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Chemically modified chiral monolithic silica column prepared by a sol–gel process for enantiomeric separation by micro high-performance liquid chromatography

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Abstract

In this work a new type of chiral monolith silica column was developed for the chiral separation by micro high-performance liquid chromatography (μ-HPLC). The chiral monolith column with a continuous skeleton and a large through-pore structure was prepared inside a capillary of 100 μm I.D. by a sol–gel process, and chemically modified with chiral selectors, such as L-phenylalaninamide, L-alaninamide and L-prolinamide, on the surface of the monolithic silica column. Based on the principle of ligand exchange, these chiral monolithic columns were successfully used for the separation of dansyl amino acid enantiomers, as well as hydroxy acid enantiomers by μ-HPLC. The chromatographic conditions, the enantioselectivity and the performance of columns are discussed.

Keywords: Monolithic columns; Chiral stationary phases, LC; Enantiomer separation; Amino acids, DNS derivatives; Hydroxy acids

1. Introduction

The development of monolithic columns for capillary electrochromatography (CEC) and μ-HPLC has received considerable attention in the recent years, because monolithic columns have many advantages over conventional packed and open-tubular capillary columns. For example, monolithic columns could eliminate the tasks of particle synthesis and the difficulty of packing columns with discrete particles, especially the need for end frits to maintain the stationary phase. Depending on the monolithic material, the monolithic columns can be classified into two categories: (i) organic polymer-based [1–3] and (ii) bonded silica-based monolithic columns [4–8]. In the first category, the fabrication of monolithic columns is accomplished through a single-step polymerization reaction of an organic monomeric precursor. One drawback associated with this type of monolithic capillary is its tendency to swell/shrink during exposure to various solvents in the running mobile phases. In the second type, the monolithic column is bonded to a silica stationary phase through the use of the sol–gel process by entrapping particles in inorganic gels [4,5] or sintering silica beds [9], as well as preparing by on-capillary sol–gel reactions

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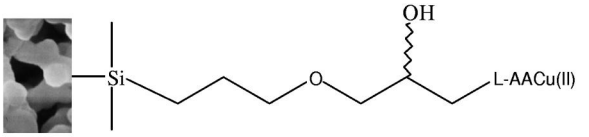
[6–8]. These papers have demonstrated the successful use of monolithic columns in the area of achiral separation. However, up to now, few works have focused on the development of chiral monolithic columns, although the research work has expanded in recent years.

Chiral separations have become an important field in separation sciences, since many drugs, agrochemicals, food additives and fragrances are chiral compounds, and their bioactivities are related to their chirality. Many discrimination principles have been used in the chiral recognitions. Chiral ligand exchange, one of chiral separation principles, has widely used in liquid chromatography (LC) [10–12], capillary electrophoresis (CE) [13,14], micellar electrokinetic chromatography (MEKC) [15–21] and recently CEC [22–24]. Over the past 30 years, a number of ligand exchange-chiral stationary phases (LE-CSP) with high enantioselectivity has been prepared by covalently bonding a chiral selector on the silica gel [10,25] and packed for enantioseparation in conventional stainless columns. Nowadays, miniaturization has become one of main objectives in analytical chemistry. In chromatographic sciences, capillary and chip based separations are receiving great attention. Therefore, it is worthwhile to study the capillary column technology for use with LE-CSPs with a high enantiselectivity in CEC and μ-HPLC. If the LE-CSPs were packed into a capillary, one would experience difficulty in packing the column, especially the frit fabrication in the CEC. Moreover, the packed capillary column usually suffers from high backpressure. In some cases, the separation cannot be carried out because of the extremely high backpressure. Therefore, monolith column technology would be a very promising approach for μ-HPLC and CEC.

This work aimed to develop chiral monolith column technology by a sol–gel process and chemical modifications for μ-HPLC. The preparation procedures consist of two steps: preparation of a monolithic silica column bed by sol–gel process and chemical modification of the silanol groups with the chiral selectors. Galli et al. reported [11] the enantioseparation of dansyl and dabsyl amino acids by (*S*)- or (*R*)-phenylalaninamide-modified 5-μm silica gel packed inside stainless-steel columns (150×4.0 mm I.D.). In this work three types of monolithic LE-CSPs, as shown in Fig. 1, were prepared by sol–gel process and chemical modifications. Our previous works [22,23] reported their applications in CEC. The present work focuses on their applications in μ-HPLC.



AA:amino acylamide
LE-CSP1: L-phenylalaninamide
LE-CSP2: L-alaninamide
LE-CSP3: L-prolinamide

Fig. 1. Chemical structures of chemically modified LE-CSPs.

2. Experimental

2.1. Chemicals

Tetramethoxysilane (TMOS) and 3-glycidoxypropyltrimethoxysilane were obtained from Shin-Etsu (Tokyo, Japan). Poly(ethyleneglycol) (PEG) (M_r 10 000), L-phenylalaninamide, L-alaninamide, L-prolinamide, dansyl amino acids and hydroxy acids were obtained from Sigma (St. Louis, MO, USA). Dehydrated toluene, dehydrated *N,N*-dimethylformamide (DMF), acetonitrile for HPLC, copper(II) acetate monohydrate and ammonium acetate were from Kanto (Tokyo, Japan). Fused-silica capillary (0.375 mm O.D., 0.10 mm I.D.) was from GL Sciences (Tokyo, Japan).

2.2. Instrumental

μ-HPLC instrumental system was set up by a LC-10ADvp pump (Shimadzu, Tokyo, Japan), a CTO-10ACvp column oven (Shimadzu), an integrator (Chromatopac C-R7A plus, Shimadzu) an Rheodyne 7520 injector with a 0.2-μl sample rotor (Supelco, Bellefonta, USA) and a CE-1570 intelligent UV–vis detector (Jasco, Tokyo, Japan). Scanning micrography was carried out on JSM-6100 scanning electron microscope (Jeol, Tokyo, Japan). Heating with a programmed temperature was per-

formed within a GC-17A oven (Shimadzu, Tokyo, Japan).

2.3. Preparation of ligand exchange monolithic columns

The monolithic LE-CSPs were prepared by sol–gel process and chemical modifications as in previous works [22,23]. Briefly, a monolithic silica network matrix was prepared inside capillary by sol–gel. It includes (i) the hydrolysis of TMOS, (ii) the condensation of the hydrated silica tetrahedra forming ≡Si–O–Si≡ bonds and (iii) polycondensation of linkage of additional ≡Si–OH tetrahedra eventually resulting in a SiO_2 skeleton having silanol groups on the surface.

The silanol groups on the skeleton surface of monolithic silica matrix were targeted for chemical modification. The chemical modifications include two steps: (i) the modification of a spacer, (3-glycidoxypropyl)trimethoxysilane and (ii) the modification of chiral selectors, such as, L-phenylalaninamide, L-alaninamide, L-prolinamide. After conditioning with $CuSO_4$ aqueous solution, the Cu(II) was grafted on the surface of LE-CSPs. The detection widow (5 mm) was made at the right after the CSP bed by burning out the polyimide coating layer of capillary.

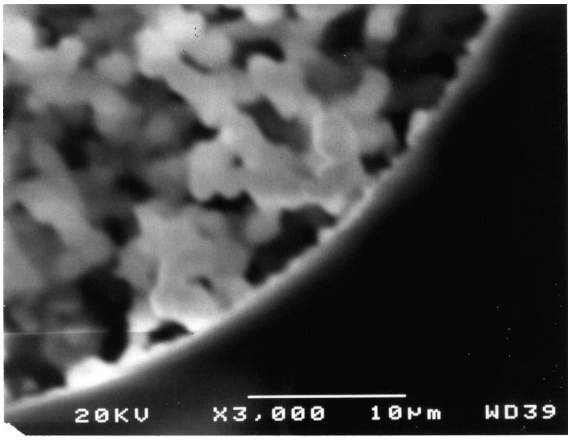


Fig. 2. SEM photograph of chemically modified monolithic column.

2.4. Chromatographic conditions

Chiral monolithic columns: chemically modified LE-CSPs. Mobile phase: mixture of acetonitrile and buffer containing 10–100 m*M* NH_4Ac and 0.10–0.50 m*M* $Cu(Ac)_2$, adjusted to the desired pH values. The mobile phases were degassed by vacuum and ultrasonication. Flow-rate: 1–20 μl/min. Column temperature was kept at 25°C, unless otherwise stated. UV detection wavelength: 254 nm. Injection volume: 0.2 μl. Sample solutions were dissolved in the mobile phase in the concentration range of 5.0–$10 \cdot 10^{-4}$ *M*.

3. Results and discussion

3.1. Morphology, permeability and the mechanical strength of the chiral monolithic columns

The SEM photographs in Fig. 2 show that the morphology of monolithic column has a continuous skeleton and large through-pore matrix. It is clearly seen that the silanol groups at the inner surface of capillary have taken part in the sol–gel reactions so that monolithic bed was bonded to capillary wall. The permeability of the column was examined by measuring the backpressure. When a mobile phase of acetonitrile–0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ (7:3, v/v) (pH 7.6) was used at the flow-rate of 5 μl/min, the backpressure with L-phenylalaninamide-modified column (42 cm total length, 32 cm CSP×100 μm I.D.) was 1470 kPa. This shows that the monolithic column has a good permeability. After frequent use during the chromatographic evaluation and sample separations for several months, no obvious decline of column efficiency was observed. This suggests that the chemically bonded CSPs were very stable under exposure to the mobile phases, and that the columns have a good mechanical strength and lifetime.

3.2. Effect of pH on the separation

pH plays an important role in chromatographic separations. The interaction between the stationary phase and the mobile phase can be controlled by pH, because the pH affects the protonation and deprotonation of the analytes and Cu(II) complexes used as

the chiral selectors. Armani et al. reported [26] the species distribution for the L-phenylalaninamide–Cu(II) system as a function of pH. In the pH range 5–10, the species of Cu(II) complexes, $[CuL]^{2+}$, $[CuL_2]^{2+}$, $[CuL_2H_{-1}]^{+}$ and $[CuL_2H_{-2}]$ are present and responsible for the enantioseparation. The species distribution changes with the change of pH. The pH effect on resolution was examined with Dns–D,L-Met and Dns–D,L-Thr as test samples in the pH range 5.5–8.5. As shown in Fig. 3, chiral resolutions were achieved throughout this pH range. The resolution (R_s) decreased with the increase of pH in the range of pH 6.5–8.5, and the highest R_s values were obtained at about pH 6.5. This results could probably be explained as follows. Judging from the isoelectric points (p*I*) of Met (5.7) and Thr (5.6), the analytes have negative charges under the experimental conditions. When the pH of mobile phase is about 6.5, however, the Cu(II) complex in CSP is mostly present in the species positively charged, which preferentially interacts with the negatively charged analytes. With the increase of pH, the ratio of positively charged species decreases, the interaction between the analytes and CSP become weak, and consequently the separation factors decrease.

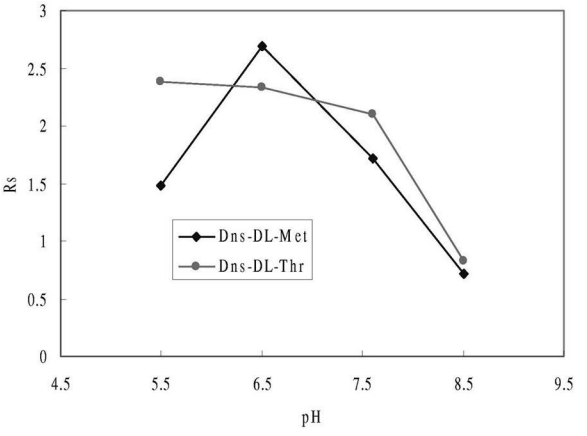


Fig. 3. Effect of pH on the resolution of Dns–D,L-Thr and Dns–D,L-Met. Mobile phase: acetonitrile–50 m*M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ (7:3) at different pH values; column: L-phenylalaninamide-modified monolithic column, 32 cm LE-CSP; flow-rate: 5 μl/min; column temperature: 25°C.

3.3. Effect of the amount of acetonitrile in the mobile phases on the separation

The effects of the acetonitrile content in the mobile phase on the separation factor (α) and migration time of D-enantiomer of Dns–D,L-Thr were investigated in the range of 50–80% (v/v). The results are shown in Fig. 4. The elution time becomes faster with the increase of acetonitrile content. The separation factor (α) increases with the increase of acetonitrile content at the concentration range of 50–70%, but very slightly decreases with the further increase of the acetonitrile content up to 80%. With the increase of acetonitrile content, the nonpolarity of the mobile phases increases. On account of retention and elution, the effect of acetonitrile content in mobile phase would be probably related to be the change of nonpolarity of the mobile phases.

3.4. Effect of buffer compositions on separation

The compositions of buffer, the concentrations of Cu(II) and NH_4Ac, were investigated by using Dns–D,L-Leu as a test sample. The NH_4Ac concentration did not significantly affect the separation of Dns–D,L-Leu at the concentration range of 20–100 m*M*. The resolutions (R_s) were calculated to be 1.30, 1.32 and

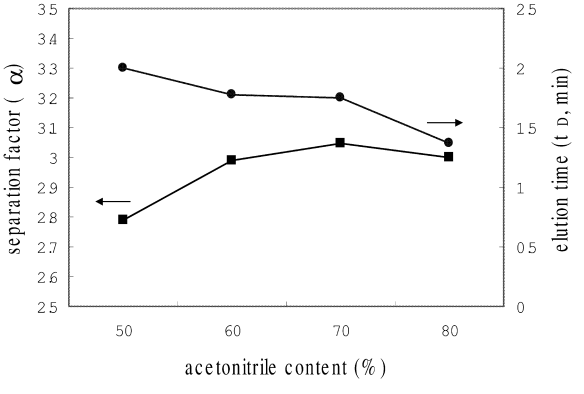


Fig. 4. Effect of acetonitrile content in the mobile phase on the separation of Dns–D,L-Thr. Mobile phase: pH 5.5, acetonitrile–50 m*M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ at different ratios. Other conditions as in Fig. 3.

1.30, respectively, when 20, 50 and 100 m*M* NH_4Ac were used in the mobile phases. The effect of Cu(II) concentrations in the range of 0.1–0.5 m*M* was very moderate. When the concentration of Cu(II) was 0.10 m*M*, the R_s value of Dns–D,L-Leu was 0.93. When Cu(II) concentration in the mobile phase was increased to 0.25 m*M*, the R_s increased to 1.32; whereas the R_s slightly decreased to 1.12, with a further increase in the Cu(II) concentration to 0.50 m*M*. This suggests that 0.25 m*M* Cu(II) is best concentration for the formation of Cu(II) complexes in CSP, which offers the ability for chiral discrimination. Cu(II) concentration higher than 0.25 m*M* may be excessive, causing the formation of diastereomeric Cu(II) complexes with analytes, which weaken the ligand exchange interaction between analytes and CSP. As a result, the R_s value decreases.

3.5. Effect of temperature on the separation

In order to investigate the interactions between the LE-CSPs and the analytes, the effect of temperature on enantioselectivity factors (α) was examined in the temperature range of 25–45°C. The effect of temperature on α is described by the following equation:

$$\ln \alpha = -\frac{\Delta\Delta H^0_{D,L}}{RT} + \frac{\Delta\Delta S^0_{D,L}}{R} = -\frac{\Delta\Delta G^0}{RT} \quad (1)$$

Where ΔH^0, ΔS^0 and ΔG^0 are the enthalpy, the entropy and Gibbs free-energy changes, respectively, associated with the analyte retention process. The subscripts D and L refer to the enantiomeric forms of a generic racemic solute. When an acetonitrile–0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ (7:3) at pH 7.6 was used as the mobile phase, the separation factors on the L-phenylalaninamide-modified CSP were determined at different temperatures. The linear inverse relationship between ln α and temperature was plotted. The linear regression equations for Dns–D,L-Asp, Dns–D,L-Met and Dns–D,L-Thr are expressed as $\ln \alpha = 929.2/T - 1.512$ ($R^2 = 0.863$), $\ln \alpha = 327.2/T + 0.0437$ ($R^2 = 0.806$) and $\ln \alpha = 466/T - 0.025$ ($R^2 = 0.8405$), respectively. Based on these experimental equations and theoretical equation (1), the thermodynamic parameters can be obtained and are given in Table 1. The molecular recognition mechanism is under enthalpic control under experimental conditions; the entropic contribution is very small and sometimes near zero. The small variation in α with the temperature suggests that the chromatography can be conveniently carried out above room temperature, taking the advantage of the increased efficiency obtained without significantly affecting the enantioselectivity.

Table 1
Thermodynamic data

Analyte	$-\Delta\Delta H^0$ (cal/mol)	$-\Delta\Delta S^0$ (cal/mol K)	$-\Delta\Delta G$ (25°C) (cal/mol)
Dns–D,L-Asp	1846	3.00	1052
Dns–D,L-Met	650	0.087	624
Dns–D,L-Thr	926	0.050	911

Column: L-phenylalaninamide-modified monolithic column, CSP: 32 cm; mobile phase: pH 7.6, acetonitrile–0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$(7:3); flow-rate: 5 μl/min.

Fig. 5 shows the chromatograms of Dns–D,L-Thr at different temperatures (25, 35 and 45°C). The results clearly show that the retention times of both enantiomers become shorter with the increase of temperature. This suggests that faster mass transfer and kinetics of complexation–decomplexation for the Cu(II) complex occurred at the higher temperatures.

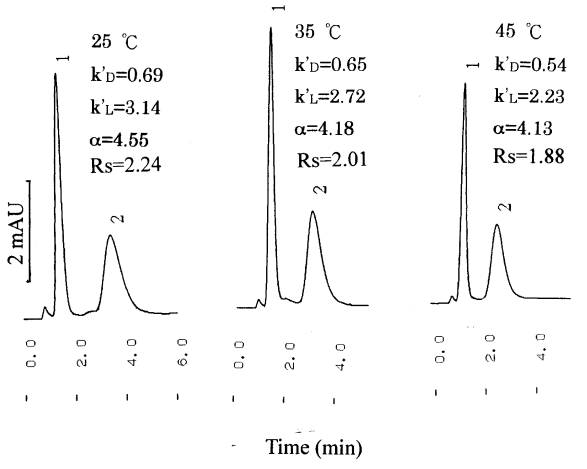


Fig. 5. Chromatograms of Dns–D,L-Thr at different temperatures. Mobile phase: acetonitrile–0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ (7:3), pH 7.6. Other conditions as in Fig. 3.

3.6. Efficiency of LE-CSPs

To evaluate the efficiency of the column, the van Deemter plot (H vs. u) for the separation of Dns–D,L-Leu is depicted in Fig. 6. Because the minimal flow-rate can only be controlled at 1 μl/min by the LC-10ADvp pump used, the effect of linear velocity on the plate height was examined in the range of 1–20 μl/min. The H value almost linearly increases with the linear velocity (u) of the mobile phase. A minimal H value was achieved at the minimal flow-rate. On the basis of the shape of H–u plot, H may be contributed to only by the A and C terms in van Deemter equation ($H = A + B/u + Cu$). The absence of the B term suggested negligible axial diffusion, probably due to the relatively high flow-rate used. Our previous work [22] demonstrated that the B term was significant when the linear velocity was controlled at less than 0.25 mm/s by the EOF.

On the basis of results in Fig. 6, only about 1 mm of minimum theoretical plate height can be obtained at the experimental conditions. The column efficiency seems not to be very high. The reasons probably are as follows: (1) the nonuniformity of monolith column bed, skeleton and through-pore (channels), causes the eddy diffusion (A term); (2) the high flow-rates result in the significant resistance to mass transfer (C term). In Fig. 6 we can extrapolate that much lower plate height (<1 mm) could be achieved if the flow-rate could be controlled at <1 μl/min. Unfortunately, the minimum flow-rate we can control by current pump was 1 μl/min. Our results in CEC [22] demonstrated 30 μm of plate height for unretained acetone could be obtained when the flow-rate was controlled by EOF at a linear velocity of 0.25 mm/s (about 0.12 μl/min for 100 μm I.D. capillary).

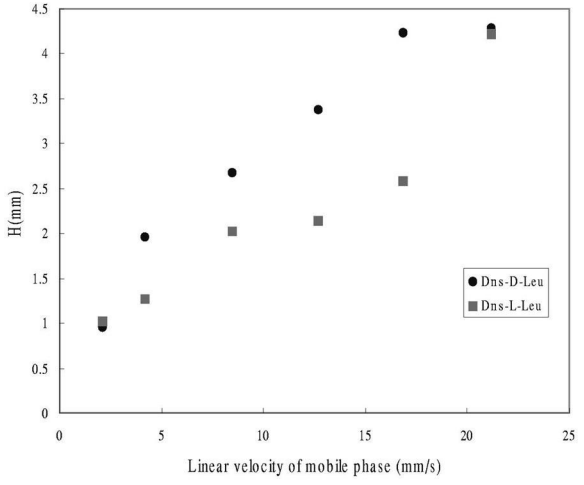


Fig. 6. Plot of plate height (H) vs linear velocity of mobile phase (u). Sample: Dns–D,L-Leu; mobile phase: acetonitrile–0.1 M NH_4Ac–0.25 mM $Cu(Ac)_2$ (7:3), pH 7.6. Other conditions as in Fig. 3.

The overall efficiency obtained in a chromatographic system depends on the efficiency of the column itself and the instrumental contributions to the band broadening, which includes contributions of the injection process, the detection volume, and the time constant of the detection system. In this work, the contribution of the detection system to the overall efficiency was neglected, since the detection was performed on-column. However, the injection volume has a considerable influence on the contribution to the overall efficiency. The injection volume controlled by the sample rotor used was usually overloaded for the capillary column. The effect of injection volume on the separation was examined; the resolution values were 0.32 and 0.75 by using 0.5 and 0.2 μl sample rotors, respectively, as shown in Fig. 7. Therefore the injection volume has a significant influence on the separation. We can also deduce that more perfect separation could be achieved if a much smaller volume of sample rotor was used. This extrapolation can be supported by CEC results [22]. A plate height of 30 μm was obtained in CEC when the volume of electrokinetic injection in CEC for 5 s at 0.25 mm/s of the EOF linear velocity was about 0.01 μl (only 1/20 of 0.2 μl sample rotor) for 100 μm I.D. capillary. Therefore, on the basis of above discussion we can draw the conclusion that LC instruments, such as pumps, having a very low flow-rate (<1 μl/min) and a small injection volume (<0.2 μl) should be beneficial for high efficiency in μ-HPLC, in addition to development of the column technology.

3.7. Enantioselectivity and enantioseparations with LE-CSPs

The enantioseparations was carried out by the use

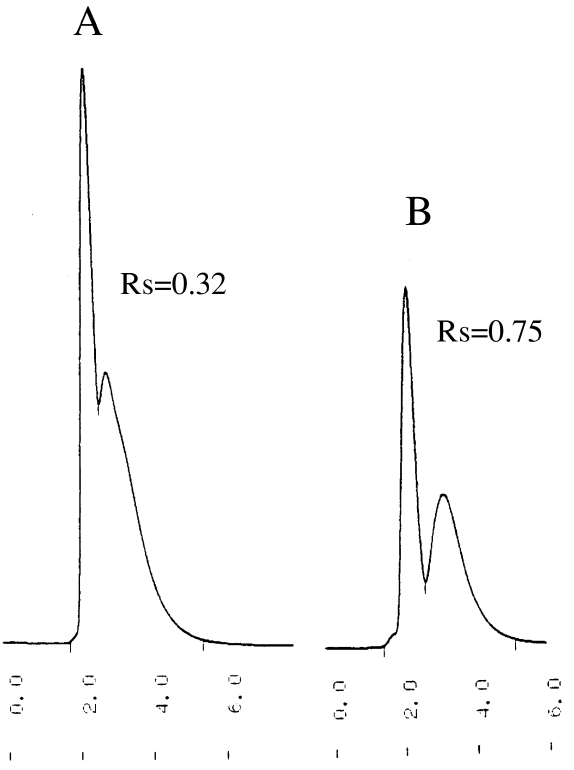


Fig. 7. Effect of injection volume controlled by 0.5 μl (A) and 0.2 μl (B) sample rotors on separation. Monolith column: L-phenylalaninamide modified CSP (20 cm); mobile phase: acetonitrile–0.1*M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$, pH 7.6; flow-rate: 2.0 μl/min; room temperature; sample: Dns–D,L-Ser.

of three types of monolithic LE-CSPs. The results are listed in Tables 2 and 3. Fig. 8 shows the representative chromatograms of dansyl amino acids. All 12 commercially available dansyl amino acids can be resolved by the L-phenylalaninamide modified monolithic silica column, and the D-enantiomers were eluted faster than the L-enantiomers. To compare the enantioselectivity of LE-CSPs, the enantioseparation of dansyl amino acids were also carried out using an alaninamide-modified monolithic column (32 cm CSP), however, only Dns–D,L-Ser (R_s = 0.92) was resolved by using the mobile phase of pH 7.6, acetonitrile–0.1 *M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$ (7:3) at flow-rate of 2 μl/min. This implies that the phenyl group in phenylalaninamide played a very important role in the chiral discrimination. Further, when we tried the separation of free amino acids, no obvious chiral resolution was observed, which suggests that the dansyl groups also play an important role in the chiral recognition. When the L-prolinamide-modified monolithic column was used, enantioseparations were achieved not only for several dansyl amino acids but also for a few hydroxy acids (Table 3). Besides, it was noticed that the L-prolinamide- and L-phenylalaninamide-modified CSPs showed a different enantioselectivity to the same analytes. L-Prolinamide-modified CSP usually shows high enantioselectivity to the analytes with a large substituent group.

Table 2
Enantioseparations of Dns–D,L-amino acids by the use of L-phenylalaninamide CSP

Samples	k'_D	k'_L	α	R_s
Dns–D,L-Ser	2.20	17.5	7.95	1.84
Dns–D,L-Glu	2.50	9.42	3.77	1.36
Dns–D,L-Thr	2.02	9.25	4.58	1.83
Dns–D,L-Asp	4.02	15.25	3.79	1.50
Dns–D,L-Met	2.54	6.18	2.43	1.13
Dns–D,L-Leu	0.56	1.88	3.35	0.88
Dns–D,L-NorLeu	1.06	2.47	2.33	0.68
Dns–D,L-NorVal	0.72	2.27	3.13	0.74
Dns–D,L-Val*	1.03	1.69	1.64	0.66
Dns–D,L-Phe*	1.87	5.82	3.11	0.47
Dns–D,L-Trp*	2.85	4.15	1.46	0.68
Dns–D,L-α-amino-*n*-butyric acid	0.84	2.50	0.98	0.80

Separation conditions: Column: L-phenylalaninamide-modified monolithic column; CSP: 32 cm; mobile phase: pH 7.6, acetonitrile–0.1*M* NH_4Ac–0.25 m*M* $Cu(Ac)_2$(7:3); flow-rate: 20 μl/min, but 5 μl/min for those marked with an asterisk (*).

Table 3
Enantioseparations using L-prolinamide CSP

Samples	k'_D	k'_L	α	R_s
Dns–D,L-Phe	0.25	0.62	2.47	0.47
Dns–D,L-Trp	0.27	1.02	3.74	0.83
Dns–D,L-NorLeu	0.38	0.81	2.13	0.70
D,L-Hydroxyphenollactic acid	0.21	0.86	4.13	1.31
D,L-Indole-3-lactic acid	0.39	1.34	3.42	1.75

Separation conditions: column: L-prolinamide-modified monolithic column; mobile phase: pH 6.5, acetonitrile–50 m*M* NH_4Ac–0.50 m*M* $Cu(Ac)_2$; flow-rate: 20 μl/min.

4. Conclusions

A new type of chemically modified chiral monolithic column was developed and successfully used for the enantioseparations of dansyl amino acids and hydroxy acids by μ-HPLC. Different enantioselectivities were obtained by different LE-CSPs like L-phenylalaninamide, L-alaninamide and L-prolinamide. Based on the thermodynamic data, the chiral recognition mechanism is mainly under enthalpic control. This work demonstrated that chemically modified monolithic silica column would be a very

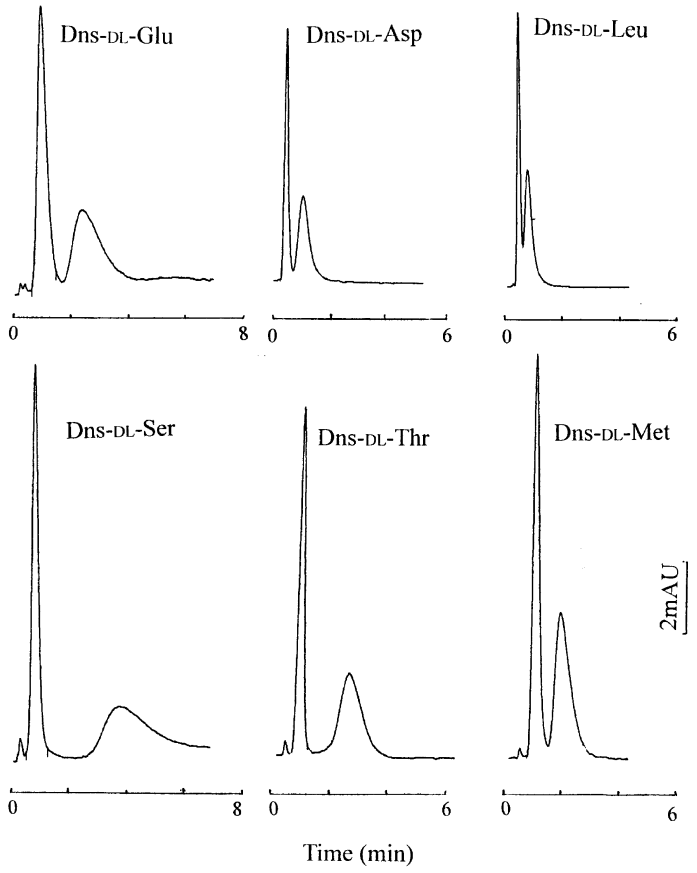


Fig. 8. Representative chromatograms for the enatioseparations of dansyl amino acids using L-phenylalaninamide modified monolithic columns. Separation conditions as in Table 2.

promising method to apply the LE-CSPs used in the conventional HPLC to the microcolumn HPLC.

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