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Functional Monomers and Polymers CLXXIII.† Immobilization of Nucleic Acid Bases on Silica Gel and Application to HPLC for Selective Separation of Nucleosides

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ABSTRACT: Nucleic acid base derivatives were immobilized on 3-aminopropylsilanized silica (APS-silica) and silica gel. The bases immobilized were thymine, uracil, cytosine, adenine, guanine, and hypoxanthine. These silica gels were proved to be useful as columns of high performance liquid chromatography (HPLC) for the selective separation of nucleosides using specific interaction between complementary bases.

KEY WORDS Nucleic Acid Base / Adenine / Uracil / Thymine / Guanine / Cytosine / Hypoxanthine / Silica Gel / Nucleoside / High Performance Liquid Chromatography /

High performance liquid chromatography (HPLC) has been used widely for the separation and analysis of nucleic acid fragments and their related compounds. HPLC systems such as ion exchange, gel permeation and reversed phase give satisfactory results for the separation of nucleosides and oligonucleotides.[1] However, the separations by these methods are not to be caused by the complementary interaction between nucleic acid bases which is widely known for nucleic acid chemistry. The specific interactions as seen for nucleic acids have been also found in synthetic analogs of nucleic acids, synthetic polymers containing nucleic acid bases.[2] The specific interactions observed in the synthetic analogs of nucleic acids can be applied to the HPLC system for separation of nucleic acid fragments. Though the nucleic acid base derivatives were immobilized on silica gel or synthetic polymers for the separation of nucleic acid bases and nucleosides.[3-9] It was, however, very difficult to separate the factors governing the separation of the components by these system.

In the previous report, we described that poly-L-lysine derivatives containing nucleic acid bases were immobilized on silica gel, and the separations were studied for oligonucleotide analogs.[10] The present paper deals with the specific separation of nucleosides by silica gels which bound nucleic acid bases, such as thymine, uracil, adenine, cytosine, guanine and hypoxanthine.

EXPERIMENTAL

Thymine Immobilized APS-Silica; Si-N-Thy (**2**)

To a solution of pentachlorophenyl 3-(thymin-1-yl)propionate[11] (**1**) (1.0 g; 2.2 mmol) and imidazole (Im) (50 mg) in dimethylformamide (DMF; 10 ml), 3-aminopropylsilanized

† For Part CLXXII, see T. Wada, Y. Inaki, and K. Takemoto, *Polym. J.*, **20**, 1059 (1988).

silica (APS-silica) (LiChrosorb NH_2, average particle size; 5 μm, nominal pore size; 60 Å, E. Merck) (2.0 g) was dispersed and kept at 70°C for 48 h. After the reaction, the silica gel was filtered and washed successively with DMF, water, methanol, ethanol, acetone, chloroform, and diethyl ether. The amino group remained in the APS-silica was protected by *p*-nitrophenyl acetate (PNPA; 0.25 g) in pyridine (10 ml) with imidazole (50 mg) catalyst at room temperature for 24 h. The silanol group in the APS-silica was endcapped by hexamethyldisilazane (HMDS; 1.0 ml) in toluene (10 ml) at room temperature for 24 h. After blocking of free amino and silanol groups, the suspension was filtered and washed thoroughly.

Uracil Immobilized APS-Silica; Si-N-Ura (**4**)

The silica gel (**4**) was prepared by the method similar to (**2**), by the reaction of pentachlorophenyl 3-(uracil-1-yl)propionate[12] (**3**) (1.0 g; 2.3 mmol) and APS-silica (2.0 g) with imidazole (50 mg). Free amino and silanol groups were endcapped with PNPA and HMDS, respectively.

Cytosine Immobilized APS-Silica; Si-N-Cyt (**6**)

The silica gel (**6**) was also prepared by the method similar to (**2**), by the reaction of pentachlorophenyl 3-(cytosin-1-yl)propionate[13] (**5**) (1.0 g; 2.3 mmol) and APS-silica (2.0 g) with imidazole (50 mg). Free amino and silanol groups were also endcapped with PNPA and HMDS, respectively.

Adenine Immobilized APS-Silica; Si-N-Ade (**9**)

The silica gel (**9**) was prepared by the reaction of the protected adenine derivatives, followed by deprotection of the protected group. Si-N-Ppt-Ade (**8**) was prepared according to the method used for the silica gel (**2**) by the reaction of pentachlorophenyl 3-(N^6-diphenylphosphinothioyl-adenine-9-yl)propionate[2] (**7**) (1.5 g; 2.2 mmol) and APS-silica (2.0 g) with imidazole (50 mg). The protecting group of adenine was removed by HBr in acetic acid (10 ml) at room temperature for 24 h. After the reaction, the silica gel was filtered and washed with sorts of solvents thoroughly, followed by treating with PNPA and HMDS.

2-(Guanin-7-yl)propionic Acid (**10**)

To a solution of guanine (2.0 g; 13.2 mmol) and KOH (0.74 g; 13.2 mmol) in water (50 ml), acrylonitrile (2.5 ml; 38 mmol) was added, stirred for 2 h at room temperature, and then refluxed until the evolution of NH_3 gas ceased. To the solution, 0.1 *N* HCl was added to adjust the system to pH 4, which then gave 2-(guanin-7-yl)propionic acid (**10**); 2.3 g, 77.8% yield. MS calcd. for $C_8H_9N_5O_3$ 223.19, found 223. UV λ_{max} (nm): 254.6, 278.0 (in H_2O); 249.6, 272.8 (pH 1); 276.0 (pH 13). ^{1}H NMR (in dimethyl sulfoxide-d_6 at 25°C, ppm): 2.77 (t, 2H); 4.13 (t, 2H); 6.47 (s, 2H); 7.59 (s, 1H); 10.64 (br s, 1H).

Guanine Immobilized APS-Silica; Si-N-Gua (**11**)

To a suspension of (**10**) (1.0 g; 4.5 mmol) in pyridine (50 ml), pentachlorophenyl trichloroacetate (2.0 g; 4.9 mmol) was added and heated until the system turned to clear solution. Then APS-silica (2.0 g) was added to the solution, kept further for 48 h at 70°C, followed by filtering and washing. Free silanol group of guanine immobilized silica was endcapped with HMDS in toluene.

Hypoxanthine Immobilized APS-Silica; Si-N-Hyp (**13**)

The silica derivative (**12**) was prepared similarly to (**2**), from the reaction of pentachlorophenyl 3-(hypoxanthin-9-yl)propionate[14] (1.0 g; 2.2 mmol) and APS-silica (2.0 g). Free amino and silanol groups on the silica were endcapped with PNPA and HMDS, respectively.

Thymine Immobilized on Silica Gel; Si-O-Thy (**15**)

To a solution of (**1**) (1.0 g; 2.2 mmol) in

DMF (50 ml), 3-aminopropyltriethoxysilane (0.51 ml; 2.2 mmol) was added and stirred for 2 h at room temperature. After the reaction, the solution was concentrated to 10 ml, and poured it into excess acetone to give the silane derivative of thymine (EtO-Si-Thy) (**14**). IR (cm^{-1}): 1640, 1540 (amide), 1110 (Si-O-Si).

To a solution of (**14**) (0.83 g; 2.0 mmol) in DMF (10 ml), silica gel (LiChrosorb Si 100, average particle size; 5 μm, pore size; 100 Å, surface area; 300 $m^2 g^{-1}$, E. Merck) (2.0 g) was dispersed and kept for 48 h at 70°C, followed by filtering and washing, and endcapping with HMDS in toluene.

Uracil Immobilized on Silica Gel; Si-O-Ura (**17**)

The silane derivative of uracil (EtO-Si-Ura) (**16**) was prepared similarly to (**14**), from the reaction of (**3**) (1.0 g; 2.3 mmol) and 3-aminopropyltreithoxysilane (0.53 ml; 2.3 mmol). IR (cm^{-1}): 1650, 1550 (amide), 1100 (Si-O-Si).

The silica gel derivative (**17**) was prepared similarly to (**15**), from the reaction of (**16**) (0.81 g; 2.0 mmol) and silica gel (2.0 g). Free silanol group was endcapped with HMDS in toluene.

Cytosine Immobilized on Silica Gel; Si-O-Cyt (**19**)

The silane derivative of cytosine (EtO-Si-Cyt; **18**) was prepared similarly to (**14**), from the reaction of (**5**) (1.0 g; 2.3 mmol) and 3-aminopropyltriethoxysilane (0.53 ml; 2.3 mmol).

The silica gel derivative (**19**) was prepared by the method similar to (**15**), by the reaction of (**18**) (0.81 g; 2.0 mmol) with silica gel (2.0 g). Free silanol groups was endcapped with HMDS in toluene.

Adenine Immobilized on Silica Gel; Si-O-Ade (**21**)

The silane derivative of adenine (EtO-Si-Ppt-Ade; **20**) was prepared by the method similar to (**14**), by the reaction of (**7**) (2.0 g; 3.0 mmol) with 3-aminopropyltriethoxysilane (0.7 ml; 3.0 mmol). IR (cm^{-1}): 1645, 1540 (amide), 1100 (Si-O-Si).

To a solution of (**20**) (1.29 g; 2.0 mmol) in DMF (10 ml), silica gel (2.0 g) was dispersed and kept at 70°C for 48 h. The silica gel obtained was treated with HBr gas in acetic acid (5 ml) to remove the protecting group in adenine, and the free silanol group was endcapped by HMDS.

Hypoxanthine Immobilized on Silica Gel; Si-O-Hyp (**23**)

The silane derivative of hypoxanthine (EtO-Si-Hyp; **22**) was prepared by the method similar to (**14**), by the reaction of (**12**) (1.0 g, 2.2 mmol) with 3-aminopropyltriethoxysilane (0.51 ml, 2.2 mmol). IR (cm^{-1}): 1640, 1540 (amide), 1110 (Si-O-Si).

The silica gel derivative (**23**) was prepared by the method similar to (**15**), by the reaction of (**22**) (0.83 g; 2.0 mmol) and silica gel (2.0 g). Free silanol group was endcapped with HMDS in toluene.

Instrumentation

IR spectra were measured with a JASCO IR-810 spectrophotometer. As the IR spectra in KBr were generally too weak, the samples were made in pellet form by direct pressurization of the silica derivatives at 600 bar for 30 min. UV spectra were measured with a JASCO UVIDEC-660 spectrophotometer. ESCA spectra were measured with a Shimadzu ESCA 750.

HPLC System

The silica gels prepared were packed into stainless steel tubes (15 cm in length, 4.6 mm i.d.) under constant flow (9 ml min^{-1}) of chloroform solvent.

The experiments were performed with a Toyo soda high speed liquid chromatograph series HLC 803D, equipped with a thermostated column oven and a UV detector operating at 254 nm. Temperature of column-oven was set at desired temperature. Methanol, ac-

Table I. Analytical data for the nucleic acid bases immobilized on silica gel

Compound	Base[a] content/ 10^{-4} mol g^{-1}	Base[b] content/ 10^{-4} mol g^{-1}
Si-N-Thy	2.87	2.56
Si-N-Ura	4.26	—
Si-N-Cyt	1.50	—
Si-N-Ade	1.59	2.41
Si-N-Gua	2.92	—
Si-N-Hyp	1.47	—
Si-O-Thy	2.49	4.40
Si-O-Ura	2.39	2.79
Si-O-Cyt	2.01	3.00
Si-O-Ade	1.80	—
Si-O-Hyp	1.29	—

[a] Calcd from UV spectra.
[b] Calcd from elementary analysis.
* All resins were before endcapping treatment.

etonitrile and water used as chromatographic solvents, were analytical-reagent grade. The amount of sample injected was 1.67 μg to 2.67 μg, dissolved in 1 μl of water–methanol mixture. Flow rate was set at 1.0 ml min^{-1}.

Determination of the Nucleic Acid Base Contents in the Silica Gel

The silica gel derivative (100 mg) was hydrolyzed in 6 *N* hydrochloric acid (10 ml) at 100°C for 48 h into the carboxyethyl derivative of the nucleic acid base and silica gel. After the reaction, the silica gel was filtered off and washed with water (90 ml). The quantitative calculation was made by the UV spectra of the filtrate using the corresponding carboxyethyl derivatives as standard sample. The nucleic acid base contents in the silica gels are tabulated in Table I.

RESULTS AND DISCUSSION

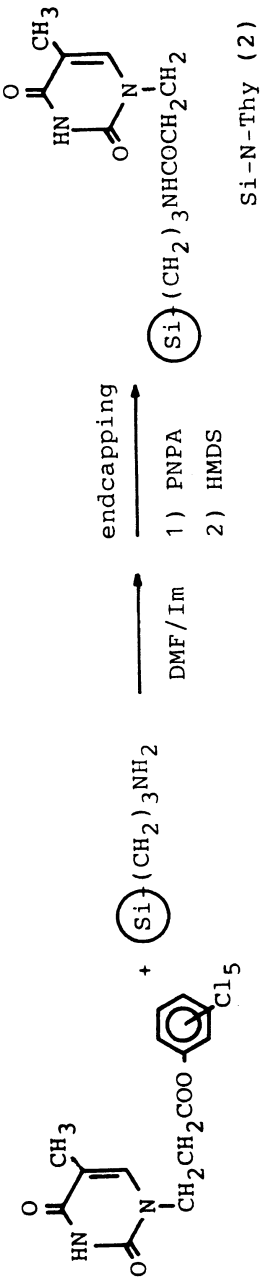
Preparation of the Silica Gels Containing Nucleic Acid Bases

APS-Silica Derivatives. The silica gels for HPLC containing the nucleic acid bases were prepared using either silica gel or APS-silica as the support materials. The APS-silica is a convenient material for immobilization of the nucleic acid base derivatives, but it is anticipated that the amino groups remained may affect the separation factors. The nucleic acid base immobilized APS-silicas was prepared according to the methods as shown in Schemes 1—6. The nucleic acid base derivatives were immobilized onto APS-silica by amide linkage using the activated ester method. By the same method, the nucleic acid base derivatives were successfully grated onto polyethyleneimine and polylysine.[2]

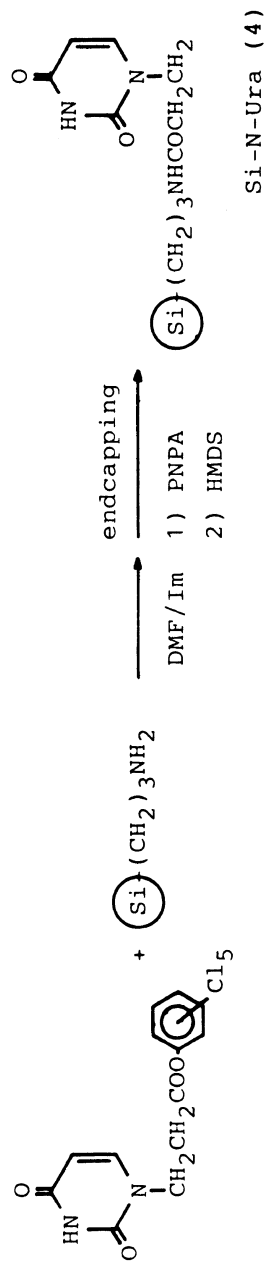
The activated ester of thymine derivative (**1**) was allowed to react with the amino group of APS-silica to give Si-N-Thy (**2**). The free silanol and amino groups remained were endcapped with HMDS and PNPA, respectively. Similar methods were applied to the preparation of silica gels containing uracil (Si-N-Ura; **4**), cytosine (Si-N-Cyt; **6**), adenine (Si-N-Ade; **9**), guanine (Si-N-Gua; **11**) and hypoxanthine (Si-N-Hyp; **13**). In the case of preparing Si-N-Ade (**9**), however, the amino group of adenine base was protected by diphenylphosphinothioyl (Ppt) group, because of the instability of the activated ester.[2]

Silica Gel Drivatives. The immobilization of the nucleic acid base derivatives onto silica gel was carried out using the triethoxysilane derivatives of the nucleic acid bases as shown in Scheme 7.

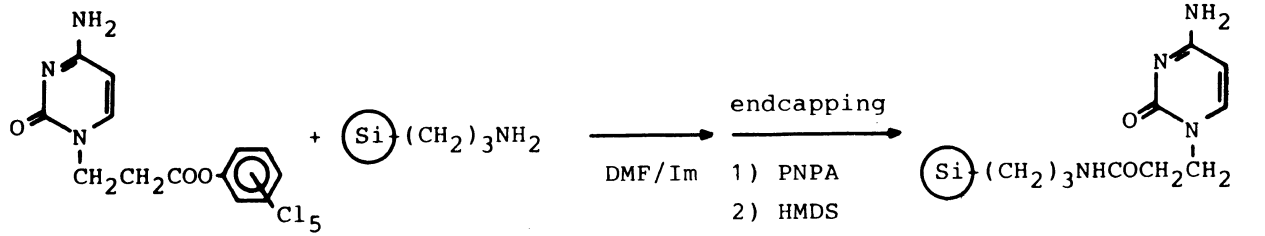
The triethoxysilane derivatives containing nucleic acid base were prepared by the reaction of the activated ester derivatives with 3-aminopropyltriethoxysilane. The reaction of the triethoxysilane derivative with silica gel gave the nucleic acid base immobilized silica gel by forming Si-O-Si bond. And the free silanol group was endcapped with HMDS. These reactions gave the silica gel derivatives containing thymine (Si-O-Thy; **15**), uracil (Si-O-Ura; **17**), cytosine (Si-O-Cyt; **19**), adenine (Si-O-Ade; **21**) and hypoxanthine (Si-O-Hyp; **23**), respectively. Immobilization of the nucleic acid base derivatives on the silica gels was confirmed by IR spectra, and ESCA spectra.



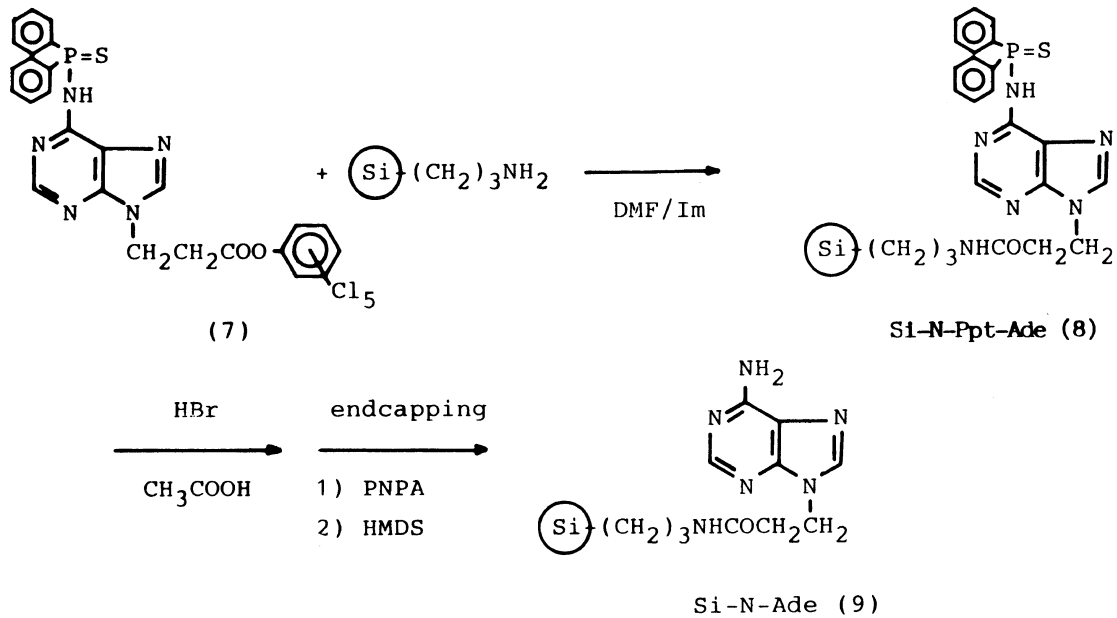
Scheme 1.



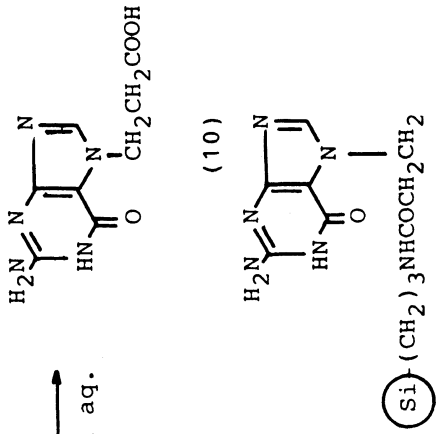
Scheme 2.

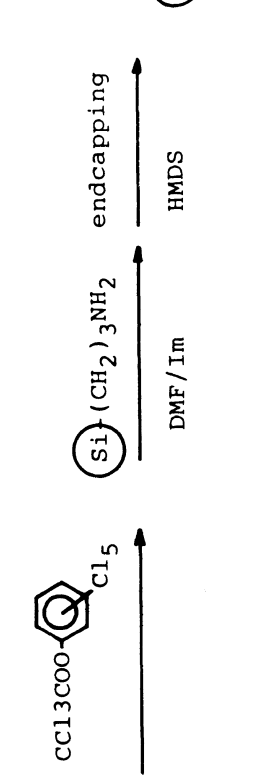


Scheme 3.

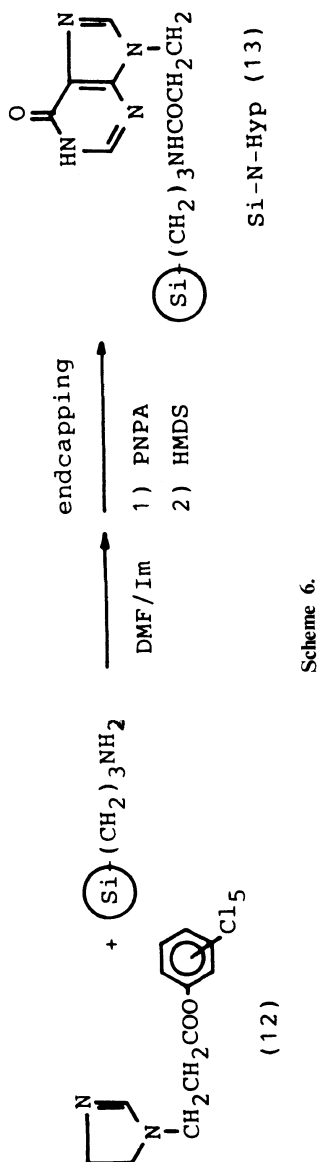


Scheme 4.

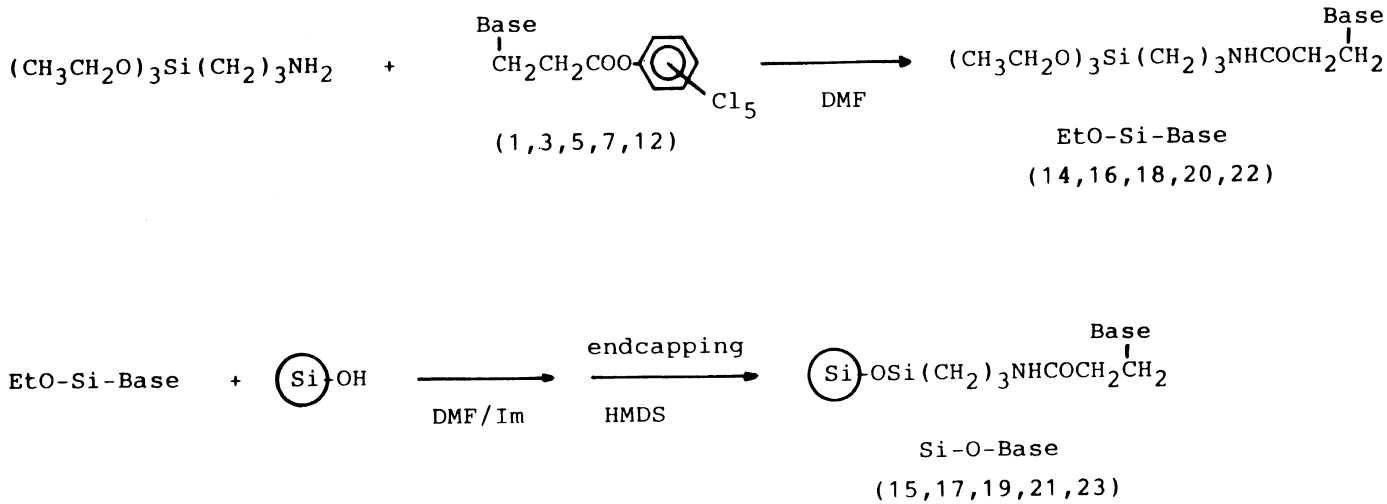




Scheme 5.



Scheme 6.


(CH3CH2O)3Si(CH2)3NH2
+
Base
CH2CH2COO
Cl5
(1,3,5,7,12)
DMF
(CH3CH2O)3Si(CH2)3NHCOCH2CH2
Base
EtO-Si-Base
(14,16,18,20,22)
EtO-Si-Base
+
Si-OH
DMF/Im
endcapping
HMDS
Si-OSi(CH2)3NHCOCH2CH2
Base
Si-O-Base
(15,17,19,21,23)

Scheme 7.

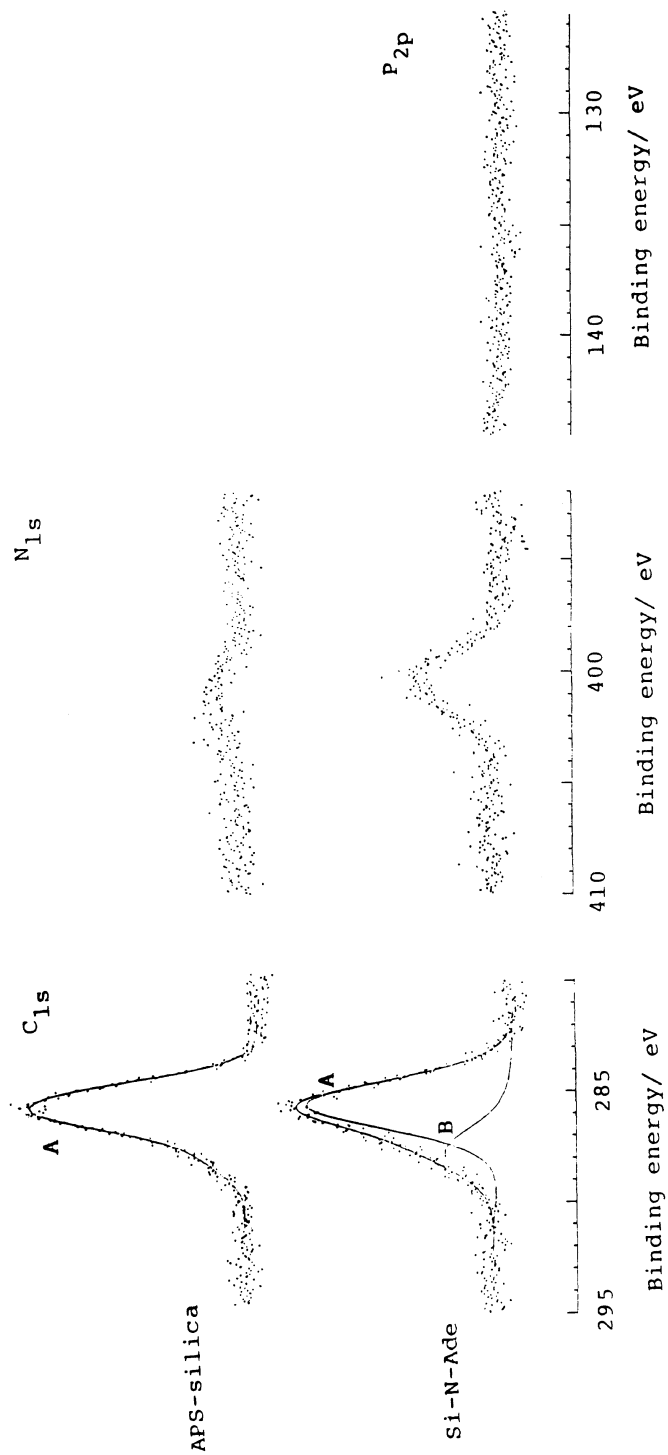


Figure 1. ESCA spectra of APS-silica and Si-N-Ade. Peaks: (A), C_{C-O} fraction; (B), $C_{C=O}$ fraction.

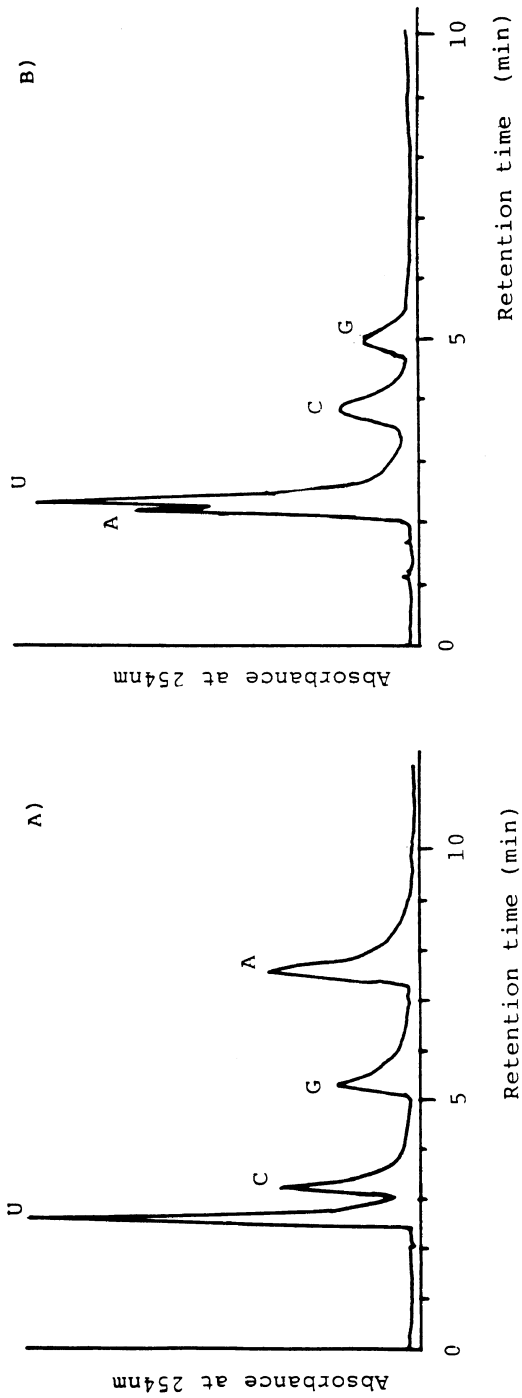


Figure 2. Chromatograms of nucleosides on Si-N-Gua with A) water and B) methanol mobile phases. Flow rate, 1.0 ml min^{-1}; temperature, 20°C; detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

The IR spectra for each resin suggested the presence of each nucleic acid base units in the silica gel derivative. Figure 1 shows the ESCA spectra of APS-silica and Si-N-Ade. In the spectra of Si-N-Ade, the C_{1s} content corresponding to the $C_{C=O}$ fraction of amide and the N_{1s} content were substantially high compared with those of APS-silica. The result indicates the immobilization of the adenine derivative onto APS-silica. The lack of the P_{2p} spectrum in the Si-N-Ade in Figure 1 suggests that the Ppt group in Si-N-Ppt-Ade was completely removed to give Si-N-Ade. These results suggest that the measurement of ESCA is effective for the analysis of the silica gel surface.

The content of the nucleic acid base units in the silica gel was quantitatively determined by the elementary analysis and the UV spectra of the hydrolyzed samples. Table I shows the results of quantitative determination of base contents in the silica gel derivatives. The content of free amino group in APS-silica was 8.01×10^{-4} mol g^{-1} from elementary analysis.

Selective Separation of Nucleoside by HPLC Si-N-Base Column Systems. Figure 2 shows the chromatograms of nucleosides on Si-N-Gua with water and with methanol mobile phases. In Figure 2A, adenosine and guanosine eluted late in water mobile phase, because of the hydrophobic interaction of adenine and guanine in the mobile phase with guanine on the silica gel. A similar chromatogram was obtained using the ODS column (TSK-gel, TOSOH Co.) which was the reverse-phase column containing C_{18} alkyl chain (Figure 3). On the other hand, in methanol mobile phase (Figure 2B), the retention time of the complementary nucleoside (cytidine) was considerably longer than that of other nucleosides. A hydrophobic interaction in methanol mobile phase is small, therefore the separation in methanol mobile phase should be caused by the specific hydrogen bonding interaction between complementary nucleic acid bases.

Figure 4 shows the chromatograms of nucleosides on Si-N-Cyt with water and with methanol mobile phases. Adenosine eluted late in water mobile phase (Figure 4A), but eluted

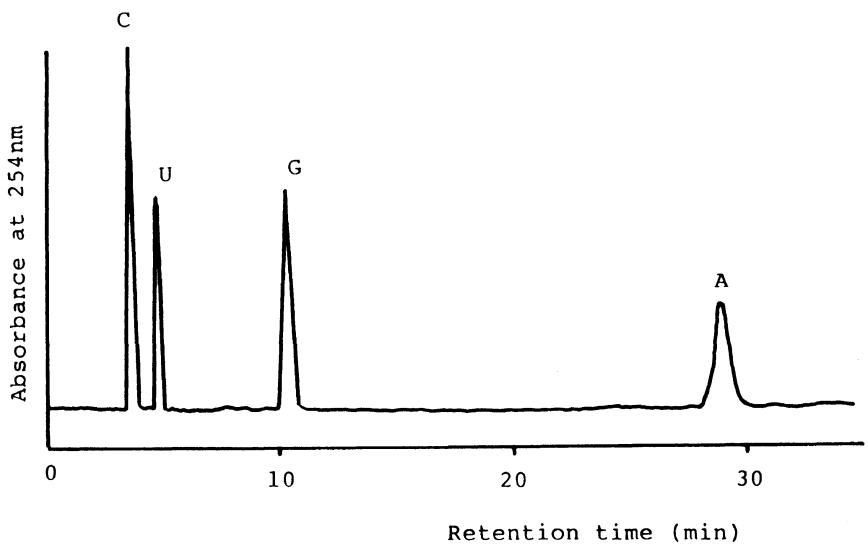


Figure 3. Chromatogram of nucleoside on ODS column (TSK-gel). Mobile phase, 1% CH_3CN aq.; flow rate, 1.0 ml min^{-1}; temperature, 26°C; detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

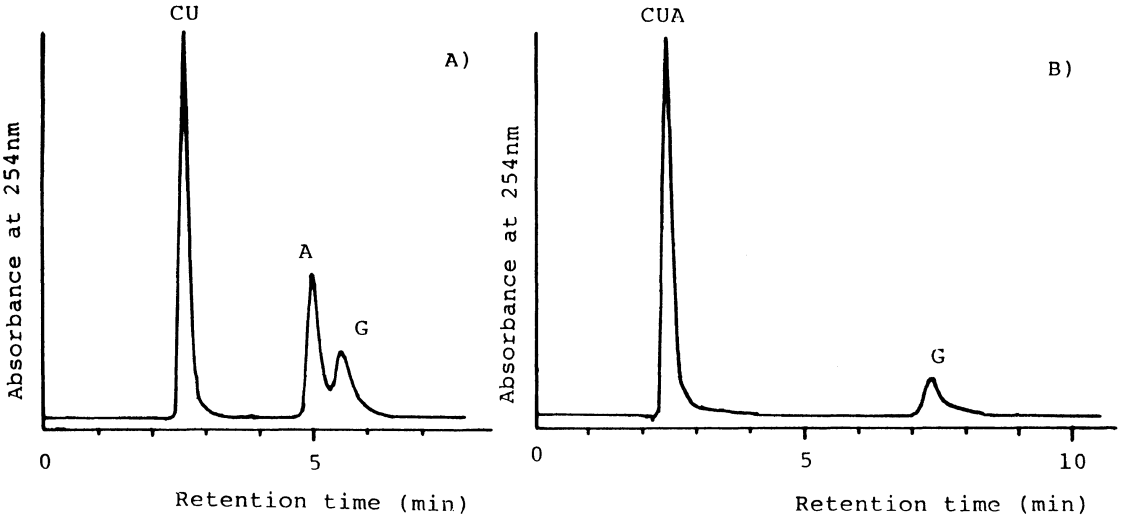


Figure 4. Chromatograms of nucleosides on Si-N-Cyt with A) water and B) methanol mobile phases. Flow rate, 1.0 ml min^{-1}; temperature, 20°C; detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

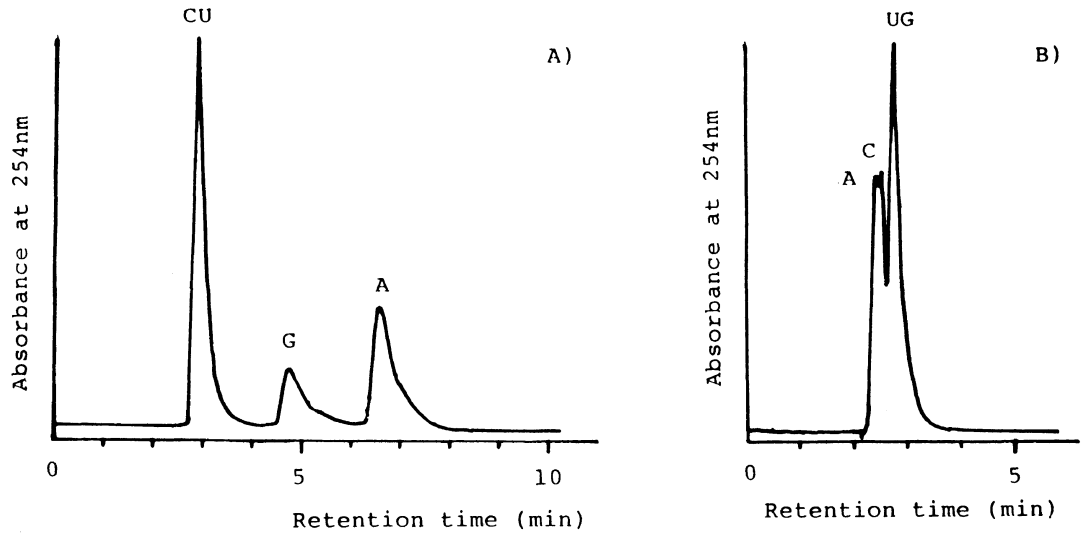


Figure 5. Chromatograms of nucleosides on Si-N-Ade with A) water and B) methanol mobile phases. Flow rate, 1.0 ml min^{-1}; temperature, 20°C; detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

fast in methanol mobile phase (Figure 4B). It can be assumed that the separation of adenosine in water is caused by the hydrophobic interaction, because adenosine is not the complementary base of cytosine. On the contrary, guanosine eluted late both in water and in methanol mobile phases. Guanosine eluted later in methanol than in water mobile phase, and only guanosine can be separated from other nucleosides in methanol mobile phase (Figure 4B). Guanosine is the complementary nucleoside of cytosine, therefore the former

was separated by the specific base-base interaction between guanine and cytosine.

Figure 5 shows the chromatograms of nucleosides on the Si-N-Ade column system. Adenosine and guanosine eluted late in water mobile phase (Figure 5A), because of the hydrophobic interaction. If the chromatogram 4A is compared with the chromatogram 5A, elution of guanosine is later than adenosine in 4A, but the chromatogram in 5A shows a reversed result. This fact suggests that the separation of guanosine on Si-N-Cyt in water (4A) was caused both by the hydrophobic interaction and by the specific base-base interaction.

In methanol mobile phase on the Si-N-Ade column system (Figure 5B), on the other hand, uridine and guanosine eluted later than other nucleosides. The chromatogram 5B is markedly different from that of 4B, suggesting strong base-base interaction (cytosine : guanine) in the 4B system. Uridine is a complementary base of adenine, therefore the elution of uridine should be later than that of guanosine in 5B. However, the retention time of uridine in Figure 5B was the same as that of guanosine. This reason may be attributed

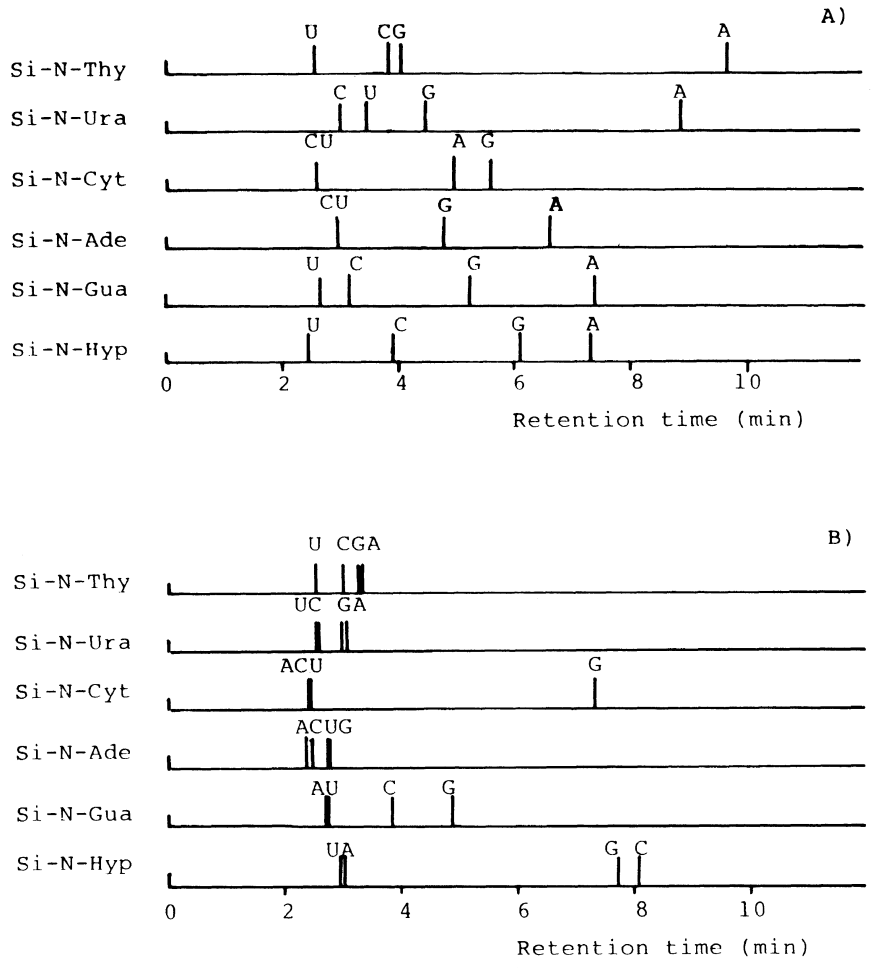


Figure 6. Retention times of nucleosides on Si-N-Base with A) water and B) methanol mobile phases. Flow rate, 1.0 ml min^{-1}; temperature, 20°C: detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

to the intramolecular interaction of adenine bases in Si-N-Ade, which may inhibit the intermolecular interaction with uracil base. The intramolecular interaction of nucleic acid bases in the silica gel derivatives was observed for purine bases (guanine and hypoxanthine).

The results of the separation study are summarized in both Figures 6A (water mobile phase) and 6B (methanol mobile phase) for six kinds of Si-N-Base column systems. When the mobile phase was water as shown in Figure 6A, two important factors should be considered; the one was hydrophobic interaction, and the other was the specific base-base interaction. In the case of the pyrimidine base bound column systems (Si-N-Thy, Si-N-Ura, and Si-N-Cyt), the specific hydrogen bonding between the complementary nucleic acid bases seems to be the predominant factor. Therefore it can be well explained that the complementary nucleoside (adenosine for Si-N-Thy and Si-N-Ura, and guanosine for Si-N-Cyt) eluted later than other nucleosides.

In the case of purine base bound column systems (Si-N-Ade, Si-N-Gua, and Si-N-Hyp), on the other hand, the profiles of the separation resemble to that of the ODS column with water mobile phase. Therefore, the predominant factors of the separation in the case of the purine base immobilized column in water mobile phase can be assumed as the hydrophobic interaction between purine bases

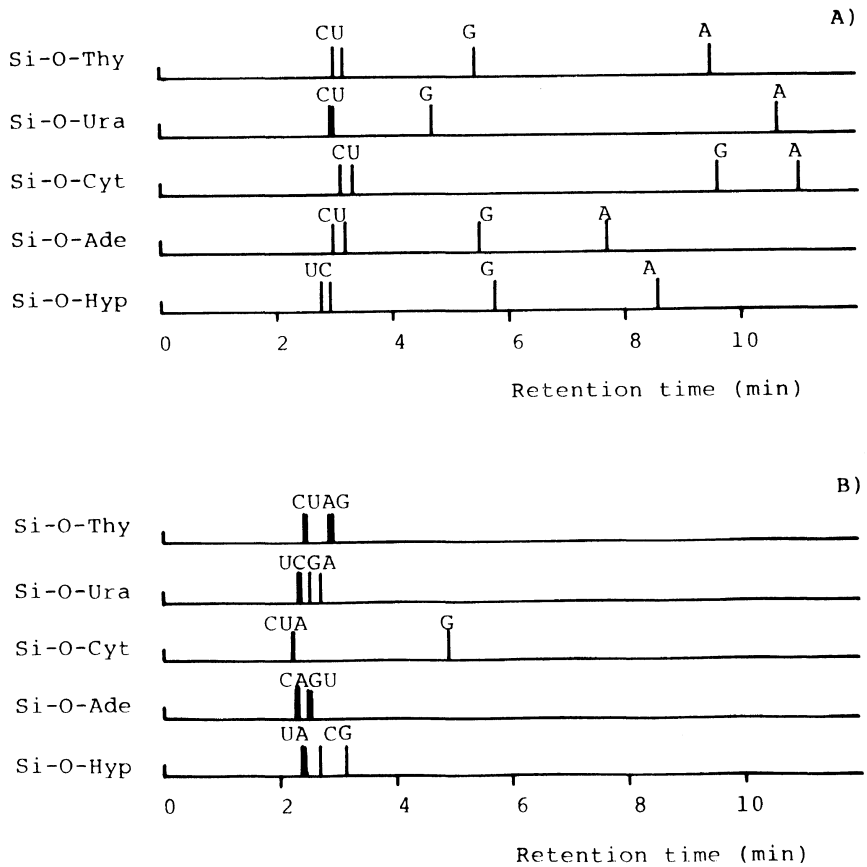


Figure 7. Retention times of nucleosides on Si-O-Base with A) water and B) methanol mobile phases. Flow rate, 1.0 ml min^{-1}; temperature, 20°C; detector, 254 nm. Peaks: (A), adenosine; (C), cytidine; (U), uridine; (G), guanosine.

in mobile phase and the purine base on the silica gel.

In methanol mobile phase (Figure 6B), the complementary nucleosides eluted later than the other nucleosides on all the column systems. In this case, the interaction between the nucleic acid bases should be caused by the specific hydrogen bonding instead of the hydrophobic interaction which is the predominant factor in water.

Si-O-Base Column System. Figure 7 shows the results of separation of nucleosides for five kinds of Si-O-Base column systems. The Si-O-Base column systems were endcapped by trimethylsilyl units, while the Si-N-Base column systems were endcapped both by trimethylsilyl units and by the 3-acetoamidopropylsilyl units. However, the separation of nucleosides on Si-O-Base was similar to that of Si-N-Base. This fact suggests that effect of endcapping groups is negligible.

The separation behavior of the nucleosides in Figures 6 and 7 can be explained by the specific interaction between complementary nucleic acid bases; adenosine for Si-Thy and Si-Ura, guanosine for Si-Cyt, uridine for Si-Ade and cytidine for Si-Gua and Si-Hyp. The latest elution of guanosine for all the column systems may be attributed to the highest ability of guanine base in the nucleic acid bases for hydrogen bonding.

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