Liquid Chromatography with Aminopropylsilica Gel Modified by Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin Derivative

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New stationary phases modified by heptakis(2,3,6-*O*-trimethyl)-β-cyclodextrin were prepared and tested as HPLC columns. The results show that these stationary phases exhibit significant chiral separation abilities for various types of aromatic compounds under reversed-phase conditions. Acetylation and propionylation of the residual amino groups on silica gel improve the retention capacity of the stationary phase and results in better chromatographic chiral separation. One of the most possible reasons for this superior chiral separation observed for the acylated stationary phases is considered to be the increased local concentration of the solutes on the hydrophobic surface of the stationary phase, which increases the possibility for the contact of solutes with the outside of cyclodextrin fixed on the silica gel.

Cyclodextrins are one of the most important host molecules for investigation of molecular recognition in an aqueous solution. These cyclic oligo-glucoses have been shown to recognize various types of organic compounds by their sizes and shapes to form so-called inclusion complexes and there have been many examples of their applications for biomimetic, organic, analytical, industrial chemistries, etc.[1] Among these examples, the applications to chromatography have been suggested as an interesting possibility of utilizations of cyclodextrins.[2] Since the scope of the separation ability of these cyclodextrins bonded stationary phase is expected to depend largely on the structural feature of fixed cyclodextrin itself, it is interesting and important to develop the stationary phases bearing various type of cyclodextrins which may more specifically interact with target compounds. For example, α- and β-cyclodextrin-bonded phases have been reported to show quite different separation effects according to their different size of binding cavities.[2]

Recently, we reported the synthesis of heptakis(2,3,6-*O*-trimethyl)-β-cyclodextrin (abb. to 2,3,6-*O*-trimethyl-β-cyclodextrin, hereafter) derivative which has carboxyl groups at specific C5 positions.[3] Since the 2,3,6-*O*-trimethyl-β-cyclodextrin is known to show higher hydrophobicity and different recognition ability compared with parent unmodified cyclodextrins,[4] it is interesting and useful to prepare the stationary phase for HPLC bearing these modified cyclodextrins. Here we report a first example of such stationary phase with chemically bonded 2,3,6-*O*-trimethyl-β-cyclodextrin. The most explicit difference between the stationary phases modified with present 2,3,6-*O*-trimethyl-β-cyclodextrin and those with simple β-cyclodextrin ever reported[2] is their hydrogen-bonding abilities, i.e., the present stationary phases completely lack hydroxyl groups of cyclodextrin which may function as hydrogen donors to form hydrogen bonds with guest molecules. The results show that the 2,3,6-*O*-trimethyl-β-cyclodextrin-modified stationary phase exhibits significant chiral separation abilities for various types of compounds, even if the hydroxy functionality of β-cyclodextrin is blocked.

Experimental

Materials. The stationary phases used in this work were prepared based on commercially available NH_2 type silica gel (Wakosil 5NH_2, 5 μ, 1.16 mmol 3-aminopropyl group/g). 5^A-Carboxy-5^A-dehydroxymethyl-2^A,2^B,2^C,2^D,2^E,2^F,2^G,3^A,3^B,3^C,3^D,3^E,3^F,3^G,6^B,6^C,6^D,6^E,6^F,6^G-icosa-*O*-methyl-β-cyclodextrin was prepared by the method reported previously.[3] *N*-(3,5-Dinitrobenzoyl)amino acids were prepared according to the standard method. Secondary alcohols used in this work were prepared by reduction of corresponding ketones by $NaBH_4$ following usual silica-gel chromatography. Flurbiprophen is kindly offered from Professor Kaneto Uekama, Kumamoto University. Commercially available HPLC grade methanol, hexane, 2-propanol, and water were used without further purification.

5^A-Chloroformyl-5^A-dehydroxymethyl-2^A,2^B,2^C,2^D,2^E,2^F,2^G,3^A,3^B,3^C,3^D,3^E,3^F,3^G,6^B,6^C,6^D,6^E,6^F,6^G-icosa-*O*-methyl-β-cyclodextrin (1). To a solution of 5^A-carboxy-5^A-dehydroxymethyl-2^A,2^B,2^C,2^D,2^E,2^F,2^G,3^A,3^B,3^C,3^D,3^E,3^F,3^G,6^B,6^C,6^D,6^E,6^F,6^G-icosa-*O*-methyl-β-cyclodextrin (5.0 g, 3.5 mmol) in THF (100 mL) was added 20 mL of thionyl chloride and the resultant solution was stirred at 50°C for 1 h. After evaporation of the solvent, the residual acid chloride was dried at room temperature in vacuo for 10 h. The obtained material was immediately used for the next reaction without further purification.

Preparation of (3-Aminopropyl) Silica Gel Modified by 2,3,6-*O*-Trimethyl-β-cyclodextrin (S.P.1). To a suspension of (3-aminopropyl) silica gel (5 g) in THF (50 mL) and pyridine (5 mL) was added a solution of **1** (5.0 g, 3.5 mmol) in THF (50 mL) at room temperature and the suspension was stirred at room temperature for 2 h. The modified silica gel, **S.P.1**, thus obtained was filtered, washed with THF, methanol, water and methanol in this order, and dried in vacuo for overnight.

Acetylation and Propionylation of Residual Amino Groups on Silica Gel (S.P.2 and S.P.3). To a suspension of **S.P.1** (2.4 g) obtained above in THF (150 mL) and triethylamine (3 mL) was added acetic anhydride (3 mL) and the suspension was stirred at room temperature for 3 h. The doubly modified silica gel, **S.P.2**, thus obtained was treated similarly to **S.P.1**. Propionylation of **S.P.1** was

similarly carried out by using propionyl chloride and the resulting stationary phase was used as **S.P.3**.

Apparatus. The stationary phases, **S.P.1**, **S.P.2**, and **S.P.3**, were packed in steel columns (4.6×250 mm) by using a usual slurry packing apparatus. A Waters Associates Model 6000 solvent delivery system equipped with Model 440 UV absorbance detector was used for HPLC experiments. The flow rate was usually 0.5 mL min^{-1} for the reversed-phase mode and 1.8 mL min^{-1} for the normal-phase mode, respectively.

Results and Discussion

The synthetic scheme of 2,3,6-*O*-trimethyl-*β*-cyclodextrin bonded phase is shown in Scheme 1. In order to modify the 3-aminopropyl groups on silica gel, the commercially available NH_2 type silica was treated with the acid chloride, **1**, prepared from the corresponding carboxylic acid and thionyl chloride. The weight analysis indicated that the silica gel thus obtained contained 0.26 g of the cyclodextrin/g of silica gel, which corresponded to the modification of ca. 16% of the entire 3-aminopropyl moiety by the present cyclodextrin. Since this cyclodextrin content was not changed by the repeated reaction with **1**, the observed content seemed to be the maximum one for the present highly bulky modification reagent, **1**. It should be noted that the reaction conditions for fixation of 2,3,6-*O*-trimethyl-*β*-cyclodextrin on the silica gel are extremely mild (at room temperature, for 2 h.) compared with those for unmodified cyclodextrin[2] and the relatively high cyclodextrin content is attained even under these mild conditions, because of the high reactivity of acid chloride, **1**, which is easily available for the present *O*-methylated cyclodextrin. For the evaluation of the effect of the residual basic amino groups, the obtained silica gel, **S.P.1**, was further treated with large excess of acetic anhydride and propionyl chloride to afford the doubly modified silica gel, **S.P.2** and **S.P.3**. These three types of 2,3,6-*O*-trimethyl-*β*-cyclodextrin bonded phase were tested as HPLC columns. Under reversed-phase conditions ($H_2O/CH_3CN=9:1$), **S.P.1**, **S.P.2**, and **S.P.3** had 10100, 7600, and 8400 plates per 25 cm for thymidine, respectively. The performances of **S.P.1**, **S.P.2**, and **S.P.3** are checked by using a mixture of *o*-, *m*-, *p*-nitroaniline which are hardly resolved by the original NH_2-type silica gel. The capacity factors, k', and separation factors, α, of these samples on the each column are summarized in Table 1. The stationary phases, **S.P.2** and **S.P.3**, which were acylated at the residual amino moiety, generally showed larger capacity factors for the same compound under the same conditions due to their higher hydrophobicity compared with original **S.P.1**. Furthermore, acylation of the residual amino moiety resulted in another advantage that the life time of **S.P.2** and **S.P.3** became significantly longer than that of **S.P.1**. After eight-month use, **S.P.2** and **S.P.3** were retaining practically same performances with those of the originals but **S.P.1** showed substantial deterioration of the performance such as a decrease in the capacity factor. These results seem to indicate that the protection of highly basic amino moieties by acylation is effective in preventing the detachment of the cyclodextrin moiety due to the hydrolysis of the amide bond.

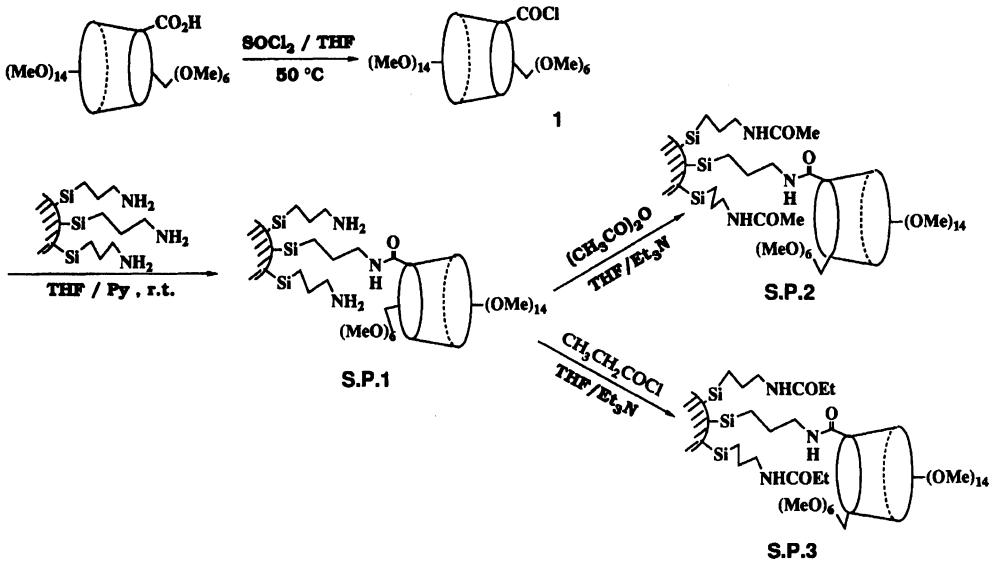
Table 1. Capacity Factors and Separation Factors of *o*-, *m*-, and *p*-Nitroanilines on **S.P.1**, **S.P.2**, and **S.P.3**[a)]

Eluent	Capacity factor (k')			Separation factor (α)	
H_2O/MeOH	$k'_{o\text{-}}$	$k'_{m\text{-}}$	$k'_{p\text{-}}$	$\alpha_{m\text{-}/o\text{-}}$	$\alpha_{m\text{-}/o\text{-}}$
S.P.1					
9/1	4.97	3.16	9.38	0.64	1.89
S.P.2					
9/1	6.05	5.26	10.41	0.87	1.85
7/3	3.49	3.74	6.08	1.07	1.63
S.P.3					
9/1	22.49	22.49	41.27	1.00	1.84
7/3	12.06	12.76	22.00	1.05	1.82
1/1	4.44	5.72	6.99	1.28	1.57

a) Flow rate: 0.5 mL min^{-1}, at 20°C.

As expected, the elution order on **S.P.1** under highly hydrophilic conditions ($H_2O/MeOH=9/1$) was *m*-<*o*-<*p*-nitroaniline which was same with that of previously reported *β*-cyclodextrin-modified stationary phase.[2a)] In contrast with **S.P.1**, however, **S.P.2** and **S.P.3** showed poorer *m*-/*o*-separation factor under same conditions in spite of their larger capacity factors. Furthermore, the elution order on **S.P.2** and **S.P.3** under more hydrophobic conditions ($H_2O/MeOH=7/3$ or 1/1) changed into *o*-<*m*-<*p*-nitroaniline. These observations suggest that separation of *o*-/*m*-nitroaniline on the present stationary phases is governed by two different mechanisms such as inclusion complex formation of 2,3,6-*O*-trimethyl-*β*-cyclodextrin with solute molecules in hydrophilic media as previously reported for unmodified *β*-cyclodextrin-bonded phase[2] and collisional interaction of solute molecules with the outside surface of cyclodextrin in relatively hydrophobic environment as observed for cellulose-coated stationary phases.[5]

Based on these results, the characteristics of **S.P.1**, **S.P.2**, and **S.P.3** as the chiral stationary phase were investigated. The typical examples of chiral separation obtained are shown in Fig. 1 and Table 2. The results showed that, although the compounds investigated here were relatively limited, these stationary phases, especially **S.P.2** and **S.P.3**, exhibited significant chiral separation abilities for *N*-(3,5-dinitrobenzoyl) derivatives of aromatic amino acid methyl esters, diphenylmethanol derivatives and so on. Among compounds investigated in this work, only enantiomers of *N*-(3,5-dinitrobenzoyl)phenylalanine methyl ester were resolved under the normal-phase conditions using **S.P.2** (hexane/2-propanol=9/1). The superior chiral separation under reversed-phase conditions indicates that the chiral separation on the present stationary phase basically



Scheme 1.

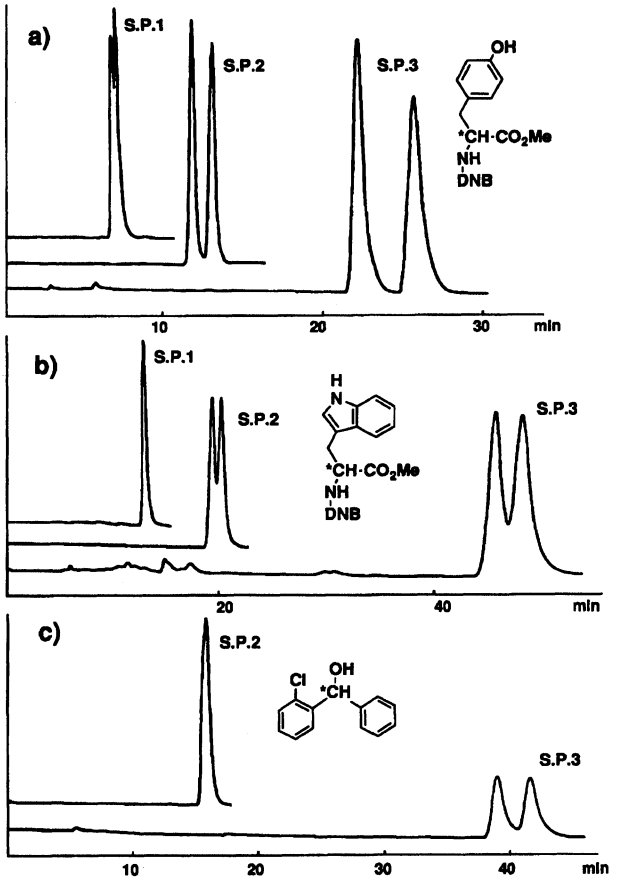
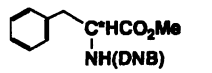
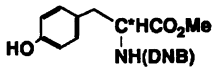
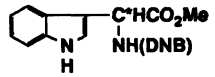
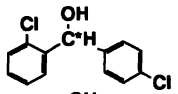
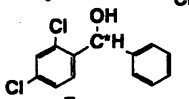
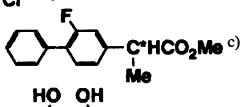
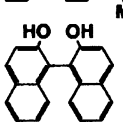
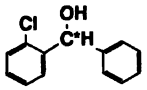
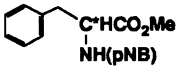
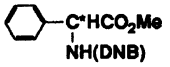
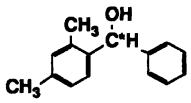


Fig. 1. Chromatogram showing chiral separation of a) *N*-(3,5-dinitrobenzoyl)tyrosine methyl ester, b) *N*-(3,5-dinitrobenzoyl)tryptophan methyl ester, c) (*o*-chlorophenyl)phenylmethanol. The chromatographic conditions are shown in Table 2.

proceeds via specific interactions between enantiomeric compounds and 2,3,6-*O*-trimethyl-*β*-cyclodextrin in hydrophobic environment. Another interesting aspect of the present results are the different chromatographic behavior between **S.P.1** and acylated stationary phases, **S.P.2** and **S.P.3**. The results shown in Table 2 clearly indicate that performances of these stationary phases for chiral separation are improved in order of their surface hydrophobicity, i.e., among compounds listed in Table 2, seven and nine enantiomers are resolved by **S.P.2** and **S.P.3** respectively, while only two by **S.P.1**. It is clear that poor chiral separation by **S.P.1** is not due to its poor capacity factor under present eluent conditions (H_2O/MeOH = 1/1), because, even if eluent conditions resulting higher capacity factor such as H_2O/MeOH = 9 : 1 are used, the performance for chiral separation is not improved and only band broadening is generally observed. Furthermore, the present chiral separation seems to be very sensitive for the structure of enantiomers, though the compounds having aromatic moieties were generally well-resolved by **S.P.2** and **S.P.3**. For example, the *N*-(3,5-dinitrobenzoyl) derivative of phenylalanine methyl ester was well resolved but that of phenylglycine methyl ester was not and (2,4-dichlorophenyl)phenylmethanol was well resolved but (2,4-dimethylphenyl)phenylmethanol was not (see Table 2). These observations suggest that the chiral resolution by present stationary phases strongly depends not only on the spatial structures but also on electronic effect of enantiomers. It should also be noticed that there seems to be no clear relationship between effectiveness of chiral separation and structural fitness of solute molecules to the cyclodextrin cavity. Thus, although it is difficult to show what is the most important specific interaction for the chiral separation by present cyclodextrin-bonded stationary phases at the present stage, the data obtained here indicate that the main driving force for observed chiral separation may

Table 2. Chromatographic Data on Resolution of Racemic Compounds on **S.P.1**, **S.P.2**, and **S.P.3**[a)]

Compounds	S.P.1			S.P.2			S.P.3		
	k'	α	R_S	k'	α	R_S	k'	α	R_S
	0.32 0.36	1.12	0.46	1.34 1.55	1.17	1.38	8.35 9.95	1.19	1.94
	1.26 1.52	1.18	0.96	1.25 1.52	1.21	1.69	8.70 10.5	1.21	2.18
	0.60 0.60	1.00	0	2.56 2.72	1.06	0.65	19.50 20.72	1.06	1.02
	2.19 2.19	1.00	0	3.48 3.68	1.06	0.91	37.75 43.25	1.15	2.53
	—	—	—[b)]	3.01 3.25	1.08	1.06	32.14 35.21	1.10	2.48
	—	—	—[b)]	5.72 6.06	1.06	1.06	38.90 44.35	1.14	2.69
	1.86 1.86	1.00	0	3.58 3.70	1.03	0.27	25.70 29.62	1.15	2.29
	—	—	—[b)]	2.32 2.32	1.00	0	16.05 17.30	1.08	1.39
	0.36 0.36	1.00	0	1.34 1.34	1.0	0	9.70 10.15	1.05	0.62
	0.26 0.26	1.00	0	1.34 1.34	1.00	0	8.5 8.45	1.00	0
	—	—	—[b)]	1.24 1.24	1.00	0	4.55 4.55	1.00	0

a) The chromatographic conditions are as follows, column: 4.6×250 mm, mobile phase: H_2O/MeOH=1/1, flow rate: 0.5 mL min^{-1}, detection: 254 nm. The abbreviations, DNB: 3,5-dinitrobenzoyl, pNB: *p*-nitrobenzoyl, k': capacity factor, α: separation factor, R_S: resolution factor. b) Not measured. c) Fluorobiprophen.

be based on a mechanism other than an inclusion complex formation. One of the most possible candidate of this mechanism is a considerable increase of local concentration of the solutes on the hydrophobic surface of the stationary phase. Such increase of local concentration enhances a possibility for contact of solutes with the outside of cyclodextrin fixed on the silica gel more effectively than that with the inside of the cyclodextrin cavity. Thus, the increase of surface hydrophobicity of the stationary phase may result in superior chiral separation, if the interaction of solute molecules with the outside of 2,3,6-*O*-trimethyl-*β*-cyclodextrin is more sensitive for molecular chirality than that with its inside.

Finally, it should be noted that, since the present cyclodextrin is attached at rather short distance from the surface of the silical gel, the molecular recognition by cyclodextrin moieties may be affected by a surface effect of the silica gel. Therefore it should be an interesting and valuable next target to develop a new stationary phase which have a longer spacer moiety between 2,3,6-*O*-trimethyl-*β*-cyclodextrin and a silica gel. Such a stationary phase is expected to reflect the characteristics of 2,3,6-*O*-trimethyl-*β*-cyclodextrin on the chiral resolution more directly. Since the present synthetic method developed here is widely applicable to the variety of such modifications, we are now continuing further investigations along this line.

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