Full Short Communication

Effects of Temperature and Mobile Phase Condition on Chiral Recognition of Poly(L-phenylalanine) Chiral Stationary Phase

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Abstract

Characteristic of chiral stationary phase (CSP) with poly(L-phenylalanine) peptide selector, which is in α-helical state, was reported. Since environmental factors affect peptide conformation, the changes of enantioselectivity were examined depending on column temperature and mobile phase conditions (ionic strength, pH, mobile phase composition). Column temperature and pH drastically affected the enantioselectivity. Based on these changes, the relation between chiral recognition and secondary structure of the peptide selector was discussed. The column stability during sequential analysis under different separation conditions was also evaluated.

Introduction

In design of a new HPLC method for the separation of enantiomers, it is preferable to use a chiral stationary phase (CSP) because of the simple operation. To date, CSPs with several chiral selectors, such as cyclodextrins [1], macrocyclic antibiotics [2], polysaccharide derivatives [3], modified amino acids [4], proteins [5] and cinchona alkaloids [6], have been reported and some of them are commercially available. We recently developed new series of the CSPs having poly(L-Phe) peptide selector and successfully applied for chiral separations of some pharmaceuticals in reversed- and normal-phase modes [7, 8]. Previous study suggests that the octapeptide selector is the best and the series of selectors are mainly in the α-helical state and this conformation partially contributes to chiral recognition [7]. In aqueous solution, the three major environmental factors that could affect the energetics of the helix-coil transition of peptides are temperature, pH and ionic strength. Therefore, in the present study, the contribution of column temperature and mobile phase condition (ionic strength, pH and mobile phase composition) to the chiral recognition of the poly(L-Phe) CSP was investigated using warfarin enantiomers as a model. Furthermore, column stability during sequential analysis under different separation conditions was also evaluated.

Experimental

Chemicals

Aminopropyl silica (APS, particle size, 5 μm; pore size, 120 Å) was a kind gift from Daiso Chemical (Osaka, Japan). Boc-L-Phe was purchased from Peptide Institute (Osaka, Japan). Dicyclohexylcarbodiimide (DCC), *N*,*N*'-diisopropylethylamine (DIEA), benzoyl chloride (BZ), triethylamine, methanol and ninhydrine were obtained from Wako Pure Chemicals (Osaka, Japan). Trifluoroacetic acid (TFA) were from Tokyo Chemical Industry (Tokyo, Japan). Dichloromethane (DCM), ethanol, sodium perchlorate, perchloric acid were from Kishida Chemicals (Osaka, Japan). HPLC grade of acetonitrile was purchased from Wako. Warfarin was obtained from Sigma (St. Louis, USA). Chirally pure warfarin was prepared from racemic warfarin, using a fractional crystallization method [9].

Preparation of Poly(L-Phe) CSP by Solid-Phase Synthesis

The synthetic procedure of the CSPs with Boc-L-amino acid was according to our previous report [7]. Boc-L-Phe (1.5 g), DCC (1.23 g) and DIEA (100 μL) were added to a suspension of APS (0.7 mmol/g, 5 g) and DCM (25 m L) and the mixture was stirred at room temperature for 2 h. Boc groups were then removed from Phe residues with TFA (25% in DCM). The same procedure was repeated seven times to obtain an octapeptide selector on APS [APS-$(Phe)_8$-H]. APS-$(Phe)_8$-H was end-capped by reaction with BZ (140 μ L) in DIEA (260 μ L) and DCM (5 m L) for 2 h.

Chromatography

The CSP was individually packed into HPLC columns (4.6 mm i.d. x 150 mm) by a slurry packing method with methanol [10].

The HPLC system included Waters LC Module 1 (Milford, USA), Waters HTR-B column oven and Rikadenki R-01 recorder (Tokyo, Japan). Flow rate was set at 0.5 ml/min with UV detection at 280 nm.

All aqueous solutions were made with the water that was deionized and distilled using WG 220 (Yamato Scientific Co, Tokyo) and then passed through a water purification system (Puric-Z, Organo Co, Tokyo). The mobile phase was a mixture of aqueous sodium perchlorate, adjusted with perchloric acid to desired pH, and acetonitrile, and was degassed thoroughly prior to use.

Results and discussion

Column Temperature

Fig. 1a shows dependence of the resolution factor of warfarin on column temperature. Resolution factor decreased as temperature increased. Especially, it dramatically decreased when temperature increased from 30 to 50 °C. Furthermore, van't Hoff plot, which is the thermodynamic relationship between retention factor and temperature in Kelvin, was not linear (Fig. 1b). Non-linear van't Hoff plots may reflect a change in the relative contributions by enthalpy and entropy of transfer of the solute from the mobile phase to the stationary phase [11]. The changes in these

relative contributions and possible changes in the retention mechanism are often thought to be related to conformation changes in the stationary phase (*e.g.* C18 stationary phases) [12, 13].

Ma *et al.* reported that the α-helical conformations of polycationic peptide melt into extended ones as the temperature increased (from 1 to 60 °C) [14]. In their study, the helical conformation most drastically decreased as the temperature was changed. Similarly, Muñoz and Serrano reported a decrease of helical content as increasing temperature using polyalanine peptide [15]. From these observations, the chiral recognition of the poly(L-Phe) peptide selector was sensitive to the column temperature probably due to change of its secondary structure.

Mobile Phase Conditions

It is known that a helix stabilization is caused by binding anions, the so-called chaotropic anions, to polycationic peptides, electrostatically shielding and thereby stabilizing partially folded conformation such as helices [16, 17]. Among the chaotropic anions, sodium perchlorate is well-known to have profound effects on helical stability and is widely used [18, 19]. On the other hand, Asciutto *et al.* reported sodium perchlorate effects on the helical stability of polyalanines that lack charged groups [20]. They explained the stabilization effects as follows: perchlorate ions compete with water molecules to solvate the peptides. The ion solvation effect is

stronger than that of hydration, and the peptide surface loses water, or gets dry. A less hydrated peptide promotes more intrapeptide hydrogen bonding, which leads to greater helical stability. In our preliminary study, chiral resolution on the poly(L-Phe) CSP was not observed when using phosphate buffer and ammonium acetate solution; however, sodium perchlorate solution provided good resolution. Therefore, perchlorate was preferable for poly(L-Phe) chiral selector (uncharged peptide) probably because of the solvation effect. The effect of sodium perchlorate concentration (0.05, 0.1, 0.3 and 0.5 M) on resolution of warfarin enantiomers was studied. The chiral resolution did not change with varying perchlorate concentration (data not shown). This might be because all the concentrations were enough to stabilize the helical state. It was reported that proteins undergo complex conformational changes in the presence of chaotropic anions at lower concentrations than the above concentrations [21, 22].

Subsequently, a series of perchlorate solution with different pH (2.0, 4.0, 6.0 and 8.0) were tested. During pH 2.0~6.0, the resolution factor was not affected significantly, and the value ranged from 1.30 to 1.42. However, at pH 8.0, the resolution drastically decreased. Although the dependence of helical content on pH was reported by some researchers, the results were varied by the type of peptide or protein [23, 24]; therefore, the decrease has yet to be resolved.

Since the retention was largely changed by slight change of perchlorate solution content in the mobile phase, the effect of mobile phase composition on retention and

resolution factor was investigated within very narrow range from 75 to 80% (Fig. 2). The retention factor significantly decreased even when only one percent of aqueous content decreased; however, resolution factor did not decrease as much as retention factor did. This indicates that hydrophobic interaction does not have a dominant role in the chiral recognition of the poly(L-Phe) peptide CSP.

Column Stability

During six months, we used several separation conditions (*e.g.*, six mobile phases, 20-50°C column temperature) in the present study. After a total of more than 100 injections under those conditions, no significant deterioritation in the resolving power of the CSP was observed (Fig. 3).

Conclusion

The experimental data have confirmed that the chiral recognition of the poly(L-phenylalanine) CSP was strongly affected by column temperature, pH as well as a type of ion in mobile phase. Low column temperature (< 30 °C), low pH (< 6.0) and perchlorate ion are preferable for the chiral separation on the CSP, which was probably because the helical conformation of peptide selector can be stabilized under such conditions. The CSP successfully separated model enantiomer (warfarin) during six months without decreasing separation performance.

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Figure captions

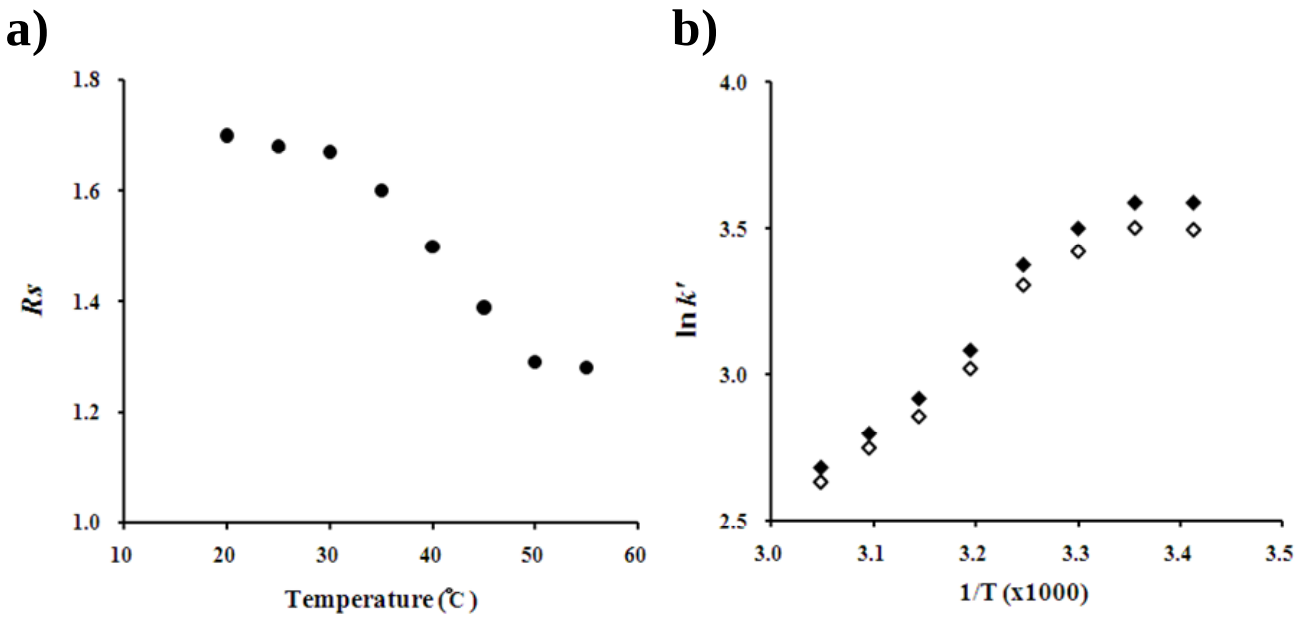

Fig. 1 a) Effect of column temperature on resolution factor and b) van't Hoff plot of warfarin enantiomers. Mobile phase: 0.5 M sodium perchlorate (pH 2.0)-acetonitrile (77/23, *v*/*v* %). Other conditions as mentioned in Section 2.

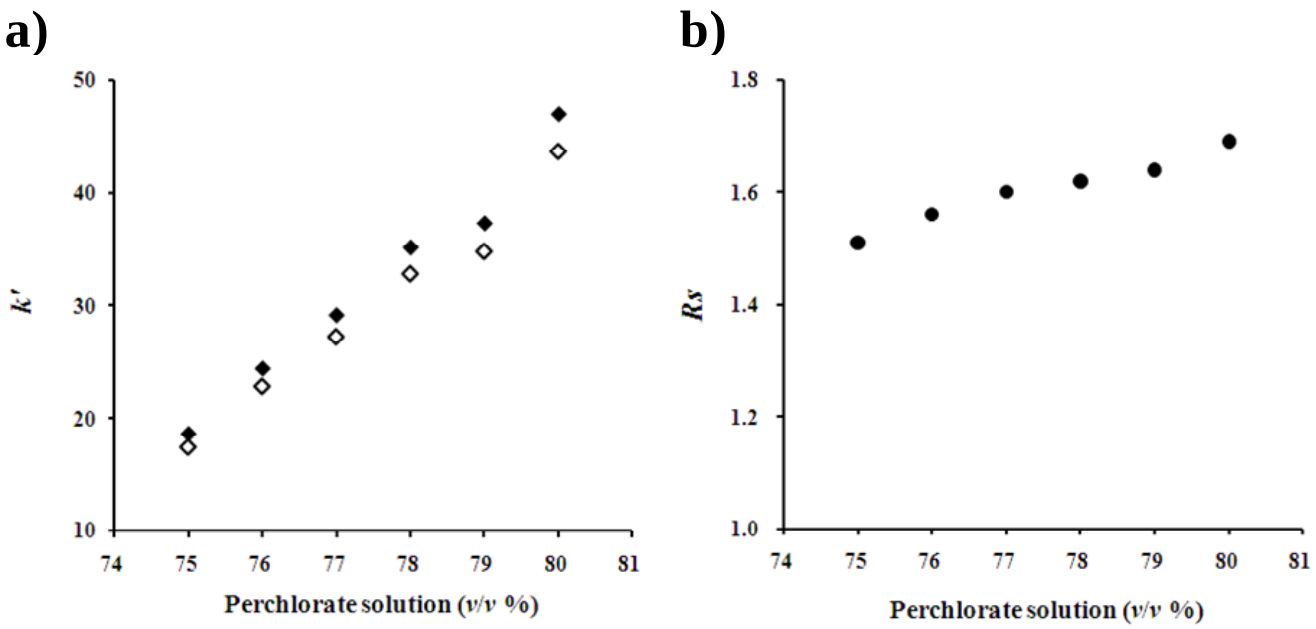

Fig. 2 Effect of perchlorate solution content in mobile phase on a) retention factor and b) resolution factor. Conditions: mobile phase, 0.5 M sodium perchlorate (pH 2.0)-acetonitrile with varying perchlorate solution content; column temperature, 35 °C. Other conditions as mentioned in Section 2.

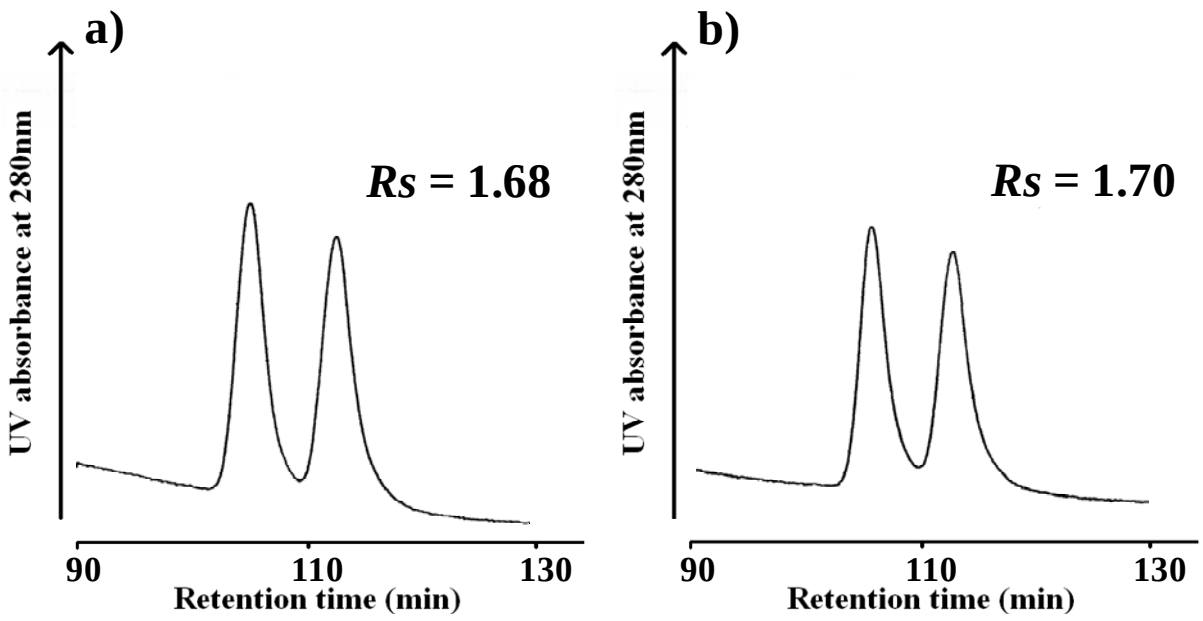

Fig. 3 Chromatograms of *R*/*S* warfarin on the CSP with poly(L-phenylalanine) peptide selector a) before and b) after more than 100 injections under several separation conditions. Mobile phase: 0.5 M sodium perchlorate (pH 2.0)-acetonitrile (77/23, *v*/*v* %); column temperature, 35°C.