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Synthesis and Performance of Ferrocene-Bonded Chiral Stationary Phase for RP-HPLC

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The preparation, characterization and potential liquid chromatographic applications of a ferrocene-bonded chiral stationary phase are presented. The phase was prepared by covalent bonding of ferrocene-derivatized alanine to aminopropylated spherical silica gel. Use of the new stationary phase enabled good separations of phenylalanine, tryptophane, clenbuterol hydrochloride and thioctic acid under reversed-phase conditions within acceptable analysis times (*i.e.*, below 20 min). The separation factors ranged between 1.07 and 1.41 and the resolutions were in no case less than 0.9. The mechanism of retention on the new stationary phase involves strong π-π interaction with each one of the cyclopentadienyl (Cp) groups of the ferrocene and simultaneously hydrogen bonding interactions between the chiral stationary phase and the analytes. The phase enables effective separation of optical isomers with a benzene ring.

Keywords: High performance liquid chromatography, Ferrocene-bonded chiral stationary phase, π-π Interactions.

INTRODUCTION

Liquid chromatographic resolution of enantiomers on chiral stationary phases (CSPs) is one of the most convenient and accurate methods for determination of optical purity. Brush-type or Pirkle-type chiral stationary phases are the most widely investigated chiral stationary phases and are present in many commercially available products[1,2]. They contain a low-molecular-weight chiral molecule (chiral selector) covalently bound to the silica gel surface and resolve racemates as a result of enantioselective interactions between the chiral stationary phase and the analytes[3]. For effective π-π interactions, Pirkle-type chiral stationary phases usually contain π-acidic or π-basic aromatic groups[4]. Rigid structural elements are often incorporated during the preparation of these chiral supports in order to create effective steric barriers triggering or amplifying their enantiorecognition ability[5]. The most relevant characteristics of brush-type chiral stationary phases include: Good kinetic performance, broad applicability, chemical and thermal inertness, compatibility with any mobile phase, elevated sample loading capacities and the availability of such chiral stationary phases in both enantiomeric forms with opposite configurations displaying reversed elution orders[6,7].

Numerous Pirkle-type chiral stationary phases are based on derivatives of α-amino acids, inexpensive and readily available enantiomerically pure materials[8-10]. For example, chiral stationary phases based on *N*-(3,5-dinitrobenzoyl)-α-amino acids (DNB-AAs), mainly *N*-(3,5-dinitrobenzoyl)-D-α-phenylglycine or *N*-(3,5-dinitrobenzoyl)-L-leucine, have been used for separation of the enantiomers of a variety of analytes[11-14]. Subtle structural modification of the chiral stationary phase has often been shown to have remarkable effects on their enantiomer-separation properties[14,15].

The number and type of brush chiral stationary phases is continuously growing. Pirkle and Lee[16] recently prepared a brush chiral stationary phase derived from α-amino β-lactam for the separation of β-blockers. They demonstrated that an enhanced rigid conformation of the chiral stationary phase gives to it enhanced enantioselectivity and greater scope with respect to its precursor derived from the α-amino phosphate. Gasparrini's group[17] synthesized the first sub 2 μm brush-type chiral stationary phase through a high reproducible synthetic methodology that allowed to assemble and surface-graft the whole chiral selector in only two steps. They compared the properties of columns packed with 1.9 μm particles with those of columns prepared with silica particles of 4.3 and 2.6 micron. Their conclusions show that the chiral stationary phases with reduced particle size and packed in short columns provide similar resolutions as those packed in standard column formats, but in considerably shorter times. Slater *et al.*[18] describe the in-column preparation of a brush-type chiral stationary phase (based on a proline-derived chiral selector) using click-chemistry (copper-catalyzed azide-alkyne cycloaddition) to functionalize the surface of monolithic silica or packed beads of 10 μm particles. The performances of the chiral stationary

phase were demonstrated by considering the enantioseparation of a series of π-acidic amino acid amide derivatives. Wei *et al.*[19] prepared two brush-chiral stationary phases by using derivatives of benzoylated tartaric acid and 1,2-diphenylethylenediamine as chiral selectors. Choi *et al.*[20] prepared a chiral stationary phase based on the antibiotics cefaclor (belonging to the family of cephalosporins). The new chiral stationary phase was demonstrated to be useful in the resolution of acidic analytes (*N*-dinitrobenzoyl-amino acid derivatives) through a molecular recognition mechanism based on enantioselective donor-acceptor interactions. Ohyama *et al.*[21] report about the preparation and the use as chiral stationary phases of peptide chiral selectors prepared by solid-phase synthesis.

In this paper, we describe the synthesis of a ferrocene-bonded high-performance liquid chromatographic Pirkle-type chiral stationary phase prepared starting from L-alanine. The chiral stationary phase was successfully applied in resolving tryptophane, phenylalanine, lipoic acidc and lenbuterol hydrochloride and found to be effective. The chiral recognition mechanism was proposed to be π-π interaction and simultaneously hydrogen bonding interactions between the chiral stationary phase and the analytes.

EXPERIMENTAL

Ferrocenol chloride was prepared from ferrocene by a procedure reported elsewhere. Methanol and acetonitrile were of HPLC grade. Water for HPLC was produced by treating deionized water with a Barnstead/Thermolyn Nanopure water purification system comprising an organic-free fine cartridge then a 0.2 μm particle filter. Other reagents were commercially available and were reagent-grade or better. The new stationary phase was prepared from Kromasil 100 A-5 μm porous 3-ammonium propyl silica (surface area 320 m 2 g^{-1}) from EKA Nobel (Bohs, Sweden). A stainless steel column (150 mm 4.6 mm i.d.) was slurry-packed with the stationary phase by use of conventional techniques.

Chromatographic conditions: HPLC was performed with a Shimadzu LC-20A liquid chromatograph equipped with an ultraviolet detector; a computer-based SSI ChemStation was used to manipulate chromatographic data. All chromatographic experiments were performed in duplicate at 30 °C at a flow rate of 1 mL min 1 and with UV detection at 254 nm, unless otherwise indicated. The dead time was determined by injection of acetone.

Preparation of the Ferrocene-Bonded Chiral Stationary Phase (Fc-CSP) (Fig. 1): 7.49 g (0.032 mol) ferrocene carboxylic acid and 200 mL anhydrous dichloromethane were added to a 250 mL three-necked bottle and then, the solution color changed from yellow to red after the addition of 4 mL (0.042 mol) oxalyl chloride and 3 drops of *N*,*N*-dimethylformamide at room temperature. The mixture became uniform, transparent and crimson after stirring constantly for 3 h. 150 mL petroleum ether was subsequently added to the solution and the procedures of filtration and rotary evaporation were then carried out. 4.61 g crimson granular crystal was finally obtained as the products with the yield of 92.5 %. m.p. 49-50 ºC. ^{1}H NMR ($CDCl_3$, 500 MHz), δ: 4.92 (s, 2H), 4.57 (s, 2H), 4.39 (s, 5H).

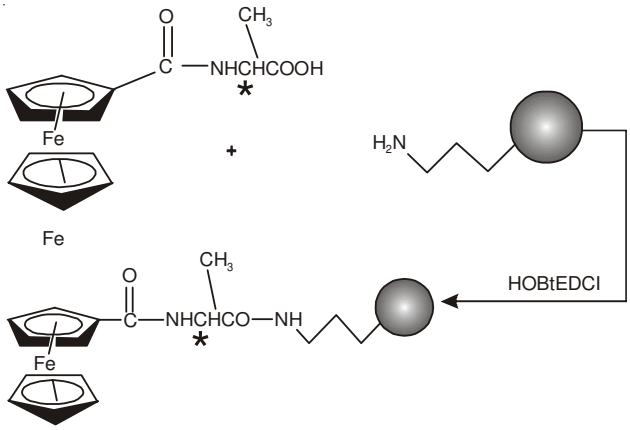

Fig. 1 Reaction scheme for the synthesis of Fc-CSP

2.4 g (0.06 mol) sodium hydroxide and 5.34 g (0.06 mol) L-alanine were firstly dissolved with 20 mL distilled water in a 250 mL three-necked flask and then 40 mL acetone was added. The solution of chlorocarbonyl ferrocene in acetone (20 mL) was slowly added into the above mixture at the rate of 5-10 drops per minute. Sodium carbonate solution (1 mol/L) was added dropwise in the process to maintain the pH values at 8-9 and the reaction temperature was simultaneously kept at 15-20 °C. The pH value of the mixture was adjusted to 2-3 by diluted hydrochloric acid after reaction for 3 h. Abundant yellow flocculent precipitation was separated out and then crude samples of *N*-dicyclopentadienyl breastplates acyl-L-alanine were prepared by suction filtration. Finally, the yellow powdered crystals were obtained after multiple washing by distilled water, recrystallization by 50 % ethanol, filtration and vacuum drying and the yield was 88.2 %. (R_f = 0.6, ethyl acetate: methyl alcohol = 6:1); m.p. 220-221 ºC; specific rotation $[\alpha]_{(l)}^{22} = -33.2°$ (c = 0.42, CH_3OH). ^{1}H NMR (CD_3CO CD_3, 500 MHz), δ: 10.56 (d, H, COOH); 7.11 (d, H, NH); 3.12 (m, H, CH); 4.69 (s, 2H, C_5H_4CO *ortho*-position); 4.32(s, 2H, C_5H_4CO *meta*-position); 4.21(s, 5H, C_5H_5); 1.50 (d, 3H, CH_3).

2 g of *N*-dicyclopentadienyl breastplates acyl-L-alanine and 60 mL dry tetrahydrofuran (THF) were introduced into a 100 mL three-necked bottle and mixed thoroughly. The solution was firstly cooled to 0 °C by ice-bath and then 1 g HOBt and 1.9 g DIEA were added. 1.5 g EDCI was subsequently added into the mixture in batches after reaction for 1 h and 3 g 3-ammonium propyl silica gel (5 μm) was finally added. The reaction was carried out at room temperature for 48 h. After suction filtration and multiple washing by THF, methanol, distilled water and acetone in turn, 3.55 g products were finally obtained with the yield of 74.5 %.

3 g of 3-Ammonium propyl silica gel was weighed accurately and the end-product was 3.55 g. The chiral selector bonded to each gram of stationary phase was calculated to be 0.76 mmol according to the results of elemental analysis, which were 7.17 % of C, 4.25 % of H and 1.20 % of N. The coverage density (α_{exp}) of chiral selector bonded to the silicone surface could be calculated as follows:

$$\alpha_{exp} = \frac{\Delta C}{100 \times M_c \times N_c \times S_p} \times 10^6 \ (\mu mol\ m^{-2})$$

where ΔC is the difference of carbon content before bonding and after bonding; M_c is the molecular weight of C element; N_c is the number of carbon atom contained in chiral selector. S_p is the superficial area of carrier matrix.

The bonding density of chiral selector bonded on the surface of 3-ammonium propyl silica gel was calculated to be 2.52 μ mol/m^2.

RESULTS AND DISCUSSION

The enantiomeric separation of D,L-phenylalanine, D,L-tryptophan, D,L-thioctic acid and clenbuterol enantiomers on ferrocene-bonded alanine chiral stationary phase was studied and the chiral resolution performance of chiral stationary phases was also investigated.

The capacity factors and a values for D,L-phenylalanine, D,L-tryptophan, D,L-thioctic acid and clenbuterol enantiomers obtained on ferrocene-bonded alanine chiral stationary phase are given in Table-1. As shown in Table-1, the D,L-phenylalanine and D,L-thioctic acid have maximal separation factor and better enantiomeric separation effect, when the composition of mobile phase is methanol/water = 80/20 (v/v); D,L-tryptophan and clenbuterol enantiomers have maximal separation factor and better enantiomeric separation effect, when the composition of mobile phase is methanol/water = 90/10 (v/v).

As shown in Table-1, the separation factor of the D, L-clenbuterol are showed that separation factor is maximal.

In addition, the influence of methanol on t_R and α value is greater than water. The polarity of methanol is stronger than water. Meanwhile, methanol is a kind of strong proton receptor and could form strong hydrogen bonding interaction with the stationary phase. This could weaken the interaction between the solute and the stationary phase. Hence, the impact of methanol on the retention time is more obvious.

As shown in Fig. 2, the D-phenylalanine (peak 1) was well separated from the L-phenylalanine (peak 2). The D-phenylalanine was eluted earlier than L-phenylalanine because of its stronger π-π interaction with chiral stationary phase.

As shown in Fig. 3, the D-tryptophan (peak 1) was well separated from the L-tryptophan (peak 2). The elution time of D-tryptophan is 12.88 min and the width of peak base is 0.20 min; the elution time of L-tryptophan is 13.15 min and the width of peak base is 0.20 min; α value of 1.35 is in 1-1.5. This indicates that the effect of resolution is well.

It could be seen from Fig. 4 that the peaks of clenbuterol enantiomers were eluted at 16.75 and 17.14 min, respectively, when the composition of mobile phase was methanol/water (v/v) = 90/10. The baseline separation was achieved. So, the chiral stationary phase can be used for the analysis and separation of enantiomeric drugs.

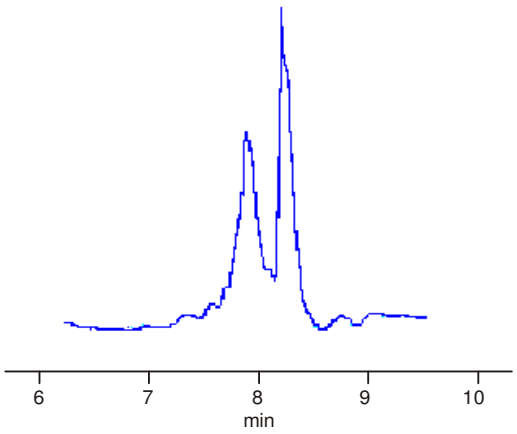

Fig. 2. Chromatogram of D,L-phenylalanine. Mobile phase, 80/20 (v/v); temperature, 25 ºC; flow rate, 1 mL min^{-1}; wavelength, 254 nm

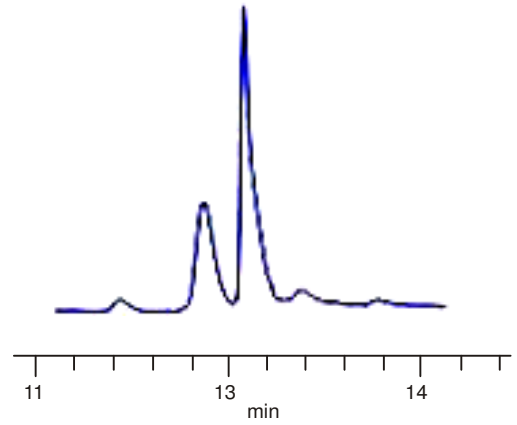

Fig. 3. Chromatogram of tryptophan enantiomers. Mobile phase, 90/10 (v/v); temperature, 25 ºC; flow rate, 1 mL min^{-1}; wavelength, 254 nm

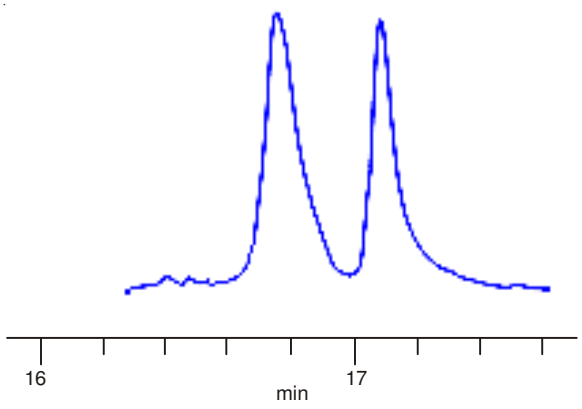

Fig. 4. Chromatogram of clenbuterol enantiomers. Mobile phase, 80/20 (v/v); temperature, 25 ºC; flow rate, 1 mL min^{-1}; wavelength, 254 nm

TABLE-1
CAPACITY FACTORS (k′) AND α VALUES FOR D,L-PHENYLALANINE, D,L-TRYPTOPHAN, D,L-THIOCTIC ACID AND CLENBUTEROL ENANTIOMERS. TEMPERATURE, 25 ºC; FLOW RATE, 1 mL MIN^{-1}; WAVE LENGTH, 254 nm

Mobile phase methanol/water (v/v)	Phenylalanine enantiomers			Tryptophan enantiomers			Lipoic acid enantiomers			Clenbuterol enantiomers		
	k_1'	k_2'	α	k_1'	k_2'	α	k_1'	k_2'	α	k_1'	k_2'	α
100				0.397	0.589	1.519				0.591	0.793	1.512
90/10	0.507	0.691	1.413	0.381	0.579	1.527	0.497	0.692	1.324	0.578	0.787	1.539
80/20	0.486	0.673	1.438	0.376	0.558	1.512	0.483	0.689	1.339	0.569	0.761	1.524
70/30	0.476	0.659	1.422				0.468	0.667	1.328			

As shown in Fig. 5, R-lipoic acid (peak 1) was well separated from the R-lipoic acid (peak 2). When the composition of mobile phase is methanol/water (v/v) = 90/10, the elution time of R-lipoic acid is 11.28 min, the width of peak base is 0.25 min; the elution time of S-lipoic acid is 11.43 min, the width of peak base is 0.36 min. The maximum of a value of 1.35 is in 1-1.5. It indicates that the effect of resolution is well.

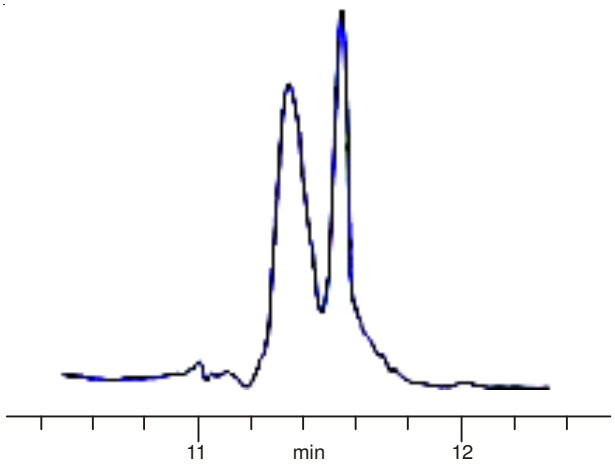

Fig. 5. Chromatogram of lipoic acid enantiomers. Mobile phase, 90/10 (v/v); temperature, 25 ºC; flow rate, 1 mL min^{-1}; wavelength, 254 nm

There existed groups which can form three-point interaction between the four molecules and the chiral stationary phase molecule. The interactions include π-π interaction, hydrogen bonding interaction, hydrophobic interaction and steric-hinerance effect. The space configuration differences of the tryptophan enantiomers could make the interaction strength different and further led to the increasement of interaction distinction between the two enantiomers and chiral stationary phase molecules. Hence, chiral resolution of the four molecules could be realized.

Conclusion

A ferrocene-bonded chiral stationary phase was prepared by covalent bonding of ferrocene-derivatized alanine to aminopropylated spherical silica gel. The new stationary phase showed good separation ability to phenylalanine, tryptophane, clenbuterol hydrochloride and thioctic acid under reversed-phase conditions within acceptable analysis times. The mechanism of retention on the new stationary phase involves strong π-π interaction with each one of the cyclopentadienyl (Cp) groups of the ferrocene and simultaneously hydrogen bonding interactions between the chiral stationary phase and the analytes. The phase enables effective separation of optical isomers with a benzene ring broadens the chromatographic applicability of the classic Pirkle **CSp**.

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