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Evaluation and application of liquid chromatographic columns coated with 'intelligent' ligands: (I) acylcarnitine column

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Abstract

Unique stationary phases of octadecylsilica (ODS) coated with acylcarnitines have been developed for liquid chromatographic columns. The ODS column coated with acylcarnitine was readily prepared by recycling the solution containing acylcarnitine through an ODS column in a closed loop. Acylcarnitine was adsorbed on the ODS surfaces by hydrophobic interaction between the acyl group of acylcarnitine and the octadecyl group of the ODS phases. The ODS column coated with stearoylcarnitine (CN-18 column) was the most stable among the four columns coated with acylcarnitines of various acyl chain lengths (decanoylcarnitine, lauroylcarnitine, myristoylcarnitine, and stearoylcarnitine) under the condition of delivery of the mobile phase, indicating that adsorption of acylcarnitine on the ODS surfaces depended on the length of acyl chains. The CN-18 column was usable for delivering the mobile phase contained less than 20% (v/v) acetonitrile, retaining almost the same separation efficiency as the intact ODS column. The retention behavior of ionic solutes on the CN-18 column could be explained by both ionic and electrostatic interactions between the solutes and the stationary phase. The CN-18 column enabled efficient separation of inorganic anions, nicotinic acids, amino acids, and nucleotides. The chiral ODS column coated with enantiomer of stearoylcarnitine, L-stearoylcarnitine (L-CN-18 column) could achieve direct enantiomeric separation of DL-tryptophan, α-methyl-DL-tryptophan and DL-3-indolelactic acid using 100% water as the mobile phase. The L-CN-18 column could also separate enantiomers of amino acids and α-hydroxycarboxylic acids by ligand-exchange chromatographic mode using a mobile phase containing copper(II) ion. The chiral recognition is discussed for enantiomeric separation on the L-CN-18 column.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Ligand exchange; Acylcarnitine; Amino acids; α-Hydroxycarboxylic acids; Stearoylcarnitine

1. Introduction

Utilizing the hydrophobic interaction between ligands and octadecylsilica (ODS) surfaces, stationary phases of ODS coated with various ligands have been developed for high-performance liquid chromatography (HPLC). These ligand-coated columns have been used to separate ionic solutes (anions and amino acids) and enantiomeric compounds (amino acids and amino alcohol) in ligand-exchange chromatography. In the 1980s, Davankov et al. described a chiral-phase column of alkylsilica coated with *N*-alkyl-L-hydroxyproline for enantiomeric separation of underivative amino acids in conjunction with a mobile phase containing copper(II) ion [1,2].

Recently, a number of reports have appeared on

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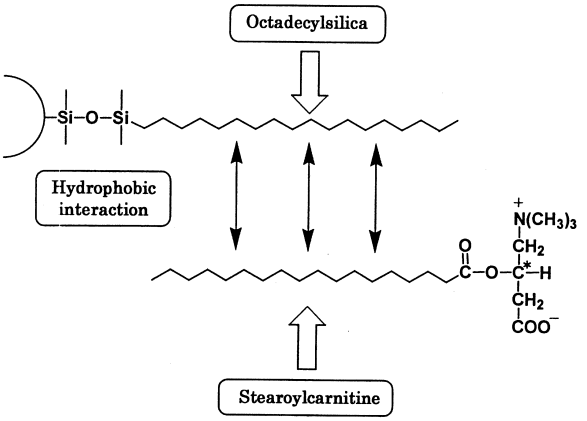
the separation of inorganic anions and amino acids using microcolumns coated with micellar bile acid derivatives [3–9]. Inorganic anions have been separated using ODS columns coated with hexadecyltrimethylammonium [10], cetyldimethylamine [11] or cetyltrimethylammonium [12]. Enantiomeric separations of amino acid analogues have been reported for ODS columns coated with various chiral ligands for ligand-exchange HPLC [13–18]. Also, enantiomeric separation of amino acids has been achieved on ODS column coated with chiral crown ether [19,20], micellar bile salt [21], or poly-(*R*)-1-(α-naphthyl)-ethyl-methacrylamide [22]. The ligands of these columns are adsorbed on the ODS surfaces by hydrophobic interaction between the hydrophobic group of ligands and the octadecyl group of ODS surfaces. The preparation of ODS columns coated with specific ligands in situ by hydrophobic interaction is convenient for preparing analytical columns to separate target compounds.

The advantages of preparing ligand-coated columns are readiness and economy, because the columns can be prepared by recycling the ligand-containing solution through an ODS column for its generation. Also, there is the merit of using desirable ligands which can raise separation selectivity. Utilizing this ligand-coating technique, we prepared acylcarnitine-coated columns [23–25], in which ionic and electrostatic interactions by the zwitterionic carnitine moiety of the ligand were anticipated to contribute to efficient separation. Carnitine, which has carboxylic and quaternary ammonium groups in its molecule, is an essential cofactor in the transport of long-chain fatty acids into the mitochondrial inner membrane for β-oxidation [24,25]. Acylcarnitines are fatty acid ester derivatives of carnitine and are specifically required for entry through the mitochondria inner membrane.

Kamimori and Konishi [26; a part of this paper was presented during the 5th Conference of the Society for Chromatographic Sciences (November, 1994, Tokyo, Japan)], found that stearoylcarnitine (CN-18) was the ligand adsorbed on the ODS surfaces by hydrophobic interaction between the acyl group of CN-18 and the octadecyl group, as shown below. As the carnitine moiety of the coated column is thought to be sticking out of the ODS phase, an inorganic anion is anticipated to be retained on the quaternary ammonium group of the coated phase by ionic interaction (Scheme 1).



Schematic concept of stearoylcarnitine-coated column (* Asymmetric carbon)

Scheme 1.

We demonstrate the preparation of the functional stationary phase of the CN-18-coated column (CN-18 column) employing a dynamic coating technique and its application to the separation of a variety of ionic compounds. We also discuss the mechanism of the retention behavior for ionic compounds on the coated column. Further, we investigate the preparation of a chiral ODS column coated with L-stearoylcarnitine (L-CN-18 column) and its application to enantiomeric separation of amino acid analogues.

2. Experimental

2.1. Apparatus

A Waters 600E Multisolvent Delivery System (Milford, MA) was used to deliver the mobile phase. Sample solutions were injected with a Shimadzu SIL-9A automatic injector (Kyoto, Japan), and a Shimadzu SPD-2A spectrophotometer was used as a UV detector. The chromatographic data were analyzed with a Shimadzu C-R4AX data processor. The column temperature was kept at 37°C with heating equipment (model 370; Chemco, Osaka, Japan).

2.2. Reagents

DL-Decanoylcarnitine (CN-10) hydrochloride, DL-lauroylcarnitine (CN-12) hydrochloride, DL-myristoylcarnitine (CN-14) hydrochloride, DL-stearoyl-carnitine (CN-18) hydrochloride, L-carnitine hydrochloride, DL-amino acids, α-methyl-DL-tryptophan and nucleotides were obtained from Sigma (St. Louis, MO). DL-3-Indolelactic acid was purchased from Aldrich (Milwaukee, WI). Stearoyl chloride was obtained from Tokyo Kasei (Tokyo, Japan). Acetonitrile and methanol were of HPLC grade, and water was purified before use with ultra-pure water generating equipment (Barnsteads, Boston, MA). All other chemicals were of reagent grade.

2.3. The ODS columns coated with acylcarnitines

2.3.1. Preparation of ODS columns coated with acylcarnitines

The ODS columns used to prepare the columns coated with acylcarnitines were an L-column ODS (150×4.6 mm I.D., 5 μm, Chemicals Evaluation and Research Institute, Tokyo, Japan) and a Develosil ODS-HG-5 (50×4.6 mm I.D., 5 μm, Nomura Chemicals, Aichi, Japan). The solution containing acylcarnitine hydrochloride was delivered through the ODS column by an HPLC pump. The temperature throughout of the column preparation was 25±1°C. The coating solution containing acylcarnitine was prepared using 10 m*M* sodium phosphate buffer (pH 6) or a mixture of 10 m*M* sodium phosphate buffer (pH 6)–methanol according to the solubility of each acylcarnitine in the coating solution. To prepare the CN-18 column, a solution containing 300 μmol of CN-18 hydrochloride in 2000 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (50:50, v/v) was pumped at a flow-rate of 1 ml/min through L-column ODS (150×4.6 mm I.D.) in a closed loop for up to 2500 ml of total delivery. Another CN-18 column was prepared by recycling a solution containing 100 μmol of CN-18 hydrochloride in 670 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (50:50, v/v) at a flow-rate of 1 ml/min through Develosil ODS-HG-5 (50×4.6 mm I.D.) for up to 840 ml of total delivery. As the delivered volumes of solution containing 300 μmol of CN-18 hydrochloride in 2000 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (50:50, v/v) increased, the coating amount of CN-18 increased. The coating amount of CN-18 (250 μmol) reached a plateau when the total delivery was more than 1.25 times the volume of coating solution (2500 ml) in a closed loop. This result would indicate an equilibrium between the packing and the recycled solutions and the status of even distribution of the ligand along the whole length of the column. The other acylcarnitine columns were prepared using a coating solution containing 300 μmol of ligand by the recycling of solution in the closed loop.

The ODS column coated with CN-10 (CN-10 column) or CN-12 (CN-12 column) was prepared by recycling a solution containing 300 μmol of CN-10 hydrochloride or CN-12 hydrochloride in 500 ml of 10 m*M* sodium phosphate buffer (pH 6) at a flow-rate of 1 ml/min through L-column ODS (150×4.6 mm I.D.) in a closed loop for up to 630 ml of total delivery. The ODS column coated with CN-14 (CN-14 column) was prepared by recycling a solution containing 300 μmol of CN-14 hydrochloride in 2000 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (75:25, v/v) at a flow-rate of 1 ml/min through L-column ODS (150×4.6 mm I.D.) for up to 2500 ml of total delivery. All of the coated columns were washed with water at 1 ml/min for 30 min.

The amounts of CN-18 coated on the ODS phase were calculated from the differences between CN-18 concentrations of the solutions before and after preparation. The concentrations were measured by HPLC with fluorescence detection after derivatization of the carboxylic acid group with 3-bromomethyl-6,7-methoxy-1-methyl-2(1*H*)-quinoxalinone [23]. The results of the coating status were confirmed by comparison of the retention factor (*k*) of sodium nitrite of the CN-18 column and the intact ODS column delivering a 10 m*M* sodium phosphate buffer (pH 3). The void volume of each column was estimated from the retention time of methanol in this study.

2.3.2. Stability of ODS columns coated with acylcarnitines

The change of retention times of nitrate was plotted against delivery volumes of 10 m*M* sodium phosphate buffer (pH 3) through each column at a flow-rate of 1 ml/min.

After measurement of the *k* values of nitrite and nitrate, delivered as 10 m*M* sodium phosphate buffer (pH 3) through the CN-18 column at a flow-rate of 1 ml/min, 50 ml of 10–50% acetonitrile were delivered into the column at a flow-rate of 1 ml/min. Subsequently the *k* values of nitrite and nitrate delivered with 10 m*M* sodium phosphate buffer (pH 3) at the same flow-rate were measured. The relationship was plotted between the content of acetonitrile in the aqueous solution and the *k* values of nitrite and nitrate after delivery of the aqueous acetonitrile solution. The retention times and *k* values were measured by injection of 10 μl of each 2 m*M* solution.

2.3.3. Retention mechanism of ionic compounds on the CN-18 column

2.3.3.1. Inorganic anions

The mobile phases used were buffer solutions of pH 2.9, 3.6, 5.2, 6.6, and 7.4 with ionic strength of 0.05, delivered at a flow-rate of 1 ml/min. The *k* values of nitrite, nitrate, iodide, and thiocyanate were measured by injection of 10 μl of each 2 m*M* solution.

2.3.3.2. Nicotinic acid analogues

The mobile phases used were 10 m*M* sodium phosphate buffer (pH 3, 4, 5, 6, and 7), delivered at the flow-rate of 1 ml/min. The *k* values of nicotinic acid, isonicotinic acid, and picolinic acid were measured by injection of 10 μl of each 0.5 m*M* solution.

2.4. The chiral ODS column coated with L-stearoylcarnitine

2.4.1. Synthesis of L-stearoylcarnitine

L-CN-18 hydrochloride was synthesized by modifying a published procedure [27]. To a solution of L-carnitine hydrochloride (5.2 g) in trifluoroacetic acid (9 ml), stearoyl chloride (11.5 g) was added to one portion, and the mixture was allowed to react at 50°C for 2 h and then allowed to stand at room temperature for 18 h. The reaction mixture was poured into diethyl ether (300 ml). The crystallization products were dissolved in methanol (80 ml), and then repeated recrystallization of crude crystals from diethyl ether yielded 9.9 g of pure L-CN-18 hydrochloride: mp, (decomposed) 172°C, $[\alpha]_D^{25} = -13.7$ ($c = 1.0\%$ in methanol). Anal. Cald. for $C_{25}H_{50}ClNO_4$: C, 64.07; H, 10.76; N, 2.99; Cl, 7.57%. Found: C, 64.12; H, 10.76; N, 3.27; Cl, 7.38%. ^{1}H NMR (CD_3OD) δ: 0.90 (3H, t, $J = 7.6$ Hz), 1.29 (28H, s), 1.63 (2H, m), 2.39 (2H, t, $J = 8.3$Hz), 2.71 and 2.79 (2H, dd, $J = 12.7$, 8.0 Hz; dd, $J = 14.7$, 8.7 Hz), 3.20 (9H,s), 3.70 and 3.85 (2H, dd, $J = 14.7$, 0.9Hz; dd, $J = 14.7$, 9.3Hz), 5.52 (1H, m).

2.4.2. Preparation of chiral ODS column coated with L-CN-18

The ODS columns used to prepare the L-CN-18 column were L-column ODS (250×4.6 mm I.D., 5 μm and 150×4.6 mm I.D., 5 μm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The solution containing L-CN-18 hydrochloride was delivered through the ODS column by HPLC pump and the L-CN-18 column was prepared throughout at a temperature of 25±1°C. The L-CN-18 column was prepared as follows: each solution containing 300 μmol of L-CN-18 hydrochloride in 2000 ml, 600 μmol of L-CN-18 hydrochloride in 4000 ml, or 1200 μmol of L-CN-18 hydrochloride in 8000 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (50:50, v/v) was pumped for recycling at a flow-rate of 1 ml/min through L-column ODS (150×4.6 mm I.D.) with a closed loop for up to 2500, 5000 or 10 000 ml of total delivery, respectively. Another L-CN-18 column was prepared by recycling a solution containing 2000 μmol of L-CN-18 hydrochloride in 13 000 ml of 10 m*M* sodium phosphate buffer (pH 6)–methanol (50:50, v/v) at a flow-rate of 1 ml/min through the L-column ODS (250×4.6 mm I.D.) with a closed loop for up to 16 500 ml of total delivery. All the coated columns were washed with water at a flow-rate of 1 ml/min after the coating procedure.

The amounts of L-CN-18 coated on the ODS phase were calculated from the differences between L-CN-18 concentrations of the solutions before and after preparation. The concentrations were measured by HPLC with fluorescence detection after derivatization of the carboxylic acid group with 3-bromomethyl-6,7-methoxy-1-methyl-2(1*H*)-quinoxalinone [23]. The results of the coating status were confirmed

by comparison of the *k* value of sodium nitrite of the L-CN-18 column and the intact ODS column delivering a 10 m*M* sodium phosphate buffer (pH 3). The void volume of each column was estimated from the retention time of methanol.

3. Results and discussion

3.1. Evaluation of ODS columns coated with acylcarnitines

3.1.1. Amount of ligands coated on ODS column

The coating amount of CN-18 was calculated to be 250 μmol on the L-column ODS (150×4.6 mm I.D.). The *k* value of the nitrite of the L-column ODS (150×4.6 mm I.D.) coated with CN-18 delivered in 10 m*M* sodium phosphate buffer (pH 3) was 6.7, although nitrate was not retained on the intact ODS column. These results indicated that nitrite was retained on the quaternary ammonium group of the carnitine moiety sticking out of the ODS phase in the CN-18 column. Similarly, the coating amount of CN-18 on the Develosil ODS-HG-5 (50×4.6 mm I.D.) was 50 μmol, and the retention of nitrate on the column delivering a 10 m*M* sodium phosphate buffer (pH 3) was sufficiently confirmed.

3.1.2. Efficiency

The separation efficiency of the CN-18 column was estimated for comparison with that of the intact ODS column for three nicotinic acid analogues. Fig. 1 shows the chromatograms of nicotinic acid analogues on the CN-18 column and the intact ODS column using the mobile phase of pH 4 buffer solution. The CN-18 column showed better separation selectivity of nicotinic acid analogues than the intact ODS column. Also, the theoretical plates of nicotinic acid were calculated to be 16 200 for the CN-18 column and 4180 for the intact ODS column, respectively, showing the better efficiency of the CN-18 column. As a result, the CN-18 column conserved the separation efficiency from the intact ODS column.

3.1.3. Stability

The effect of acyl length of the acylcarnitine on the stability of the coated column was estimated from the parameters of retention time and retention factor of inorganic anions. Fig. 2a shows the relationship between the volumes of the buffer solution as the mobile phase delivered and the retention times of nitrate for each column. The retention times of nitrate for the CN-10 and the CN-12 columns decreased as the volume of the mobile phase delivered increased, indicating that the ligands became detached from the ODS surfaces. On the other hand, the retention times of nitrate for the CN-14 and CN-18 columns remained unchanged even after the buffer solution had been delivered through the columns up to a total volume of 1500 ml. It was proved that the stability of the acylcarnitine coated on the ODS surfaces mainly depended on the strength of the hydrophobic interaction between the

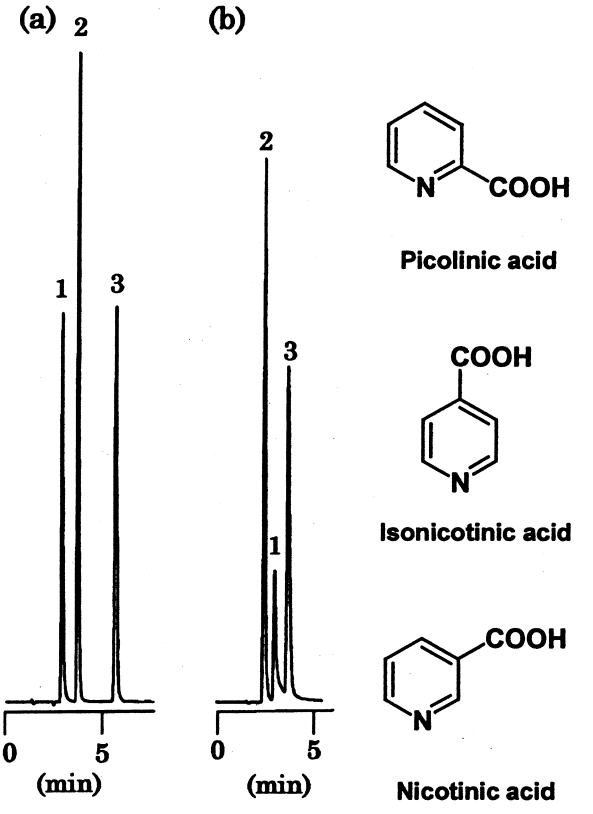


Fig. 1. Chromatograms of separation of nicotinic acids on the CN-18 column (a) and the ODS column. Mobile phase, 10 m*M* sodium phosphate buffer (pH 4); flow rate, 1 ml/min; detection, 230 nm; column, (a) L-column ODS (150×4.6 mm I.D.) coated with CN-18, (b) L-column ODS (150×4.6 mm I.D.). Peaks: (1) picolinic acid; (2) isonicotinic acid; (3) nicotinic acid.

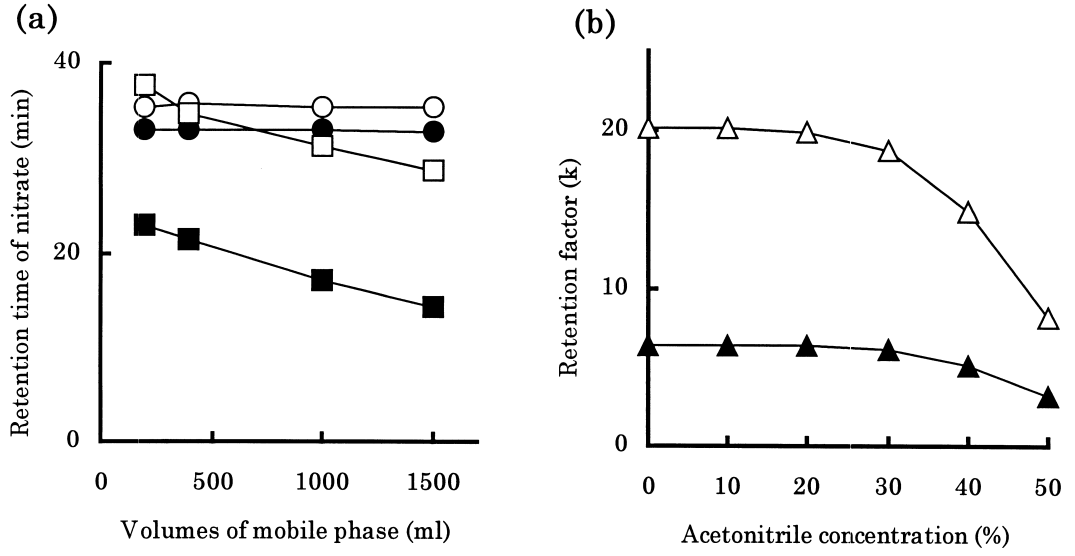


Fig. 2. Stability of the ODS column coated with acylcarnitines for aqueous mobile phase (a) and the CN-18 column for aqueous–organic mobile phase (b). Key: (a) CN-18 column (○), CN-14 column (●), CN-12 column (□), CN-10 column (■), (b) nitrate (△), nitrite (▲). Mobile phase, 10 m*M* sodium phosphate buffer (pH 3); flow rate, 1 ml/min; detection, 230 nm; column, (a) L-column ODS (150×4.6 mm I.D.) coated with each CN-10, CN-12, CN-14, and CN-18, (b) L-column ODS (150×4.6 mm I.D.) coated with CN-18.

acyl group of acylcarnitine and the octadecyl group. Consequently, the CN-18 column was found to be the most stable among these columns.

Fig. 2b shows the relationship between the content of acetonitrile in aqueous solution and the *k* values of nitrate and nitrite after delivery of the aqueous acetonitrile solution through the CN-18 column. Even after delivery of 50 ml of the mobile phase containing 20% (v/v) acetonitrile through the column, the *k* values of both anions were not affected. The *k* values of both anions gradually decreased as the acetonitrile content rose above 20% due to elimination of the coated CN-18 from the ODS surface. Thus, the CN-18 column can be used with a mobile phase containing up to 20% acetonitrile.

3.1.4. Reproducibility of column preparation and regeneration

To test the reproducibility of the column preparation method, three columns coated with CN-18 were prepared in separate experiments and their *k* values for nitrate, nitrite, iodide, and thiocyanate were measured with the mobile phase of 50 m*M* sodium phosphate buffer (pH 5). The relative standard deviation of the *k* values of these inorganic anions were calculated to be less than 2.5%. The results indicated that the coating procedure was reproducible.

The intact ODS column from which CN-18 had been removed by washing with methanol was again coated with CN-18 and the regeneration of the coated columns using the original ODS column was estimated by measuring the *k* value of nitrate with the mobile phase of 10 m*M* sodium phosphate buffer (pH 3). The *k* values of nitrite were 6.7 for the initial CN-18 column and 6.4 for the regenerated CN-18 column. Thus, the CN-18 column could be regenerated using the original ODS column.

3.1.5. Retention mechanisms of ionic compounds on the CN-18 column

3.1.5.1. Inorganic anions

The characteristics of the anionic exchanger of the CN-18 column were observed from the retention behavior of inorganic anions. The chromatogram of a mixture of inorganic anions on the CN-18 column with the mobile phase of pH 5.2 buffer solution is shown in Fig. 3. Inorganic anions were completely separated with the CN-18 column.

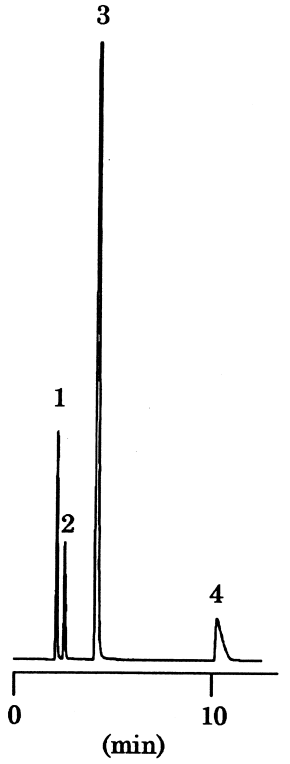


Fig. 3. Chromatogram of separation of inorganic anions on the CN-18 column. Mobile phase, pH 5.2, buffer solution; flow rate, 1 ml/min; detection, 230 nm; column, L-column ODS (150×4.6 mm I.D.) coated with CN-18. Peaks: (1) nitrite; (2) nitrate; (3) iodide; (4) thiocyanate.

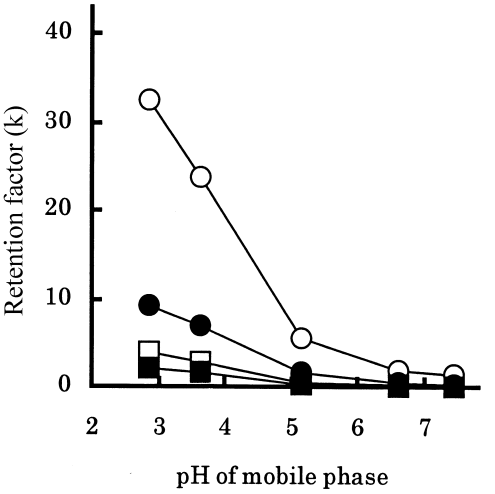


Fig. 4. Effect of mobile phase pH on retention factors of inorganic anions on the CN-18 column. Key: thiocyanate (○), iodide (●), nitrate (□), nitrite (■). Mobile phase, pH 2.9, 3.6, 5.2, 6.6, and 7.4 buffer solution; flow rate, 1 ml/min; detection, 230 nm; column, L-column ODS (150×4.6 mm I.D.) coated with CN-18.

Fig. 4 shows the relationship between the *k* values of inorganic anions and the pH of mobile phases under constant ionic strength. The retention of anions on the CN-18 column decreased as the mobile phase pH increased. The retention behavior of anions on the CN-18 column would arise from the ionic interaction between inorganic anions and the quaternary ammonium moiety in aqueous solution. For the CN-18 molecule, part of the intramolecular ionic bonds between carboxylic and quaternary ammonium groups must increase as the mobile phase pH increases due to dissociation of carboxylic acid. Consequently, the observed proportion of the quaternary ammonium cation which is likely to interact with solute anion will decrease. As a result, the retention of anions might be weakened as the mobile phase pH increases. The largest decrease of *k* values of anions observed at above pH 3, indicates that the intramolecular ionic interaction of carboxylic and quaternary ammonium groups became predominant because the pK_a of the carboxylic group is 3.80 for carnitine [28,29]. These results suggest the successful coating of CN-18 on the ODS surfaces.

3.1.5.2. Nicotinic acid analogues

Fig. 5 shows the relationship between the *k* values of the nicotinic acid analogues and the pH of mobile phases on the CN-18 column. Clearly, the *k*–pH profiles of nicotinic acid analogues are as different as those of the anions in Fig. 4. The retention order of the nicotinic acid analogues was nicotinic acid> isonicotinic acid>picolinic acid in the range of pH 3–7, which was consistent with the magnitude of their pK_a of 5.40, 4.96, and 4.85, respectively, based on the acid dissociation of the carboxylic group. The maximum *k* values were observed at pH 4 for nicotinic acid and isonicotinic acid, and pH 5 for picolinic acid. On the other hand, the *k* values of these nicotinic acid analogues on the intact ODS column were unchanged in the range of pH 3–7. The *k*–pH profiles of nicotinic acid analogues on the CN-18 column were attributed to the ionic interaction between the ligand acylcarnitine and the

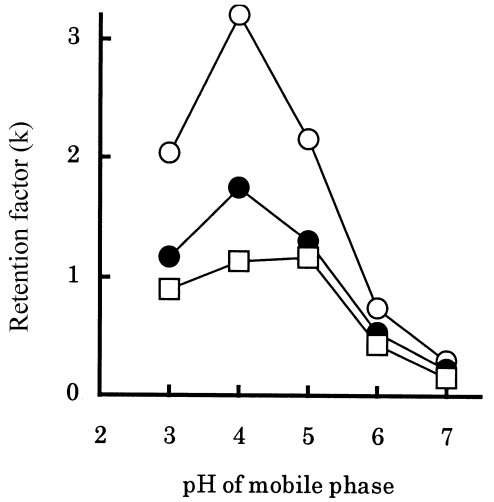


Fig. 5. Effect of mobile phase pH on retention factors of nicotinic acid analogues on the CN-18 column. Key: nicotinic acid (○), isonicotinic acid (●), picolinic acid (□). Mobile phase, 10 m*M* sodium phosphate buffer (pH 3, 4, 5, 6, and 7); flow rate, 1 ml/min; detection, 230 nm; column, L-column ODS (150×4.6 mm I.D.) coated with CN-18.

solutes, which involved the ionic carboxylate–quaternary ammonium interaction at pH above pK_a of the solute and the ionic repulsion between the protonated nitrogen and quaternary ammonium at pH below pK_a of the solute. The rapid decrease of the *k* values of the solutes above the maximum pH of retention is likely to be due to the increasing ratio of intramolecular interaction in acylcarnitine structure as discussed above. The specific retention characteristics of the acylcarnitine-coated phase may result in selective separation of nicotinic acid analogues as shown in Fig. 1.

3.1.6. Separation of amino acids and nucleotides on the CN-18 column

Fig. 6a shows the chromatogram of a mixture of five amino acids on the CN-18 column using 100% water as the mobile phase. The amino acids were completely eluted from the CN-18 column within 18 min, while 26 min was required for the ODS column (data not shown). We assume that two factors would influence the retention of amino acids with the CN-18 column. One is that the hydrophobic interaction should be weakened by the introduction of CN-18 compared with that of the intact ODS column, and

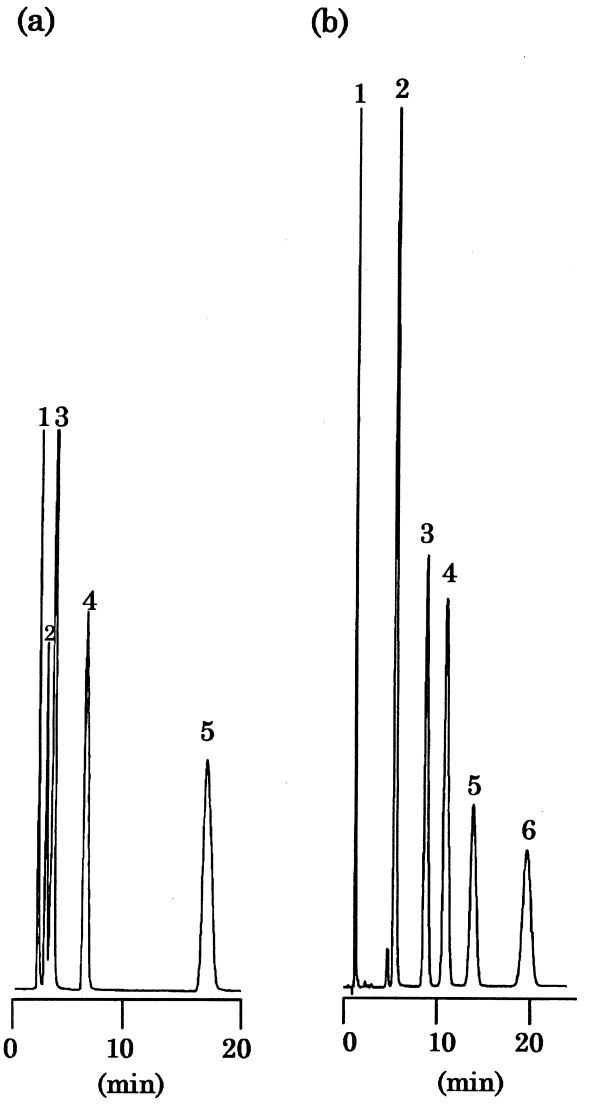


Fig. 6. Chromatograms of separation of amino acids (a) and nucleotides (b) on the CN-18 column. Mobile phase, (a) 100% water, (b) 5 m*M* sodium phosphate buffer (pH 3); flow rate, 1 ml/min; detection, (a) 210 nm, (b) 260 nm; column, (a) L-column ODS (150×4.6 mm I.D.) coated with CN-18, (b) Develosil ODS-HG-5 (50×4.6 mm I.D.) coated with CN-18. Peaks: (a): (1) valine; (2) isoleucine; (3) tyrosine; (4) phenylalanine; (5) tryptophan; (b) (1) 5′-CMP; (2) 5′-AMP; (3) 5′-UMP; (4) 5′-GMP; (5) 5′-IMP; (6) 5′-XMP.

another is the formation of an electrostatic interaction between amino acids and the CN-18 stationary phase. Electrostatic attraction and repulsion may occur simultaneously between two molecules in

100% water. If electrostatic attraction were limited to the solutes and the stationary phase, the solute should not have been eluted from the column by 100% water. Under this condition, as the repulsion force may be greater than the attraction, the retention of solutes on the CN-18 column would be weakened. As a result, amino acids on the CN-18 column could be separated by combining moderate hydrophobic interaction and electrostatic repulsion between the ligand and the solutes.

Fig. 6b shows the chromatogram of six nucleotides (adenosine-5′-monophosphate (5′-AMP), guanosine-5′-monophosphate (5′-GMP), inosine-5′-monophosphate (5′-IMP), uridine-5′-monophosphate (5′-UMP), xanthosine-5′-monophosphate (5′-XMP), and cytidine-5′-monophosphate (5′-CMP)) obtained with the CN-18 column (50×4.6 mm I.D.) using pH 3 buffer solution as the mobile phase. These nucleotides were completely separated within a reasonable analysis time. The separation of nucleotides with the CN-18 column was as effective as that with the conventional anionic exchange column.

3.2. Evaluation of chiral ODS column coated with L-stearoylcarnitines

3.2.1. Column stability

The quality of the L-CN-18 column used in this study was found to be unchanged for the chiral separation of racemic compounds even after intermittent use for at least 2 months.

3.2.2. Direct enantiomeric separation

Enantiomeric separation for racemic amino acid analogues was investigated using the L-CN-18 column with various mobile phases. As shown in Fig. 7, the enantiomeric DL-tryptophan, α-methyl-DL-tryptophan and DL-3-indolelactic acid were separated with the L-CN-18 column (250×4.6 mm I.D., 906 μmol of coated L-CN-18) using 100% water as the mobile phase. Separation factors (α) were 1.06 for DL-tryptophan, 1.05 for α-methyl-DL-tryptophan and 1.04 for DL-3-indolelactic acid, respectively. Enantiomeric separation was similarly achieved with 10 m*M* sodium phosphate buffer (pH 5, 6, and 7) as the mobile phase.

Table 1 shows the effect of the coating amounts of L-CN-18 on the *k* values and α values for nitrite, DL-tryptophan using the mobile phase of 10 m*M* sodium phosphate buffer (pH 3) or 100% water. The *k* values of nitrite on each column were 7.2, 11.0, and 14.4 for 270, 415, and 544 μ*M* of coating, respectively, indicating that the *k* value of nitrite increased as the coating amount increased. The α value for tryptophan increased with increasing amounts of L-CN-18. These results indicate that the coating amounts of L-CN-18 is the most important factor for enantiomeric separation. The *k* values of tryptophan decreased as the coating amounts increased, while the *k* value of nitrate increased. Two factors which decrease the retention must be considered as reducing the hydrophobicity of the coated phase and the electrostatic repulsion between the solutes and the stationary phase. Since the carnitine moiety and the amino acid are likely to form a zwitterion structure, electrostatic attraction and repulsion which occur simultaneously between the stationary phase and the solute should affect the retention behavior under 100% water. If there is only electrostatic attraction, the solutes may not be eluted from the column with 100% water. The repulsion would be greater under this condition. As a result, the retention of solutes on the L-CN-18 column might be weakened by this combination of reducing the hydrophobicity and the electrostatic repulsion.

3.2.3. Chiral recognition of direct enantiomeric separation

To estimate the chiral recognition of the L-CN-18 column under 100% water for racemic tryptophan analogues, the structural characteristics of those molecules were considered to explain the interaction between the solutes and the chiral-coated phase. For the L-CN-18 molecule of the coated phase, the quaternary ammonium groups and the carbonyl oxygen of stearoyl ester are located in these functional groups which suitably form both an ionic bond interaction with the carboxylic acid and a hydrogen bond interaction with the amino groups against tryptophan analogues, respectively. The capability for direct enantiomeric separation was conserved with a mobile phase of pH 5–7; however, the enantiomeric selectivity disappeared with a mobile phase below pH 4. The results indicated that the ionic bond between the solutes and the chiral-coated phase offers a major contribution to this separation,

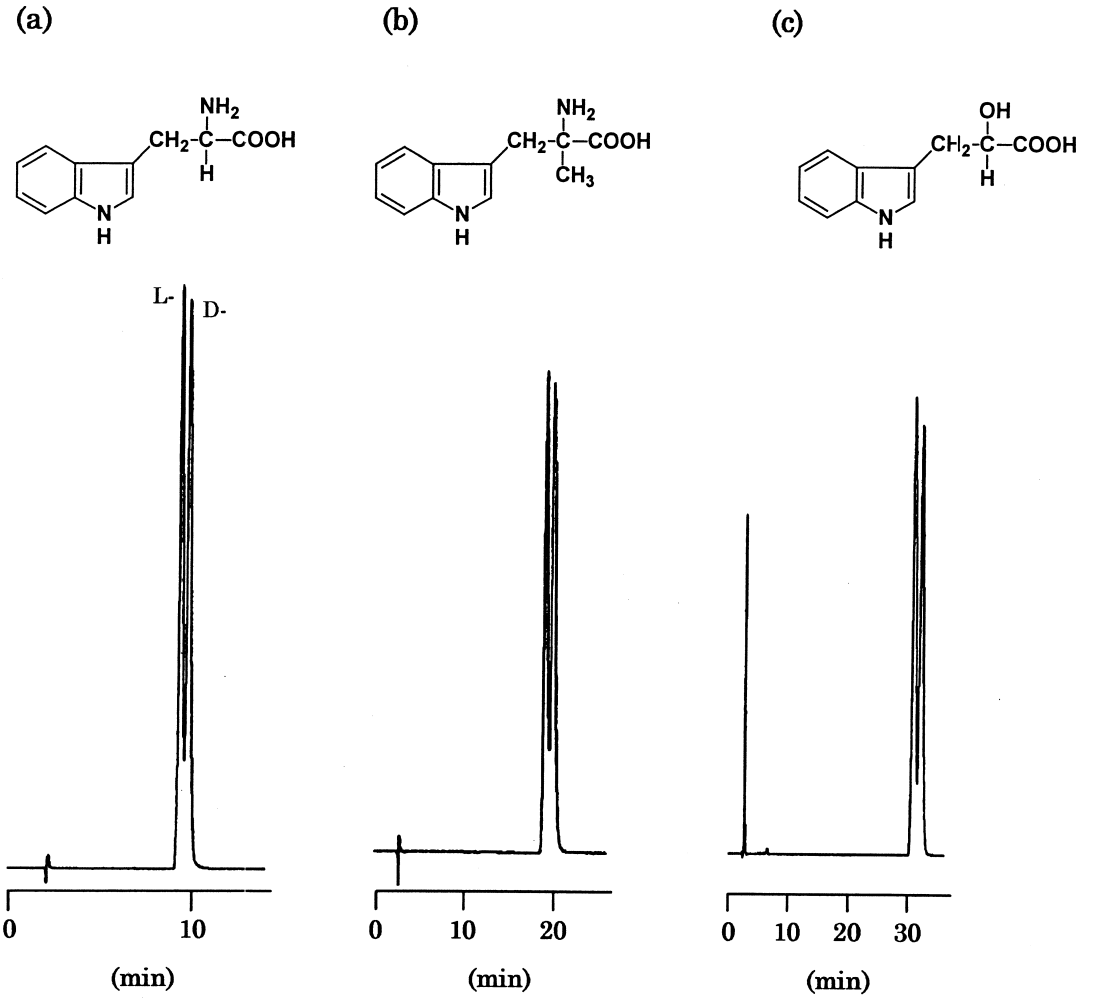


Fig. 7. Enantiomeric separation of DL-tryptophan (a), α-methyl-DL-tryptophan (b) and DL-3-indoleacetic acid (c) using 100% water as mobile phase. Mobile phase, 100% water; flow rate, 1 ml/min; detection, 210 nm; column, L-column ODS (250×4.6 mm I.D.) coated with L-CN-18.

because the carboxylic acid groups of these solutes are completely dissociated at pH between 5 and 7 (tryptophan: $pK_a = 2.38$). Based on this, we conclude that the enantioselectivity of L-CN-18 is based on the extent of the ionic and hydrogen bond interactions between the solutes and the coated phase, in addition to the hydrophobic interaction.

3.2.4. Enantiomeric separation by ligand exchange mode

The L-CN-18 column was used for enantiomeric separation by ligand exchange mode. As shown in Fig. 8, enantiomers of isoleucine and lactic acid could be completely separated on the L-CN-18 column using the mobile phase containing aqueous copper(II) acetate solution. The selectivities of the enantiomeric separation for various amino acids and α-hydroxycarboxylic acids are summarized in Table 2. Eight of the 15 amino acids tested could be well separated with the L-CN-18 column with α values of 1.06–1.20. Moreover, enantiomers were effectively separated with the coated column for α-hydroxycarboxylic acid. The chiral recognition is probably based on the thermodynamic stability of the com-

Table 1
Effect of coating amounts of L-CN-18 on retention factor and separation factor of tryptophan

Amounts of L-CN-18 (μmol/column)	Tryptophan		Nitrite
	$k^{a,c}$	α^{b}	k^{d}
270	6.6	1.04	7.2
415	4.7	1.06	11.0
544	3.2	1.06	14.4

Flow rate, 1 ml/min; detection, 210 nm; column, L-column ODS (150×4.6 mm I.D.) coated with L-CN-18.
[a] Retention factor of first eluted enantiomer.
[b] Separation factor ($k_{\text{second eluted enantiomer}}/k_{\text{first eluted enantiomer}}$).
[c] Mobile phase, 100% water.
[d] Mobile phase, 10 m*M* sodium phosphate buffer (pH 3).

plexation consisting of the carboxylic acid groups, the ester groups in the L-CN-18 and the solutes via copper(II) ion as a metallic ligand, as previously discussed elsewhere [1,2,30–40]. The functional groups directly related to the formation of the complex are amino or hydroxy groups for amino acid

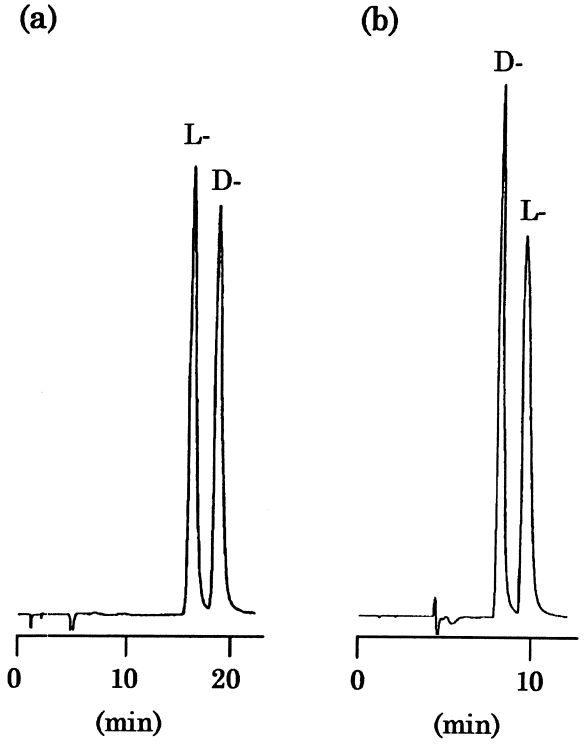


Fig. 8. Enantiomeric separation of DL-isoleucine (a) and DL-lactic acid (b) using a mobile phase containing copper(II) ions. Separation conditions as in Table 2.

Table 2
Enantiomer separation of amino acids and α-hydroxycarboxylic acids on L-CN-18 column by ligand exchange mode with mobile phase containing copper(II) ion

Compound	α^{a}	Mobile phase
Amino acids:		
Proline	1.15	I
Valine	1.20	I
Methionine	1.09	I
Leucine	1.11	I
Tyrosine	1.07	II
Isoleucine	1.17	II
Phenylalanine	1.08	III
N-Acetyltryptophan	1.06	IV
α-Hydroxycarboxylic acids:		
Lactic acid	1.22	II
Mandelic acid	1.19	V

Mobile phase: (I) 0.5 m*M* aqueous copper(II) acetate solution; (II) 1 m*M* aqueous copper(II) acetate solution; (III) 0.5 m*M* aqueous copper(II) acetate solution–acetonitrile (90:10); (IV) 1 m*M* aqueous copper(II)acetate solution–acetonitrile (80:20); (V) 0.1 m*M* aqueous copper(II)acetate solution–acetonitrile (80:20). Flow rate, 1 ml/min; detection, 230 and 254 nm; column, L-column ODS (150×4.6 mm I.D.) coated with L-CN-18.
[a] Separation factor ($k_{\text{second eluted enantiomer}}/k_{\text{first eluted enantiomer}}$).

or α-hydroxycarboxylic acid in addition to the carboxy group of both compounds. The hydrophobicity of the solute would be one of the important factors for enantiomeric separation of amino acids with the L-CN-18 column, since hydrophilic amino acids, such as alanine and serine, are too weak to be retained on the coated column.

4. Conclusion

The CN-18 column could be readily prepared by recycling the solution containing CN-18 through an ODS column. The preparation of the CN-18 column was reproducible, and the coated column could be repeatedly prepared using the original ODS column. The CN-18 column was capable of delivering the mobile phase containing 20% acetonitrile, and reversed the separation efficiency of the intact ODS column. Efficient separation on the CN-18 column was observed for inorganic anions, nicotinic acids, amino acids, and nucleotides. The retention mechanisms of ionic compounds on the CN-18 column

could be explained on the basis of ionic and electrostatic interactions between the solutes and the carnitine moiety of the stationary phase. Also, the L-CN-18 columns could be readily prepared by recycling the solution containing L-CN-18 through an ODS column; the column thus enables direct enantiomeric separation of DL-tryptophan, α-methyl-DL-tryptophan, and DL-3-indolelactic acid using 100% water as the mobile phase. The chiral recognition was shown to be based on the interaction via ionic and hydrogen bonding between the solutes and the ligand molecule. The L-CN-18 column could also be used for enantiomeric separations of amino acids and α-hydroxycarboxylic acids by ligand-exchange chromatographic mode using a mobile phase containing copper(II) ion.

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