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Chiral Separation of Tryptophan Enantiomers by Liquid Chromatography with BSA-Silica Stationary Phase

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Abstract The separation of tryptophan enantiomers was carried out with medium-pressure liquid chromatography using BSA (bovine serum albumin)-bonded silica as a chiral stationary phase. The influence of various experimental factors such as pH and ionic strength of mobile phase, separation temperature, and the presence of organic additives on the resolution was studied. In order to expand this system to preparative scale, the loadability of sample and the stability of stationary phase for repeated use were also examined. The separation of tryptophan enantiomers was successful with this system. The data indicated that a higher separation factor (α) was obtained at a higher pH and lower temperature and ionic strength in mobile phase. Addition of organic additives (acetonitrile and 2-propanol) in mobile phase contributed to reduce the retention time of L-tryptophan. About 30% of the separation factor was reduced after 80 days of repeated use.

Keywords: BSA-silica, chiral separation, chiral stationary phase, liquid chromatography, tryptophan enantiomers

INTRODUCTION

The separation of enantiomers has long been a challenging field, since isomeric impurities may have unwanted toxicity, pharmacological actions, and metabolism in living systems. Chromatographic separation methods are considered most useful for optical resolution of such enantiomers [1]. Traditionally, derivatization of a given enantiomeric mixture with a chiral reagent, leading to a pair of diastereoisomers, indirectly allows resolution by achiral stationary phase. A direct method using chiral mobile phase (CMP) or chiral stationary phase (CSP) is an alternative procedure that has come into use more recently. The resolution of enantiomers is accomplished in CMP mode through the formation of diastereoisomeric complexes with a chiral molecule added to the mobile phase, which is often used in capillary electrophoresis [2]. In CSP mode, enantiomers are resolved by the interactions between the analytes and the chiral molecule that is bound to the stationary phase.

The use of CSP is a fast growing area of chiral separations in the fields of capillary electrophoresis (CE) and HPLC. The CSPs commercially available today are subdivided into several categories (shown in Table 1) according to the type of major stereoselective interaction between chiral selector and analytes. The stereoselectivity of a chiral selector is the combined result of the different degrees of multiple interactions (hydrogen bonding, π-π interaction, hydrophobic interaction, cavity inclusion, steric interaction, complex formation, *etc.*) with different enantiomers [7].

Recently, proteins have received increased attention as a new generation of chiral selectors, since proteins are high-molecular weight polymers composed of multiple chiral subunits which have shown to bind stereoselectively to chiral molecules. They can perform all kinds of interactions (listed in Table 1) due to their complicated three dimensional structures [8]. These properties have been utilized in the development of CE and HPLC, employing a phase based upon proteins such as bovine serum albumin (BSA) [9], α_1-acid glycoprotein (AGP) [10], and cellobiohydrolase (CBH) [11]. Yet the detailed mechanisms of chiral recognition by these proteins are largely unknown.

Although the CSPs with BSA and AGP have been successful in resolving a wide number of chiral compounds of pharmacological interest, their applications have been limited to analytical tools like CE and small scale HPLC [12-14]. In this study, we investigated the potential of using BSA-silica CSP for the separation of tryptophan enantiomers, on preparative scale under medium-pressure conditions. As a "preparative" stage, any separation of enantiomers for further use is considered. The roles of various experimental factors such as pH and ionic strength of mobile phase, separation temperature, and the presence of organic additives were considered. Loadability of the analyte sample and the stability of BSA-silica CSP for repeated use were also discussed.

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Table 1. Types of traditional chiral selectors used in chiral stationary phase

Category	Major stereoselective interaction	Typical structure of chiral selector
Brush type	Multiple hydrogen bonding, π-π interaction, dipole stacking, hydrophobic interaction, *etc.*	Derivatized amino acids [3]
Helix type	Attraction to steric structure of polysaccharides	Derivatized cellulose or amylose [4]
Cavity type	Formation of inclusion complex in chiral cavity	Functionalized cyclodextrins [5]
Ligand exchange type	Formation of diastereomeric metal complex	Amino acids-copper [6]

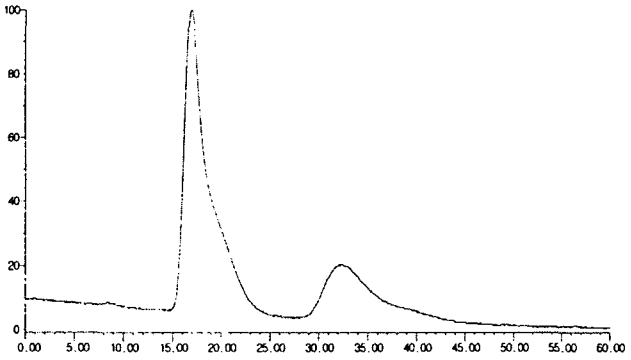

Fig. 1. Typical chromatogram of the separation of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: 0.01 M phosphate buffer at pH 6.5 and 25°C. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan. First peak: D-tryptophan. Second peak: L-tryptophan. Horizontal axis: elution time (min).

MATERIALS AND METHODS

Materials and Reagents

The BSA (Cohn fraction V, 96%) and D- and L-tryptophan were from Sigma (St. Louis, MO, U.S.A.). The Davisil 663XWP spherical silica gel (35-75 μm diameter, 500Å pore size) was obtained from Supelco (Bellefonte, PA, U.S.A.). The reagents for silica derivatization and BSA immobilization on the silica, such as 3-glycidoxy-propyltrimethoxysilane, triethylamine, periodic acid, and sodium cyanoborohydride were from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile (EM Science, NJ, U.S.A.) and 2-propanol (Showa, Japan) were used as mobile phase additives.

Preparation of BSA-Silica

Covalently bonded BSA-silica CSP was prepared according to Larsson's procedure [15] with some modifications. Twenty grams of silica was dried for 4 h at 90°C to remove moisture. After cooling, the silica was suspended in a mixture of 300 mL of toluene, 10 mL of 3-glycidoxy-propyltrimethoxysilane, and 0.25 mL of triethylamine. The mixture was reacted at 70°C for 16 h and filtered on a glass filter. The product slurry was washed successively with toluene, acetone, and ether, then dried under reduced pressure. The resulting epoxy-silica was converted to diol-silica by suspending in 500 mL of 10 mM sulfuric acid for 1 hr at 90°C and then was filtered and washed. The diol-silica was oxidized with 2 g of periodic acid in 250 mL and the resulting aldehyde-silica was washed with sodium phosphate buffer at pH 7.0 and dried under reduced pressure.

Five grams of aldehyde-silica in 20 mL at pH 7.0 buffer solution was mixed with 500 mg of cyanoborohydride and sonicated for 3 minutes with an Ikasonic U50 sonicator (IKA Labortechnik, Germany). BSA 250 mg was added and stirred very gently for 6 days at 4°C. The resulting mixture was filtered and washed with sodium phosphate buffer at pH 7.0. BSA concentration in silicarbonded phase was estimated by comparing the UV absorbance of aqueous phase at 280 nm before and after the reaction. The amount of BSA bonded to silica was about 40 mg per g of silica.

Chromatographic Separation

A low pressure liquid chromatographic system was used equipped with a Gilson 306 pump, a Gilson 118 UV/Vis detector, and the Rheodyne 7725i injection valve. A glass column with a diameter of 1.0 cm and a height of 30 cm was slurry packed with prepared BSA-silica. The resulting packed bed volume was 15.7 cm^3. For the separation of tryptophan enantiomers, a racemic solution was prepared by dissolving equimolar D- and L-tryptophan in a phosphate buffer, which was also used as a mobile phase. All LC experiments were carried out under isocratic conditions, and the detector wavelength was fixed at 214 nm. Applied pressure was in the range of 70-100 psi throughout all experiments when the flow rate of the mobile phase was 1 mL/min.

A typical chromatogram obtained from the separation of the mixture of D- and L-tryptophan is shown in Fig. 1. According to the results of separate experiments with each pure tryptophan enantiomer (data not shown), D-tryptophan eluted earlier than L-tryptophan through the above-mentioned BSA-silica column. Therefore, the separation factor (α) as an index for the separation efficiency is defined as

$$\alpha = t_L / t_D \quad (1)$$

where t_L and t_D represent the retention times of L- and D-tryptophan, respectively, in the separation of the mixture. Larger α values mean that the BSA-silica stationary phase has a greater enantioselectivity for L-tryptophan than for D-tryptophan.

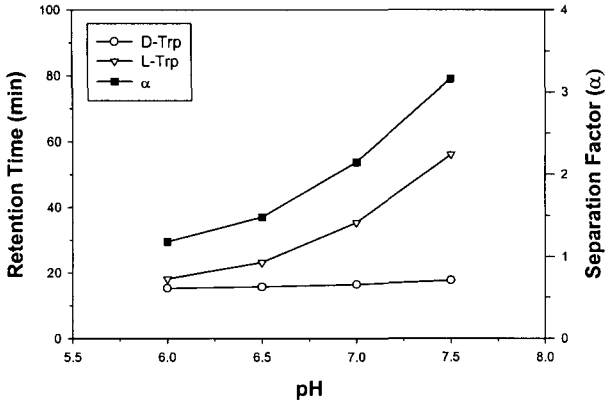

Fig. 2. Effect of mobile phase pH on the resolution of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: 0.01 M phosphate buffer at 25°C. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan.

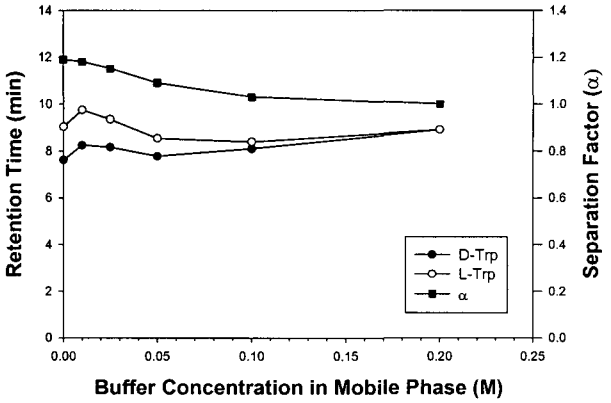

Fig. 3. Effect of buffer concentration in mobile phase on the resolution of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: phosphate buffer at pH 6 and 25°C. Flow rate: 2 mL/min. Loading sample: 5 μL of 1 mM L- and D-tryptophan.

RESULTS AND DISCUSSION

Effect of Mobile Phase pH

Fig. 2 shows the effect of mobile phase pH on the resolution of tryptophan enantiomers. The feed was 5 μL of 1 mM equimolar racemic mixture of D- and L-tryptophan (total 2 mM). The mobile phase was a 0.01 M phosphate buffer in the range pH 6 to 7.5, while its flow rate was fixed at 1 mL/min. Column temperature was maintained at 25°C. The retention time for D-tryptophan was almost not influenced by the pH change, whereas the retention time for L-tryptophan increased from 18.1 min to 56.0 min as the pH increased. As a result, the separation factor (α) increased from 1.18 to 3.16 as the pH increased. The retention times of two enantiomers were not different at acidic pH lower than 6.0. Alkaline pH was not included in the figure because the BSA-silica stationary phase became unstable in pH higher than 8.0 as reported elsewhere [15].

It is known that the capability of chiral recognition (stereoselectivity) of chiral selector is based on the combined results of multiple interactions, such as hydrogen bonding, electrostatic interaction, π-π interaction, dipole and hydrophobic interactions, and cavity inclusion between protein and analytes [7,8]. Therefore, the explanation for the resolving power of the protein-based chiral stationary phase concerning the viewpoint of the structures of protein and analyte molecules is an extremely complex subject which requires further study. A BSA-type chiral selector is known to be useful for the resolution of anionic racemic analytes with one or two benzene rings [13,14]. Fig. 2 implies only that, in the neutral pH range, the high pH is favorable for the BSA-silica stationary phase to interact differently with D- and L-tryptophan, and that the selectivity for L-tryptophan in particular becomes stronger at a higher pH.

Effect of Buffer Concentration in Mobile Phase

The concentration of phosphate buffer used as the mobile phase was varied up to 0.2 M (Fig. 3). The feed was 5 μL of 1 mM equimolar mixture of D- and L-tryptophan. The mobile phase was at pH 6.0, while its flow rate was 2 mL/min. Zero concentration in the figure means that distilled deionized water was used as a mobile phase. The retention times of D- and L-tryptophan fluctuated as buffer concentration increased. However, the separation factor steadily decreased as a higher buffer concentration was used, while the difference in retention times between enantiomers became smaller. It is likely that the high polarity or high ionic strength of the mobile phase is not favorable for the resolution, perhaps because it reduces the electrostatic interaction or hydrogen bonding [9,16] between the BSA-silica stationary phase and tryptophan and thus affects stereoselectivity and retention.

Effect of Temperature

The effect of temperature on resolution is shown in Fig. 4. Column temperature was maintained constant by using a water jacket. The feed was 5 μL of 1 mM equimolar mixture of D- and L-tryptophan. The mobile phase was 0.01 M phosphate buffer at pH 7.0, while its flow rate was 1 mL/min. As the temperature decreased, the retention time of L-tryptophan and the separation factor (α) increased, while the retention time of D-tryptophan remained constant. Low temperature is known to provide stable binding of protein to silica. Also it has been reported that low temperature is favorable for protein-based chiral selectors to maintain stereoselective characteristics of protein structure, and thus to enhance the degree of chiral interaction [17].

Effect of Mobile Phase Additives

The retention and selectivity of CSP can be influenced by the existence of an organic modifier in the mobile phase. The mobile phase additives are sometimes required in chiral separation to enhance resolu-

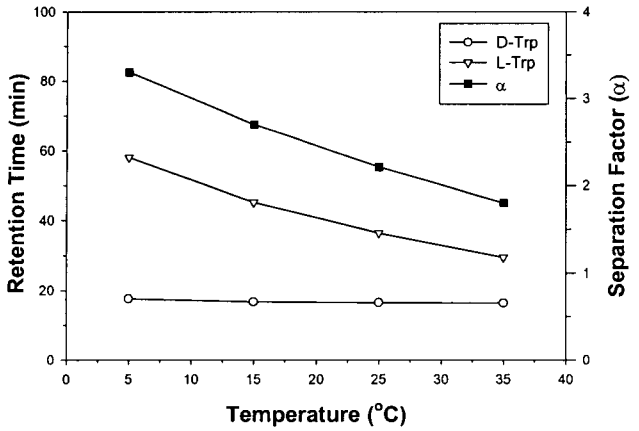

Fig. 4. Effect of separation temperature on the resolution of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: 0.01 M phosphate buffer at pH 7. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan.

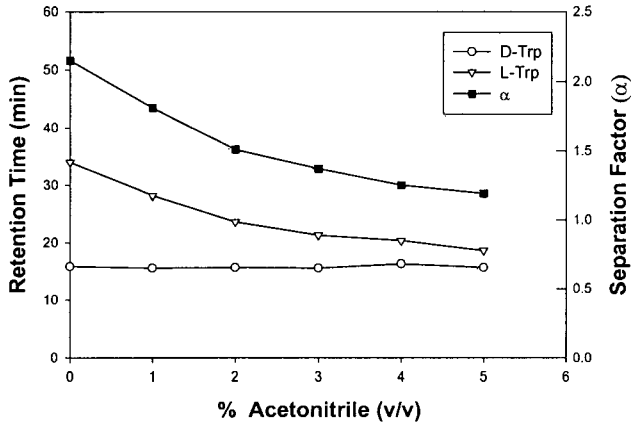

Fig. 5. Effect of addition of acetonitrile as a mobile phase modifier on the resolution of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: 0.01 M phosphate buffer at pH 7 and 25°C. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan.

tion when enantiomers are not easily separated, or to decrease retention time when the stereoselectivity of the stationary phase to enantiomers is too strong. Figs. 2 through 4 show that the separation of tryptophan enantiomers by using BSA-silica CSP was successful, and that the retention times were large enough. Therefore, for the purpose of saving the separation time, it would be beneficial to shorten the retention time within the range as long as the resolution is achieved.

In Figs. 5 and 6, the effects of two organic modifiers, acetonitrile and 2-propanol, on the separation were examined. They were added up to 5%(v/v) to the mobile phase of a 0.01 M phosphate buffer at pH 7.0. The flow rate was 1 mL/min and the operating temperature was 25°C. The feed was 5 μL with a racemic mixture of 1 mM each of D- and L-tryptophan. In both cases, the retention time of L-tryptophan was decreased as the concentration of additives increased, and little change

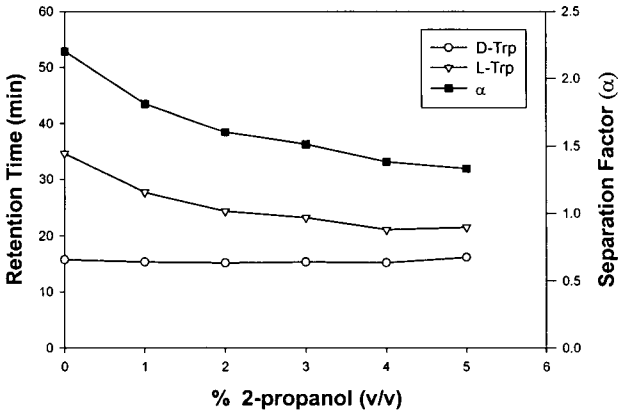

Fig. 6. Effect of addition of 2-propanol as a mobile phase modifier on the resolution of tryptophan enantiomers by BSA-silica stationary phase. Mobile phase: 0.01 M phosphate buffer at pH 7 and 25°C. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan.

was observed in the retention time of D-tryptophan. Some organic additives present are suspected to interact non-specifically with protein, which weaken the hydrophobic interaction or hydrogen bonding between protein and analytes [18,19]. It is reasonable to postulate that a competitive adsorption exists between the tryptophan and organic modifier to a hydrophobic domain of BSA, since adsorption of alcohols to hydrophobic support is known [10]. In the case of separating tryptophan enantiomers with BSA-silica, adding a judicious amount of nonpolar additive (acetonitrile) or polar additive (2-propanol) to a mobile phase can save separation time, although the separation factor was reduced.

Loadability

In general, preparative chromatography for pharmaceuticals refers to a separation that can load more than 150 mg [20]. The chromatographic system used in this study is a kind of small scale preparative LC under medium pressure in terms of columnar dimension (1 cm in diameter) and applied pressure (less than 100 psi). In order to investigate the potential for developing the BSA-silica stationary phase to preparative chiral separation, a loadability test was carried out. Loadability, or loading capacity, is the maximum loading concentration in which the complete resolution of enantiomers is achievable. Loadability can be correlated with the degree of resolution (R_S) which is defined as

$$R_S = (t_{Lf} - t_{Di}) / (W_L + W_D) \quad (2)$$

where W_L and W_D are peak widths of L- and D-tryptophan, respectively, in time dimension. t_{Lf} is the elution finishing time of the second eluting enantiomer (L-tryptophan), and t_{Di} is the elution starting time of the first eluting enantiomer (D-tryptophan). An R_S value greater than 1 means that two enantiomers are

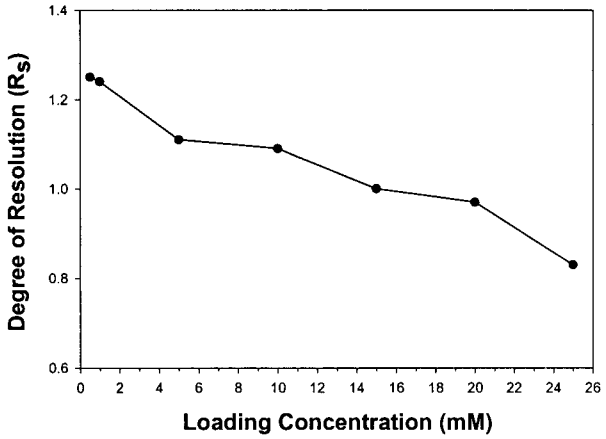

Fig. 7. Loading capacity of BSA-silica column for the resolution of tryptophan enantiomers. Mobile phase: 0.01 M phosphate buffer at pH 7 and 25°C. Flow rate: 1 mL/min. Loading: 5 μL. Concentration represents that of each enantiomer in racemic mixture.

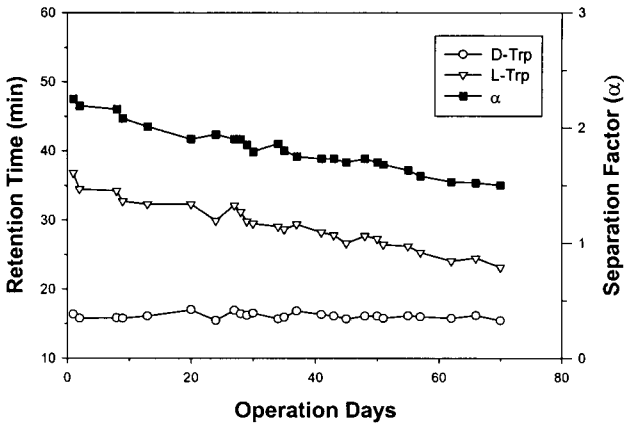

Fig. 8. Long term stability of BSA-silica column for the resolution of tryptophan enantiomers. Mobile phase: 0.01 M phosphate buffer at pH 7 and 25°C. Flow rate: 1 mL/min. Loading: 5 μL of 1 mM L- and D-tryptophan.

completely resolved; less than 1 means that peaks are overlapped. Two peaks are baseline resolved when R_S is 1. In Fig. 7, separations were carried out with different loading concentrations of tryptophan racemic mixture. The mobile phase was a 0.01 M phosphate buffer at pH 7 and 25°C, while its flow rate was 1 mL/min. The sample injection volume was 5 μL. A smaller R_S value was obtained when higher loading concentration was used, since the peak widths (W_L and W_D) of enantiomers increased. The complete resolution could be achieved up to loading 15 mM of each enantiomer (total 30 mM) under the above experimental conditions. Loadability can be further improved if the difference in the retention times of two enantiomers is enlarged by judicious selection of operating conditions such as pH, ionic strength, temperature, mobile phase additives, and the amount of loading.

Stability of Stationary Phase

To examine the long term stability of the BSA-silica stationary phase, the separation of tryptophan enantiomers was repeated for 80 days under identical conditions (Fig. 8). At least one run per day was carried out by loading 5 μL of a 1 mM racemic mixture (total 2 mM), a 0.01 M phosphate buffer at pH 7, a temperature of 25°C, and 1 mL/min flow rate. Although the retention of D-tryptophan was nearly unchanged, that of L-tryptophan was shortened to 23% of the initial time after 80 days. In addition, the separation factor was lowered from 2.2 to 1.5 for 80 days. It is not yet clear whether there was any loss of protein from the silica particle by wash-out or whether the stereoselectivity of the stationary phase was weakened by any structural change of protein after repeated use.

CONCLUSIONS

The chiral stationary phase in which BSA was covalently bonded to aldehyde-derivatized silica was prepared and successfully utilized in the resolution of tryptophan enantiomers under a preparative medium-pressure liquid chromatography. The capability of chiral recognition or stereoselectivity of the BSA chiral selector is based on the combined results of multiple interactions, such as hydrogen bonding, electrostatic interaction, π-π interaction, dipole and hydrophobic interactions, and so forth between protein and analytes.

A high pH level in the neutral range was favorable for BSA-silica CSP to interact differently with D- and L-tryptophan. Thus, the selectivity for L-tryptophan became stronger at a higher pH level. High ionic strength of mobile phase was inhibitory to the resolution because it possibly reduced the electrostatic interaction or hydrogen bonding between BSA-silica CSP and tryptophan. Low temperature was favorable toward maintaining the stereoselective characteristics of protein, and thus to enhance the separation factor. The existence of organic additives like acetonitrile and 2-propanol weakened the hydrophobic interaction or hydrogen bonding between protein and analytes, since competition might exist between the tryptophan and organic modifier for adsorption to a hydrophobic domain of BSA.

In order to expand this system to a real preparative-scale chromatography, the loading capacity should be further improved. This can be done if the difference of retention times of the two enantiomers are enlarged by judicious selection of operating conditions such as pH, ionic strength, temperature, or mobile phase additives. Moreover, there must be some improvement in the long-term stability of BSA-silica CSP, since about 30% of the separation factor was lost after 80 days of repeated use.

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