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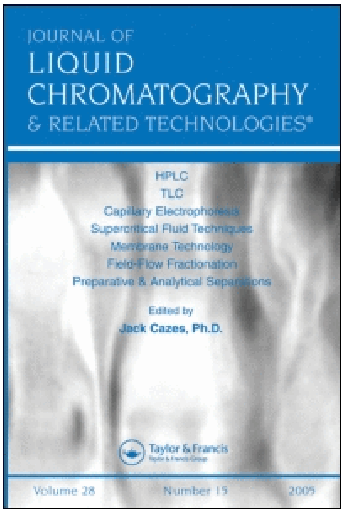

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Facile Enantiomer Analysis by Combination of *N*-Dansyl Amino Acid as Diastereomerizer and Molecular-Shape Recognitive RP-HPLC Using Comb-Shaped Polymer-Immobilized Silica

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ABSTRACT

Poly(octadecyl acrylate)-grafted silica has been developed as a special stationary phase showing molecular-shape recognition in high-performance liquid chromatography. The bonded polymer phase is characterized by the fact that the polymer forms highly-ordered structures similar to a solvated

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crystalline state on silica and multiple $\pi-\pi$ interaction between carbonyl groups in the polymer and aromatic solutes is promoted. This paper shows that the selectivity for DL-amino acids diastereomerized with *N*-dansyl L-proline can be enhanced by using the polymer stationary phase instead of octadecylated silica (ODS).

Key Words: Liquid chromatography; Diastereomer; Chiral discrimination; Dansyl amino acid; Proline; $\pi-\pi$ interaction; Polymeric stationary phase; Molecular shape selectivity.

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INTRODUCTION

Diastereomerization of enantiomeric isomers is useful for chiral discrimination. When this method is combined with reversed-phase liquid chromatography (RPLC), it yields very convenient and quick analysis of enantiomer mixtures, although not suitable for fractionating. Many diastereomerizing reagents for RPLC have been developed: e.g., *o*-phthaladehyde-*N*-acetyl-L-cystein,[1] (+)-1-(9-fluorenyl)ethyl chloroformate,[2] (a2)-2′-methoxy-1,1′-binaphthyl-2-carboxylate,[3] (*S*)-(−)(2,3-naphthalene dicarboximidyl)-propionyl fluoride,[4–6] and chiral monohalo-*s*-triazines,[7] 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole,[8,9] (*1R,2R*)-*N*-[(2-*iso*-thiocyanato)cyclohexyl]-6-methoxy-4-quinolinylamide),[10] and *O*-(4-nitro-benzyl)-tyrosine methyl ester[11] for racemic amines/or amino acids. These diastereomerizing reagents can be characterized by the facts that chromophoric groups are included for sensitive detection, and chiral separation is realized by discriminating the hydrophobicity (or polarity) difference between the resulting diastereomers. Therefore, simply-hydrophobized silica, such as octadecylated silica (ODS), has been mainly used for organic stationary phase in RPLC. However, it is fairly certain that ODS does not show high selectivity in this method because the hydrophobicity difference of resultant diastereomers is rather small. One of the solutions of this problem would be to use an alternate stationary phase with subsidiary recognition mode.

For this purpose, we have developed new packing materials, comb-shaped polymer grafted silica, for RPLC, which are based on the fact that the organic phase can form highly-ordered structures similar to a crystalline state on silica (Fig. 1), and the carbonyl groups can enhance the selectivity through $\pi-\pi$ interaction with aromatic solutes.[12–17] Therefore, we assumed that our new RPLC column would be useful for diastereomer separation because almost all diastereomerizing reagents are π-electron-rich compounds, which can be used for multiple $\pi-\pi$ interaction sources. Using these features, we have reported that the combination of (*S*)-(−)(2,3-naphthalenedicarboximidyl)propionyl fluoride[5,6] or 4-nitro-7-(3-amino-pyrrolidin-1-yl)-2,1,3-benzoxadiazole[9] as

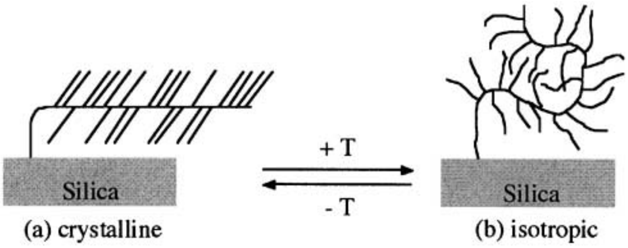

Figure 1. Proposed physical states of immobilized organic phases on silica estimated by DSC and x-ray analyses.

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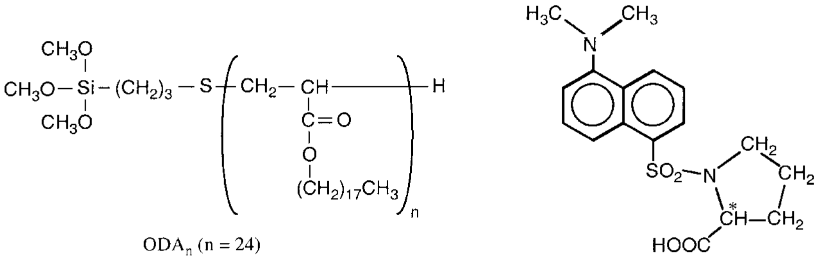
commercially available diastereomerizing reagents with our column, showed better selectivity in racemic substances than ODS.

In this communication, we report that *N*-dansyl-L-proline is more facile for enantiomer discrimination of α-amino acids by the combination with the comb-shaped polymer grafted silica, Sil-ODA$_n$ (Sch. 1).

EXPERIMENTAL

Poly(octadecyl acrylate) with terminal reactive groups, ODA$_n$, was prepared according to the telomerization method reported previously.[13,14] The average degree of polymerization (n) of the polymer was determined by ^{1}H-NMR spectroscopy to be 24. ODA$_{24}$ was readily grafted onto porous silica by mixing in tetrachloromethane at reflux temperature. Fuji Silysia Super Micro Bead silica gel (100A-5D, diameter 5 µm, pore size 146 Å, specific surface area 330 cm^2 g^{-1}) was used as porous silica. The amount of ODA$_{24}$ immobilized was determined by elemental analysis to be 20.1 wt%. Simply-



Scheme 1.

ODS (C 13.7%) was prepared from Fuji Silysia Super Micro Bead silica gel 100A-5D with octadecyltrimethoxysilane and trimethylchlorosilane by the ordinary procedure.[14] The silica-supported poly(octadecyl acrylate) (Sil-ODA_{24}) and ODS were packed into stainless-steel columns (4.6 mm I.D. × 250 mm).

N-Dansyl derivatives of L-proline as diastereomerizing reagents were purchased from Sigma-Aldrich Co., St. Louis, MO Methyl esters of L-alanine, L-valine, L-leucine, L-phenylalanine, and their DL-derivatives were used for target samples. They were purchased from Kokusan Chemical Co. Ltd. or Nacalai Tesque Inc. and used without further purification. Diethyl phosphorocyanidate (DEPC) was purchased from Wako Pure Chemical Inc., Japan, and used as a coupling reagent between a dansyl derivative and a methyl ester of amino acid.

A typical sample preparation for HPLC was carried out as follows: 5.0 mg (0.014 mmol) of *N*-dansyl L-proline and 15.5 mg (0.072 mmol) of DL-phenylalanine methyl ester were dissolved to 5 mL of dimethylformamide at room temperature. Triethylamine (20 μL) and 9 μL of DEPC were added to the solution and then stirred for 15 min at room temperature. This solution was used as an assay sample for HPLC without any treatment.

The chromatograph included a JASCO 980 PU pump and a Jasco MD-910 UV-visible photodiode array detector. Five μL of the sample was injected through a Reodyne Model 7125 injector. Liquid chromatography was carried out with a methanol–water mixture as a mobile phase at flow-rate $1.0\,\mathrm{mL\,min^{-1}}$. The retention factor ($k'$) was determined by $(t_e - t_o)/t_o$, where t_e and t_o are retention times of samples and methanol, respectively. The separation factor (α) was given by the ratio of retention factors.

Thermograms by differential scanning calorimetry (DSC) of ODA_{24} and their silica-supported derivatives were obtained with a heating rate of $1^\circ\mathrm{C\,min^{-1}}$ using Seiko I & E SSC-580 with a DSC-10 instrument. X-ray diffraction of ODA_{24} and this silica-supported derivative were obtained by the reflection method (2θ–θ scan) and by the transmission method (edge view) with a Rigaku Denki RAD-IB x-ray diffractometer.

RESULTS AND DISCUSSION

Physical State of ODA_{24} as Bonded Organic Phase

The physical states of ODA_{24} should be investigated before applying for chromatography because the comb-shaped polymer often possesses several physical states including a mesophase. DSC thermograms showed that ODA_{24} had a sharp endothermic peak around 49°C (T_c, peak-top temperature) with a

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shoulder around 42°C. By a polarization microscopic observation of ODA_{24}, these transitions were characterized to be liquid crystalline-to-isotropic and crystalline-to-liquid crystalline phase transitions, respectively. This estimation was supported by the fact that the x-ray analysis at room temperature (25°C) provided a sharp diffraction with 4.15 Å at $2\theta = 21.4°$. This indicates that ODA_{24} forms an ordered structure at the side long-chain alkyl groups. Similar phase transition behavior was observed after grafting onto porous silica. The peak-top temperature (T_c) of silica-supported ODA_{24} (Sil-ODA_{24}) was at 48°C, while a mesophase was not clearly detected. The x-ray analysis at room temperature provided a diffraction with 4.16 Å at $2\theta = 21.3°$. These values are very close to that in original ODA_{24}. This indicates that ODA_{24} undergoes a crystalline-to-isotropic phase transition on silica and is in a crystalline state at room temperature (Fig. 1).

On the other hand, when Sil-ODA_{24} was placed in methanol–water (60 : 40), which is corresponding to the mobile phase in this study, the peak-top temperature decreased from 48°C to 33°C with peak-broadening. This is attributable to a solvation to the bonded phase, but indicates that ODA_{24} can maintain a crystalline state at room temperature even in a chromatographic condition. According to these observations, chromatography was mainly carried out at -10°C, at which the organic phase was in a crystalline phase.

Time Course of Diastereomerization

Dicyclohexylcarbodiimide (DCC), or some other carbodiimides, has been widely used as condensing reagents for peptide synthesis. However, it is known that these reagents are often accompanied by undesirable racemization for *N*-acyl amino acids. We also observed, that the resultant diastereomer was accompanied by the corresponding D-phenylalanine derivative when the coupling reaction was carried out by *N*-dansyl L-proline and L-phenylalanine methyl ester with DCC at room temperature. The racemization was almost over 90%. To avoid this racemization, it is usual to use an additive such as *N*-hydroxysuccinimide, but this leads the resultant composition to be too complicated to analyze. In view of this, we adopted DEPC[18,19] for condensation of amino acid methyl esters with *N*-dansyl L-amino acids, because DEPC has not only been known as a condensing reagent with few racemizations, but also has almost no absorption at higher wavelength than 250 nm.

Figure 2 shows an example of the time course of chromatograms for methyl ester of DL-phenylalanine diastereomerized by *N*-dansyl-L-proline with the Sil-ODA_{24} column. The chromatogram, at a minute after addition of DEPC to the mixture of DL-phenylalanine and *N*-dansyl-L-proline, provided two new peaks with λ_{max} of 350 nm at 6.8 min and 8.5 min; those were attributable to the absorption based on a dansyl group. It was also confirmed that the

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first peak at 6.8 min was the same retention time as that obtained by the diastereomer of L-phenylalanine methyl ester with N-dansyl-L-proline. Therefore, it is evident that DEPC promotes the coupling of N-dansyl-L-proline with DL-phenylalanine, and that Sil-ODA$_{24}$ discriminates the chirality difference between the resultant diastereomers. Also confirmed was that the coupling reaction was almost completed in 10 min, and DEPC did not include racemization during the coupling reaction for L-phenylalanine methyl ester.

Application to Various Amino Acids

We focus on a proline derivative as a diastereomerizer compared with the other amino acids such as alanine and leucine, because its unique chemical structure is rigid through a closed cyclic structure, and thus, L-proline derivatives have been adopted as chiral ligands for enantioselective HPLC.[20–23] In

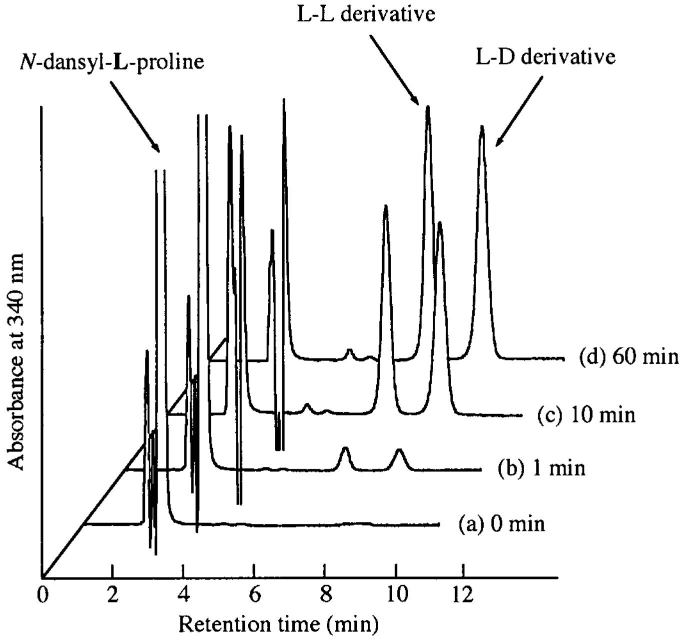

Figure 2. Time course of diastereomerization of DL-phenylalanine with N-dansyl-L-proline using DEPC. The chromatograms (a), (b), (c), and (d) were obtained with a Sil-ODA$_{24}$ column for the resultant samples after 0, 1, 10, and 60 min, respectively. Mobile phase: MeOH–H_2O (70 : 30), 25°C. Flow rate: 1.0 mL min^{-1}.

addition, *N*-dansyl amino acids have been often investigated as target samples[24,25] because *N*-dansyl groups work not only as a sensitive fluorescent group, but the rigid planar structure of the naphthyl group increases the selectivity. Probably, its π-electron-rich structure would be useful for effective $\pi-\pi$ interaction with our polymeric stationary phase. As a result, we confirmed that *N*-dansyl-L-proline provided better selectivity than *N*-dansyl derivatives from L-alanine, L-valine, L-leucine, and L-phenylalnine. The detailed data will be reported elsewhere. Therefore, we focus on the applicability of *N*-dansyl-L-proline as a diastereomerizing reagent towards methyl esters of the other amino acids.

Figure 3 shows a simple comparison of the separation factors between Sil-ODA$_{24}$ and ODS towards the diastereomerized DL-phenylalanine methyl esters with *N*-dansyl-L-proline. In Sil-ODA$_{24}$, the L–L diastereomer is shorter and the L–D diastereomer is longer in their retention times than in ODS and thus, the separation factor is much higher in Sil-ODA$_{24}$ than in ODS.

Such better results were obtained against the other methyl esters of DL-amino acids, as summarized in Table 1, while the separation factor depended on the chemical structure of the residual group: Val > Leu, Phe > Ala.

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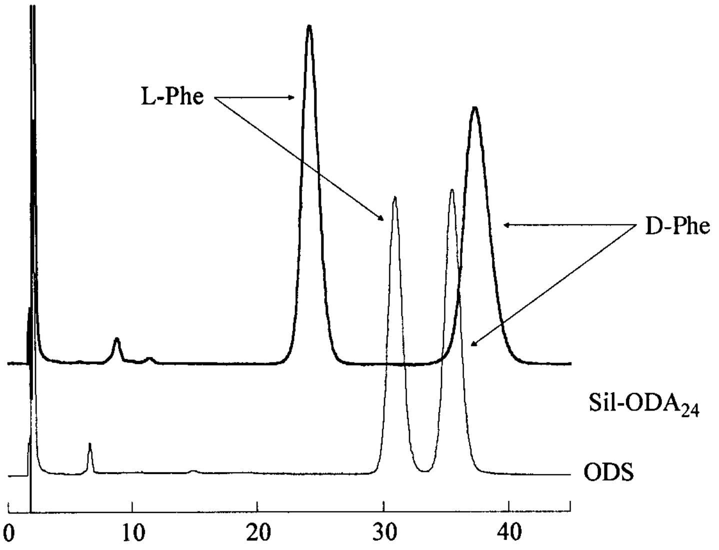

Figure 3. Chromatograms of diastereomerized DL-phenylalanine methyl ester with *N*-dansyl-L-proline. Mobile phase: MeOH/H$_2$O = 60/40, −10°C. Detection wavelength: 340 nm.

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To discuss the better selectivity with Sil-ODA$_{24}$ compared with ODS, the temperature dependency was examined, because the ODA$_{24}$ phase underwent a phase transition between crystalline and isotropic states.

Figure 4 indicates that the selectivity is remarkably temperature-dependent, especially in Sil-ODA$_{24}$. Similar selectivity increases with decreasing temperature was observed in DL-alanine, valine, and leucine methyl esters. In our recent study, we have reported that ODA$_n$, which is in a crystalline state, shows distinct molecular shape recognition against polyaromatic hydrocarbons,[13–15] and this ability is through a carbonyl-π interaction.[6,14] As shown in Fig. 5, molecular shape recognition by Sil-ODA$_{24}$ can be distinctly realized in the separation among the mixture of triphenylene and *o*-terphenyl.[13] The retention factor is not only much larger for triphenylene as a planar compound than *o*-terphenyl as a bending compound, but the separation factor also increases remarkably at temperature below T_c of the immobilized polymer phase: $\alpha = 4.6$ and 2.4 at 15°C and 60°C, respectively, in Sil-ODA$_{24}$, while ODS provides almost constant and smaller α value ($\alpha = 1.4–1.3$) independent of temperature. Similarity in their patterns between Figs. 4 and 5 indicates that Sil-ODA$_{24}$ is sensitive to molecular shape even for diastereomers, especially at crystalline temperatures. However, it was observed that addition of 20 v/v% acetone as an inhibitor[6] against π–π interaction[6,26] to the mobile phase, reduced the selectivity by ca. 20%, but 2-propanol did not show any decrease. Similar decreases of the selectivity have been reported for polyaromatic hydrocarbons.[13–15] Therefore, it is estimated that π–π interaction will be included in this selectivity.

In conclusion, we have reported a facile method for enantiomer analysis with RP-HPLC. Good results should be derived from the facts that (1) DEPC as a condensing reagent was chosen, (2) a highly-ordered organic phase was used as an immobilized phase, and (3) a dansyl group in a diastereomerizer

Table 1. Comparison of the separation factor (α) of methyl esters of DL-amino acids after diastereomerized with *N*-dansyl-L-proline.

	Separation factor (α)	
Amino acid	With Sil-ODA$_{24}$	With ODS
DL-Alanine	1.31	1.20
DL-Valine	1.57	1.33
DL-Leucine	1.46	1.28
DL-Phenylalanine	1.45	1.16

Note: Mobile phase: MeOH : Water = 60 : 40 at −10°C.

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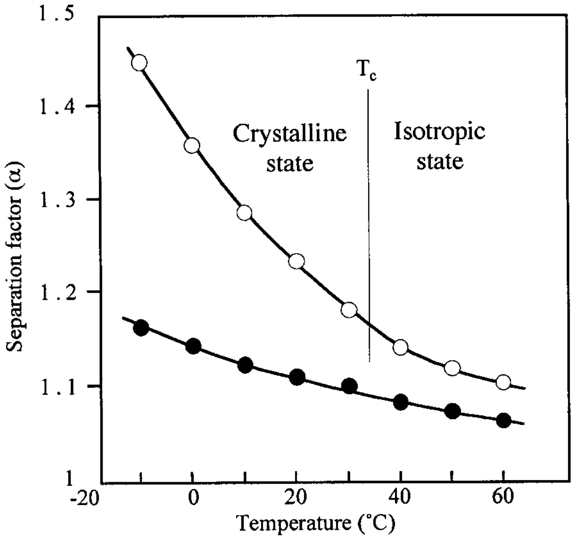

Figure 4. Temperature dependencies of separation factor for *N*-dansyl-L-Pro-DL-Phe-OMe with Sil-ODA$_{24}$ (○) and ODS (●). Mobile phase: MeOH/H_2O = 60/40.

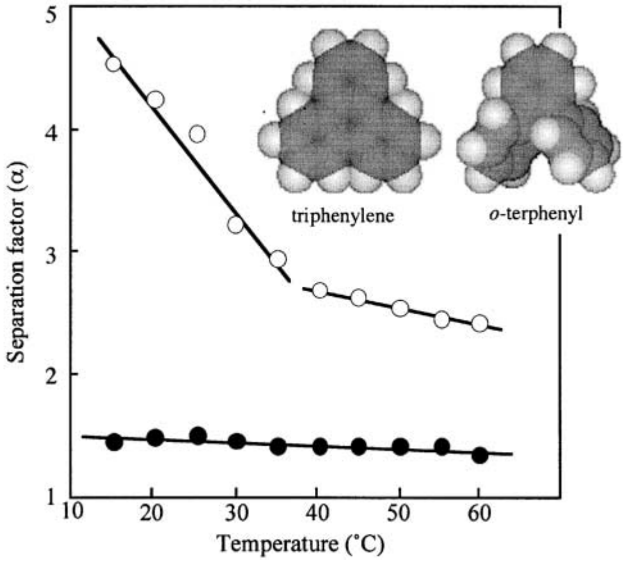

Figure 5. Temperature dependencies of separation factor for triphenylene and *o*-terphenyl with Sil-ODA$_{24}$ (○) and ODS (●). Mobile phase: MeOH/H_2O = 9/1.

works not only as a chromophoric group for sensitive detection, but also as a planar and π-electron-rich group.

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