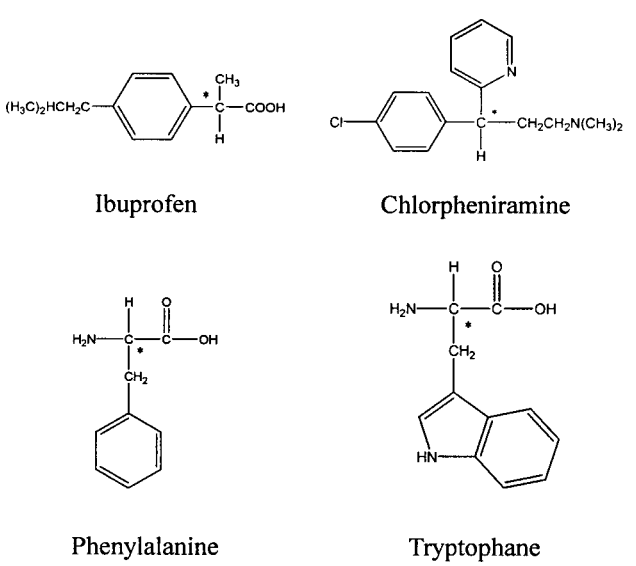

Figure 6 Chiral compounds used in this study.

TABLE I
The Chiral Separation of Chiral Compounds on β-CD-BSA Chiral Stationary Phase in Sodium Phosphate Buffer with Various pH Value

Enantiomers	pH									
	3.5		4.0		5.0		6.0		7.0	
	Rs	α	Rs	α	Rs	α	Rs	α	Rs	α
Tryptophan	–	–	0.71	1.34	0.30	1.10	–	–	–	–
Phenylalanine	0.62	1.45	–	–	–	–	–	–	–	–
Chlorpheniramine	1.41	1.86	0.78	1.46	0.46	1.41	–	–	–	–
Ibuprofen	–	–	0.55	1.13	0.70	1.11	0.37	1.22	–	–

Conditions in Table I: 60 cm fused silica capillary. (a) Tryptophan: sodium phosphate 0.020 mol/L, operation voltage −15 kV, column pressure 0.1MPa, detection wavelength 214 nm; (b) Phenylalanine: sodium phosphate 0.020 mol/L, operation voltage −15 kV, column pressure 0.1 MPa, detection wavelength 214 nm; (c) Chlorpheniramine: sodium phosphate 0.020 mol/L, operation voltage −18 kV, column pressure 0.1 MPa, detection wavelength 214 nm; (d) Ibuprofen: sodium phosphate 0.020 mol/L, operation voltage −15 kV, column pressure 0.1 MPa, detection wavelength 214 nm.

Effect of operation voltage was performed using 0.020 mol/L sodium phosphate buffer at pH 3.5. Chlorpheniramine and tryptophan, the chiral compounds were used for studying the effect of operation voltage. Good chiral separation was obtained at higher voltage.

The operation voltages had the significant affect on the separation results. When β-CD-BSA was used to separate racemic chlorpheniramine and tryptophan, the high resolution and large relative retention time were obtained at high operation voltage. In the separation of chlorpheniramine, when the operation voltage is −12 kV, the resolution was 0.51, the relative retention time was 1.14; when the operation voltage was −15 kV, the resolution was 1.05, the relative retention time was 1.70; when the operation voltage was −18 kV, the resolution was 1.51, the relative retention time was 1.86, the baseline separation was obtained for two isomers. In the separation of tryptophan, when the operation voltage is −10 kV, the resolution was 0.50, the relative retention time was 1.30; when the operation voltage is −12 kV, the resolution was 1.02, the relative retention time was 1.33; when the operation voltage is −15 kV, the resolution was 1.45, and the relative retention time was 1.41.

Comparison of chiral separation results on three kinds of β-CD, BSA, and β-CD-BSA stationary phases

Using racemic tryptophan as chiral sample, the comparisons of chiral separation results on β-CD, BSA and β-CD-BSA were discussed, as shown in Figure 8. All the analysis was performed under the same conditions, namely, sodium phosphate buffer, pH 4.0, operation voltage −15 kV, pressure 0.1 MPa, and detection wavelength 214 nm.

Figure 8(a) showed the chiral separation data of racemic tryptophan on β-CD, Figure 8(b) showed the

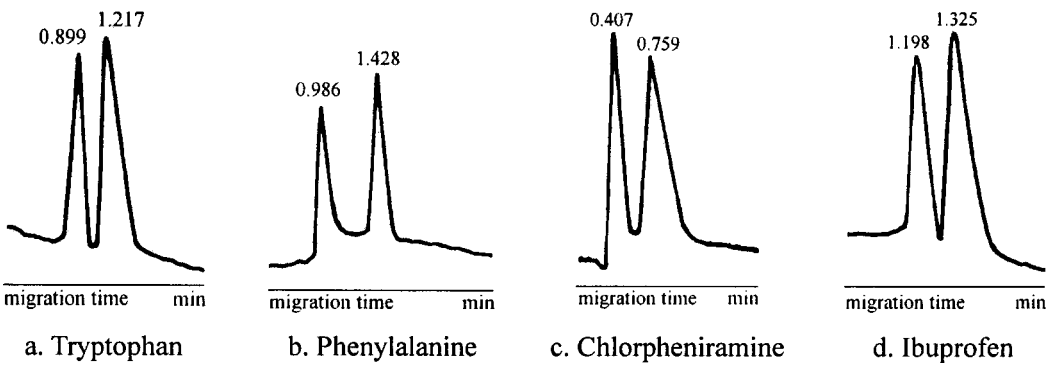

Figure 7 The electrochromatograms of tryptophan, phenylalanine, chlorpheniramine, and ibuprofen. Conditions: 60 cm fused silica capillary. (a) tryptophan: sodium phosphate 0.020 mol/L, pH 4.0, operation voltage −15 kV, column pressure 0.1 MPa, detection wavelength 214 nm; (b) phenylalanine: sodium phosphate 0.020 mmol/L, pH 3.5, operation voltage −15 kV, column pressure 0.1 MPa, detection wavelength 214 nm; (c) chlorpheniramine: sodium phosphate 0.020 mol/L, pH 3.5, operation voltage −18 kV, column pressure 0.1 MPa, detection wavelength 214 nm; (d) ibuprofen: sodium phosphate 0.020 mol/L, pH 5.0, operation voltage −15 kV, column pressure 0.1 MPa, detection wavelength 214 nm.

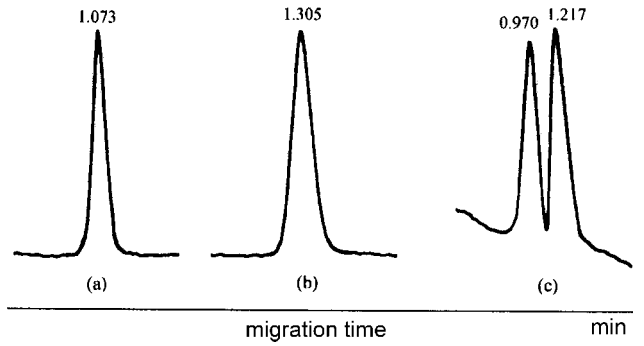

Figure 8 The electrochromatograms of tryptophan on β-CD, BSA, and β-CD-BSA. Conditions: 60 cm fused silica capillary. Sodium phosphate buffer 0.020 mol/L, pH 4.0; column pressure, 0.1 MPa; detection wavelength, 214 nm. Operation voltage: −15 kV. (a) on β-CD, (b) on BSA, (c) on β-CD-BSA.

chiral separation data of racemic tryptophan on BSA and Figure 8(c) showed the chiral separation data of racemic tryptophan on β-CD-BSA. Good separation was observed on β-CD-BSA, the resolution was 1.45, and the relative time was 1.25. No separation was observed on β-CD and BSA. The only difference in electrochromatograms of β-CD and BSA was the retention time. The retention time of tryptophan on BSA was longer than that on β-CD. The results indicated that a good chiral separation was obtained when β-CD-BSA was used as a chiral selector.

CONCLUSION

A new chiral selector, β-cyclodextrin derivatized BSA was synthesized and further used in pressurized capillary electrochromatography for the separation of ibuprofen, tryptophan, phenylalanine, and chlorpheniramine. The synthesis of the chiral selector was carried out by crosslinking β-cyclodextrin and BSA using EGDE, and then the synthesized compound was covalently bonded to the capillary internal surface. The β-CD-BSA has the ability to separate chiral compounds, and good separation results can be obtained at higher voltage.

Using racemic tryptophan as a model chiral sample, chiral separation results on β-CD, BSA, and β-CD-BSA were compared. Only on β-CD-BSA, racemic tryptophan was separated. This indicated that β-CD-BSA is an efficient chiral selector and can result in a good separation for chiral compounds.

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