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Synthesis of a silica-bonded bovine serum albumin *s*-triazine chiral stationary phase for high-performance liquid chromatographic resolution of enantiomers

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Abstract

A novel method of synthesizing protein chiral stationary phase (protein–CSP) is proposed with 2,4,6-trichloro-1,3,5-triazine as the activator. The bovine serum albumin (BSA) based chiral columns (150×4.6 mm I.D.) were prepared successfully within 8 h. With tryptophan as the probe solute, it was observed that the BSA immobilized by this method had a better ability to distinguish enantiomers than that activated by glutaric dialdehyde. This may be due to the well-maintained BSA conformation and the larger amount of BSA immobilized on the silica gel. The BSA–CSP prepared by this method was relatively stable under experimental conditions, and the resolution of 13 chiral compounds was achieved. The coupling reaction in this method is mild, reliable and reproducible; it is also suitable for the immobilization of various biopolymers in the preparation of bioreactor, biosensor and affinity chromatography columns.

Keywords: Enantiomeric separation; Chiral stationary phase; Albumin stationary phase; Triazine stationary phase

1. Introduction

Enantiomer separation is one of the most important areas in chemistry. Several methods have been successfully used to achieve chiral separation. To date the chromatographic method is one of the most effective. In the past decades several kinds of chiral stationary phases (CSPs) have been developed [1–4] based on the various chiral selectors such as celluloses, cyclodextrins, synthetic enantiomers, synthetic polymers and proteins. Proteins are natural high-molecular-mass polymers composed of L-amino acids which have been shown to stereoselectively bind chiral molecules [5–10], and it has been proved that the protein–chiral stationary phases (protein–CSPs) usually showed better enantiomer discrimination in general, because proteins possess complex and changeable conformations. This property has been used to synthesize a number of CSPs including the phases based upon bovine serum albumin (BSA), human serum albumin (HSA), α_1-acid glycoprotein (AGP), avidin, cellulase, ovoglycoprotein and enzymes such as trypsin, α-chymotrypsin and pepsin [4,11–16]. Since maintaining the natural conformation of proteins is essential for chiral separation, the methods for the immobilization of proteins must be

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mild and quick. Several different procedures have been proposed to synthesize protein–CSPs [17–20]. The reactive groups through which the protein was bonded to matrix are the amino, carboxyl and sulphur groups etc., but the amino group is mostly used, e.g. in the glutaric dialdehyde, 1,1-carbonydiimidazole, epoxide, and N,N-disuccinimidyl carbonate methods. However, most of above processes have either complex or rigid reaction conditions.

s-Triazine is an intermediate that was originally used as the linker in reactive-dye synthesis [21]. One of its most important properties is that it can react with an amino group progressively according to the change of temperature, although it can also react with an hydroxyl group. The reaction can be taken place rapidly and almost quantitatively in both aqueous and non-aqueous solutions. The carbon–nitrogen bonding is very stable under normal experimental conditions, because of the induction and resonance effects on triazine ring. Recently, the *s*-triazines have been used to synthesize various CSPs with the amino acids and peptides as the chiral selectors [22–24]. In this work, the aminopropyl–silica gel was activated by 2,4,6-trichloro-1,3,5-triazine and the immobilization of protein with BSA as the chiral selector on silica gel is described for the first time. Furthermore, BSA was also bonded onto the silica gel by the glutaric dialdehyde method, and the chiral separation of D,L-tryptophan as the probe solute on both kinds of BSA–CSPs was compared. Resolution of various kinds of chiral compounds on an aminopropylsilica-bonded BSA *s*-triazine column was carried out successfully.

2. Experimental

2.1. Materials

HPLC grade of acetonitrile was purchased from the Shanghai Bioengineering Center (China). (3-Aminopropyl)triethoxysilane, sodium cyanoborohydride, benzoin, atropin, Dns-D,L-threonine, DNP-D,L-glutaric acid, DNP-D,L-methionine, warfarin, ketoprofen and fenoprofen were purchased from Sigma (St. Louis, MO, USA). α-Methylphenylacetonitrile, α-ethylphenylacetonitrile, α-propylphenylacetonitrile, α-butylphenylacetonitrile were the gifts from the Institute of Chemistry (CAS, Beijing, China). Propanol, 2-propanol, toluene, acetone, glutaric dialdehyde, sodiumdihydrogen phosphate, disodiumhydrogen phosphate and other reagents were of analytical- reagent grade. Silica (5 μm, 300 Å) was purchased from Chrom Expert (Sacramento, CA, USA). 2,4,6-Trichloro-1,3,5-triazine was supplied by San Zheng (Yingkou, Liaoning, China). The deionized water was purified in-house using a Milli-Q system (Millipore, Belford, MA, USA).

The procedure for synthesis of BSA bonded *s*-triazine chiral stationary phase from silica gel is shown in Fig. 1.

2.2. Synthesis of aminopropylsilica gel

Silica (2 g) was added to 50 ml 20% HCl solution. The mixture was stirred gently at 110°C. After 4 h the silica was filtered and rinsed with water until pH 7.0. Then the silica was dried in vacuum at 110°C overnight. The dried silica was dispersed in 50 ml toluene under N_2, subsequently 3 ml (3-aminopropyl)triethoxysilane was added and the temperature was raised to 110°C. The reaction mixture was refluxed for 12 h under N_2 and then the toluene was filtered. The obtained aminopropylsilica was washed three times with toluene and dried under N_2.

2.3. Preparation of BSA bonded s-triazine chiral stationary phase

The aminopropylsilica gel (2 g) was added to 40

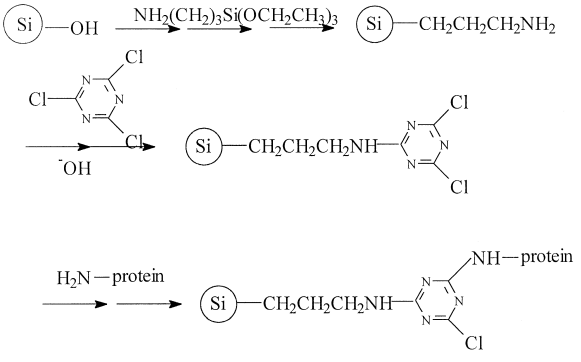

Fig. 1. Procedure for synthesis of BSA bonded *s*-triazine chiral stationary phase from silica gel.

ml water, the mixture was cooled to 0°C by an ice-bath. Under agitation, 0.36 g of 2,4,6-tricholoro-1,3,5-triazine was added slowly, during which $NaHCO_3$ was added gradually to keep the pH of the reaction solution at ~4, and the temperature was never above 5°C. After 2 h the mixture was filtered and the activated silica was washed several times with ice–water to guarantee that no excessive 2,4,6-tricholoro-1,3,5-triazine was adsorbed on the silica-gel. The obtained *s*-triazine activated silica can be stored at desiccator or used immediately.

The activated silica was then packed into a 150× 4.6 mm I.D. stainless steel column using slurry packing techniques. Then the column was linked to a HPLC instrument. A solution of 50 m*M* phosphate buffer (pH 7.0) was delivered through the column at 0.5 ml/min, which was monitored at 254 nm. About 1 h later the baseline was horizontal, a solution of BSA in 50 m*M* phosphate buffer (pH 7.0) was passed through the column set on the recirculation at 25°C. The immobilization of BSA was followed by UV detection at 280 nm. There was no significant change in the UV absorbance about 2 h later, and the amount of the BSA immobilized on the support was calculated from the difference in UV absorbance as reported by Domenici et al. [17]. The column was washed with a glycine ethyl ester solution (1% w/w, pH 6.7) at 25°C for another 2 h, and successively with 50 ml of the following solutions: 50 m*M* phosphate buffer (pH 7.0), 25 m*M* phosphate buffer (pH 7.0) containing 25 m*M* NaCl, deionized water and 50 m*M* phosphate buffer (pH 7.0) again. The column was then stored at 4°C until use.

2.4. Preparation of glutaric dialdehyde activated BSA chiral stationary phase

BSA was immobilized on the aminopropyl-modified silica gel by the procedure reported by Miva et al. [11], except that the glutaric dialdehyde activated silica was packed into a 150×4.6 mm I.D. column directly, after that BSA was immobilized on the silica support in situ.

2.5. Chromatography

The liquid chromatographic system consisted of two P200 pumps controlled by a WDL-95 workstation, a UV 200 multiple wavelength detector (National Chromatographic R&A Center, Dalian, China) and a model 7125i Rheodyne injection valve with a 20-μl loop. The column temperature was controlled by a ZW-Isotemp Controller (National Chromatographic R&A Center). The phosphate buffer was prepared by mixing 50 m*M* Na_2HPO_4 with 50 m*M* NaH_2PO_4 solution to adjust the pH of buffer to the desired value. The mobile phases were obtained by the addition of the organic modifier to the buffer in proportion. The mobile phases were filtered and degassed before use.

3. Results and discussion

In our work, BSA was immobilized successfully on silica via a two-step process which consisted of an initial derivatization to yield dichloro-*s*-triazine activated silica followed by protein coupling. The process was very simple and reproducible. The BSA–CSPs were synthesized in parallel with the glutaric dialdehyde and 2,4,6-tricholoro-1,3,5-triazine as the linkers for the immobilization of BSA on the silica gel, and HPLC columns of the same dimension were filled with the prepared BSA–CSPs. Tryptophan was selected as the probe solute to be separated at the same mobile phase used to examine the difference between those BSA–CSPs. The chromatograms obtained for the separation of tryptophan on the CSPs are shown in Fig. 2. It can be seen that the separation factor (α) for tryptophan on the BSA–CSP prepared by the linker of glutaric dialdehyde is 2.46, but that on the BSA–CSP by the linker of 2,4,6-tricholoro-1,3,5-triazine could reach to 3.27. It has been reported that the D,L- tryptophan binds the native BSA differently [25], the L-tryptophan bound the protein tighter when the eluent pH at 7.6 and the α value was larger. Also the amount of the immobilized BSA has a positive contribution to the α value [26]. The larger α value for the resolution of D,L-tryptophan on the silica-bonded BSA *s*-triazine CSP indicated that (1) the natural conformation of BSA was better preserved during the immobilization process, because each protein molecule had only one amino group to link with silica due to the space and temperature restriction and the whole process was rapid and mild. In particular, the reduction reagent

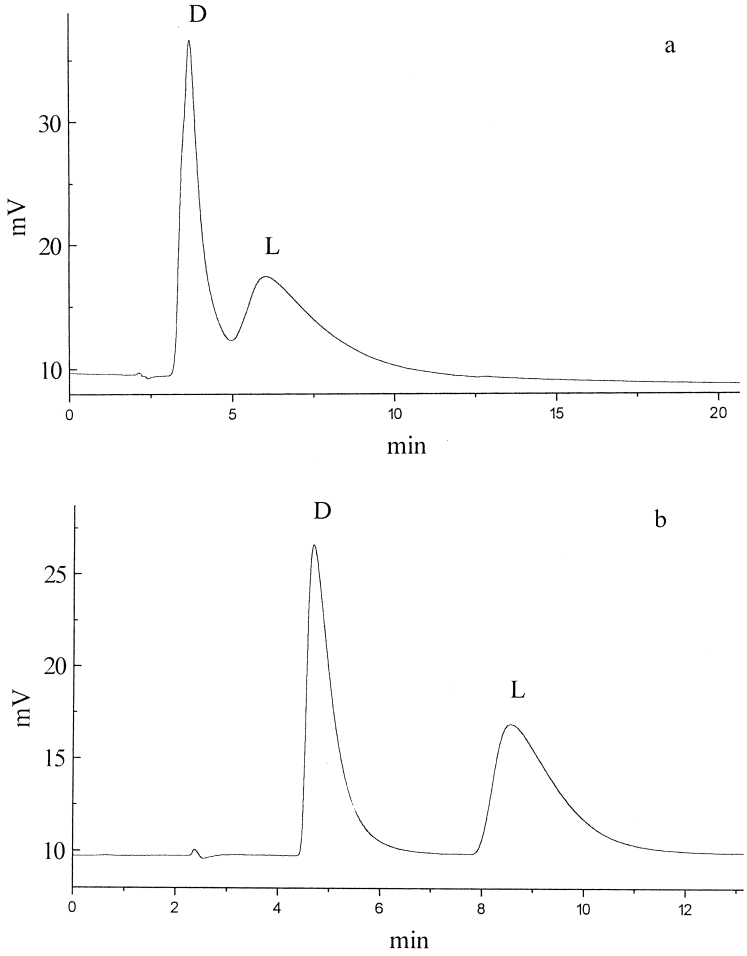

Fig. 2. Resolution of tryptophan on the BSA–CSPs prepared by the (a) glutaric dialdehyde method and (b) *s*-triazine method. Experimental conditions: mobile phase, 50 m*M* phosphate buffer (pH 7.4); flow-rate, 0.8 ml/min; column temperature, 25°C; UV detection wavelength, 280 nm.

used for the immobilization of protein in the glutaric dialdehyde method was not needed. (2) As shown in following section, the amount of BSA immobilized on silica gel by the linker of 2,4,6-tricholoro-1,3,5-triazine was much higher than that by the linker of the glutaric dialdehyde.

Three batches of BSA *s*-triazine CSPs were synthesized, and none of them failed. The reproducibility of BSA–CSPs from batch to batch tested by resolution of tryptophan is listed in Table 1. The average amount of BSA immobilized on the silica gel calculated from the change in UV absorbance during the circulation of BSA solution was about 1.45 μmol BSA per gram of silica, which is much higher than that of BSA–CSP prepared by the glutaric dialdehyde method [18] and nearly equal to

Table 1
Reproducibility of *s*-triazine BSA–CSPs tested by resolution of tryptophan[a]

No.	Amount of BSA immobilized on silica support (μmol/g)	α	R_s
1	1.45	3.27	4.96
2	1.40	3.19	4.85
3	1.42	3.21	4.89

[a] Experimental conditions: the mobile phase was 50 m*M* phosphate buffer (pH 7.4), flow-rate was 0.8 ml/min.

Table 2
Stability of *s*-triazine BSA–CSP tested by resolution of tryptophan[a]

Run time (days)	k_1'	α	R_s
1	0.85	3.17	4.90
30	0.80	3.05	4.74
140	0.68	2.14	3.28

[a] Experimental conditions as in Table 1.

that by the 1,1-carbonyldiimidazole method [17]. The anhydrous conditions in the activation in latter method was harmful for the protein coupling. In our hands the reaction of 2,4,6-trichloro-1,3,5-triazine with silica is not sensitive toward water, virtually the same results could be reached when the reaction takes place in aqueous solution. The CSP with aminopropylsilica bonded BSA *s*-triazine is stable under experimental conditions since the amino groups on both aminopropyl and protein are resonated with the triazine ring [27], and the stability of prepared BSA–CPS should be well kept in acidic and basic eluents even a chlorine atom was remained in *s*-triazine ring. Such an expectation has been supported by the synthesis of bis [carbamoyl (alkyl) methylamino]-6-chloro-*s*-triazine derived chiral stationary phases by Lin and Yang [23]. We tested the stability of BSA–CSP several times by resolution of tryptophan during its use for the resolution of various chiral compounds, and the obtained data are listed in Table 2. The results indicate that over the period of 140 days, there was about 18% decrease in the k' of D-tryptophan, but about 35% decrease in the separation factor of enantiomers, which may be caused by conformation changes of the immobilized BSA. The above results indicate that the BSA–CSPs prepared with a linker of 2,4,6-tricholoro-1,3,5-triazine give more reliable and reproducible results.

Table 3
Retention factors of the first eluted enantiomers (k_1'), separation factors (α), resolution factor (R_s) and the eluents used for chromatography of various chiral compounds on *s*-triazine BSA–CSP

No.	Sample	Mobile phase	Flow-rate (ml/min)	k_1'	α	R_s[a]
1	D,L-Tryptophan	50 m*M* phosphate (pH 7.4)	0.8	0.85	3.27	4.96
2	DNP-D,L-methionine	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 6.0)	0.8	0.95	1.32	2.04
3	DNP-D,L-glutaric acid	2% acetonitrile–1% 1-propanol in 50 m*M* phosphate (pH 8.0)	0.8	0.39	3.17	3.77
4	Dns-D,L-threonine	10% acetonitrile–1% 1-propanol in 50 m*M* phosphate (pH 7.4)	1.0	1.95	1.18	1.72
5	α-Methylphenyl acetonitrile	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	0.8	0.93	1.39	2.41
6	α-Ethylphenyl acetonitrile	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	0.8	1.70	1.41	2.72
7	α-Propylphenyl acetonitrile	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	0.8	3.02	1.56	3.82
8	α-Butylphenyl acetonitrile	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	0.8	5.23	1.60	3.85
9	Fenoprofen	15% acetonitrile–4% 1-propanol in 50 m*M* phosphate (pH 7.4)	1.0	1.62	1.25	1.50
10	Ketoprofen	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	1.0	4.88	1.12	1.71
11	Atropin	10% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 7.4)	1.0	3.69	1.14	1.43
12	Benzoin	15% acetonitrile–1% 1-propanol in 50 m*M* phosphate (pH 6.7)	1.0	2.92	1.24	1.15
13	Warfarin	5% acetonitrile–2% 1-propanol in 50 m*M* phosphate (pH 6.7)	1.8	17.9	1.28	2.53

[a] Resolution factor (R_s) was calculated by equation $R_s = 2 \times (t_2\text{-}t_1)/(W_{1\ 1/2} + W_{2\ 1/2})$, where t and $W_{1/2}$ are the retention time and peak width at half height for a solute.

Furthermore, the synthesis method developed is very useful for the immobilization of biopolymers on the solid media in situ, because the activated silica is relatively stable under normal conditions and the support can be prepared in 8 h.

CSPs based on proteins have been used to resolve a wide number of chiral compounds of pharmacological interest [28,29] and to probe the binding of a drug to proteins [30,31]. The effects of the various factors such as the mobile phase composition, the

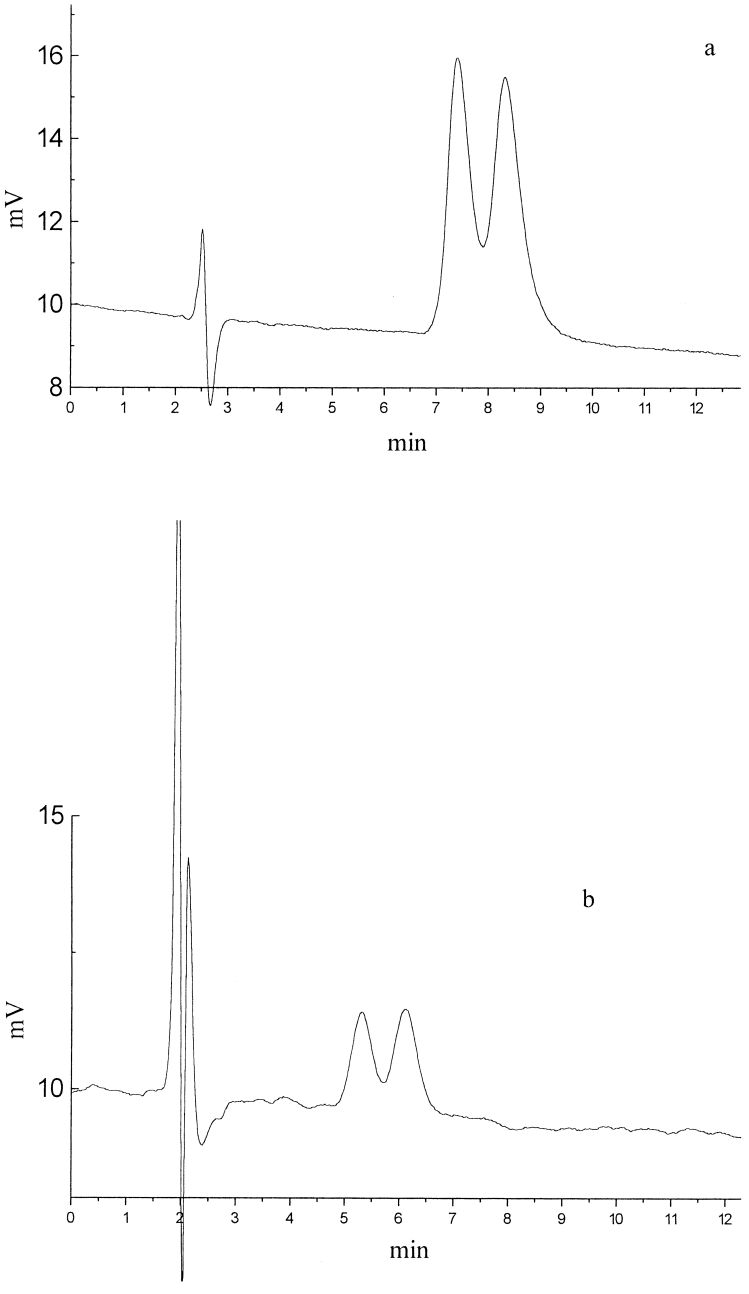

Fig. 3. Selected chromatograms for resolution of some chiral compounds on the *s*-triazine BSA–CSP. Experimental conditions as in Table 3. Solutes: (a) Dns- D,L-threoine (b) fenoprofen (c) ketoprofen (d) warfarin and (e) α-methylphenylacetonitrile.

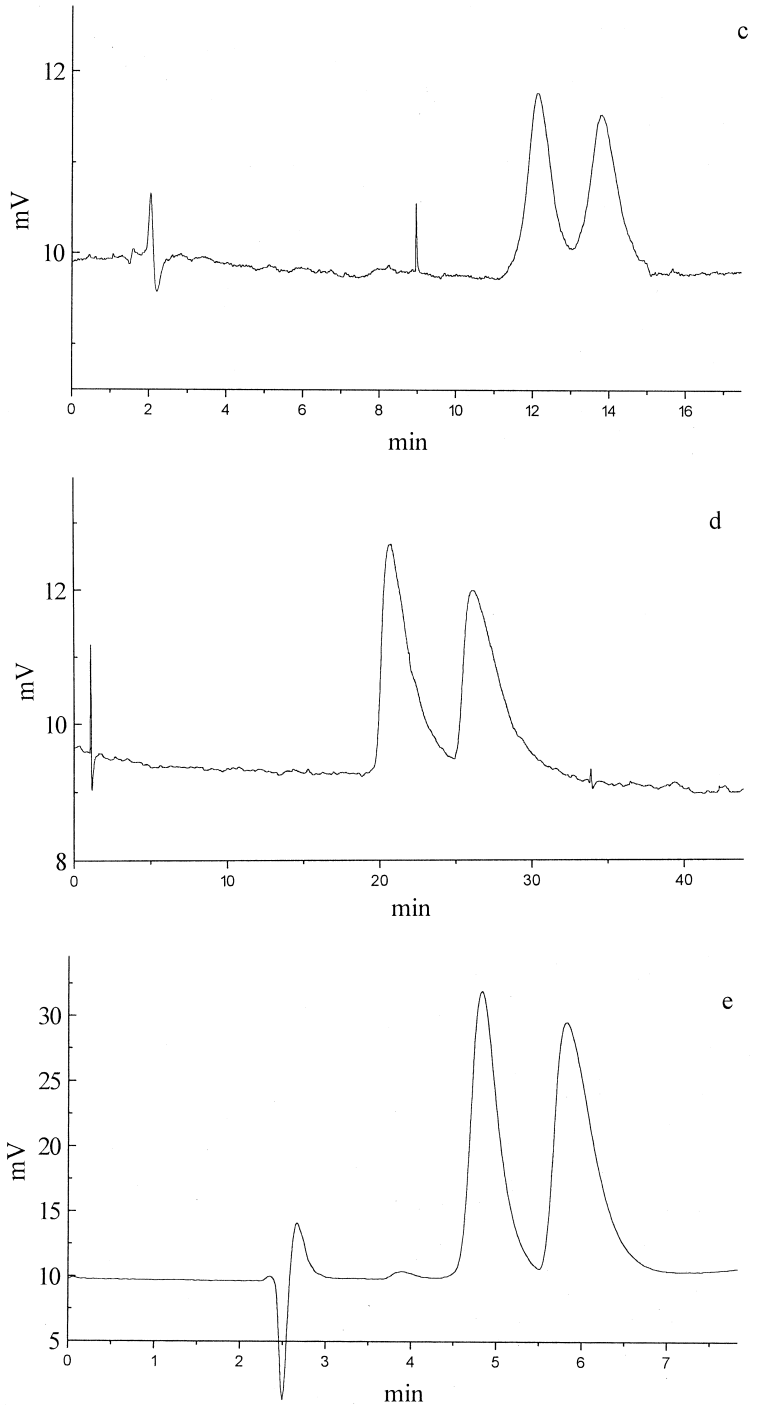

Fig. 3. (*continued*)

eluent pH value and the column temperature on the separation of chiral compounds have been investigated extensively [17,18,32]. In this study, the separations of some typical chiral compounds on the aminopropylsilica bonded BSA *s*-triazine column were performed. Table 3 lists the retention factors for the first eluted enantiomers, separation factors (α), resolution factor (R_s) and eluents used for chromatography of various chiral compounds. The selected chromatograms for separation of some chiral compounds are shown in Fig. 3. From the results shown in Fig. 3 and Table 3, the following conclusions can be drawn: (1) the aminopropylsilica bonded BSA *s*-triazine column is capable of resolving various kinds of chiral compounds; good resolution of enantiomers can be achieved for the 13 chiral compounds listed in Table 3. (2) For the chiral compounds of homologous α-alkylphenylacetonitriles, both the retention factors and separation factors increase with the number of carbons (N_C) in the solutes. The results for linear regression analysis between the log k' for the first and lately eluted compounds and N_C values are shown as follows

$$\text{Log } k_1' = -0.835 + 1.42\,N_C \quad r = 0.975 \quad n = 4$$
$$\text{Log } k_2' = -1.65 + 2.34\,N_C \quad r = 0.971 \quad n = 4$$

The above equations indicate that the retention of solutes on the BSA–CSP increases with their hydrophobicity, and the separation factors for homologous chiral compounds also increases with the length of α-alkyl chain. It has been widely recognized that hydrophobic interactions play an important role in the binding of drugs to proteins such as HSA and BSA; this is supported by the decrease of the retention of drug compounds on those protein CSPs with an increase of organic modifier concentration in the mobile phases [17,32]. (3) The method for synthesis protein–CSPs reported here is a reliable and general procedure for the immobilization of biopolymers on the solid matrices, which may be useful to covalently bound biopolymers in situ for preparation of bioreactors, biosensors and affinity chromatography supports, etc.

4. Conclusion

A new method to synthesis protein CSP was developed, using 2,4,6-trichloro-1,3,5-triazine as the activator. The coupling reaction in this method was rapid and reproducible. The BSA immobilized on silica by this method held its native conformations very well, and had a better ability for chiral separation than the BSA–CSP prepared by the glutaric dialdehyde method, using tryptophan as the probe solute. Different kinds of enantiomers were separated successfully on the aminopropylsilica-bonded BSA *s*-triazine CSP. The BSA–CSPs prepared by this method showed relatively good stability and reproducibility.

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