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Novel Stationary Phases for Affinity Chromatography. Nucleobase-Selective Recognition of Nucleosides and Nucleotides on Poly(9-vinyladenine)-Supported Silica Gel[1)]

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Poly(9-vinyladenine) (PVAd) was immobilized or coated on macroporous silica gel to be used as a stationary phase for HPLAC of nucleosides and nucleotides. The stationary phases showed nucleobase-selective recognition ability for nucleic acid constituents and gave practically useful HPLAC columns. On the basis of the results, the relationships between separation ability and the structure of PVAd are discussed.

High-performance liquid affinity chromatography (HPLAC) has become a practically useful method for separation and purification of nucleic acid related compounds, and has been used to study the nature of biological interactions between nucleic acid base derivatives. Ion-exchange and reversed-phase HPLCs give good separation for nucleosides and oligonucleotides.[2)] However, these methods present certain problems, as it is difficult to predict the elution properties and almost no information is afforded on the study of the specific interaction between nucleic acid base derivatives such as hydrogen bonding interaction and base-stacking hydrophobic interaction. These interactions have been expected to result in effective separation of nucleic acid related compounds. Utilizations of nucleic acid bases substituted synthetic polymers have been reported by Y. Kato, et al.,[3)] and Kondo, Takemoto, et al.[4)] Previously, we found that adenine- and uracil-immobilized silica gels showed an effective separating ability for nucleosides and alkyl derivatives of nucleic acid bases.[5)]

In the present study, we synthesized poly(9-vinyladenine) (PVAd)-immobilized (I) or -coated (II) macroporous silica gel and used them as stationary phases for HPLAC. These two, particularly I, offered a practically useful column for the effective separation of nucleosides and nucleotides.[6)] The recognition abilities of those PVAd-supported silica gel columns were compared to that of adenine-immobilized silica gel (Si-A).

The preparation of Si-A column was previously reported.[5)] PVAd was chemically bonded to silica gel (Wakogel LC-10K, particle size 10 μm) pretreated with 3-trimethoxysilylpropyl methacrylate by free radical copolymerization with 9-vinyladenine (VAd) (Si-PVAd(I)). The typical copolymerization method is as follows. To a solution of 0.75 g (4.7 mmol) 9-vinyladenine and 1.55 g silica gel pre-

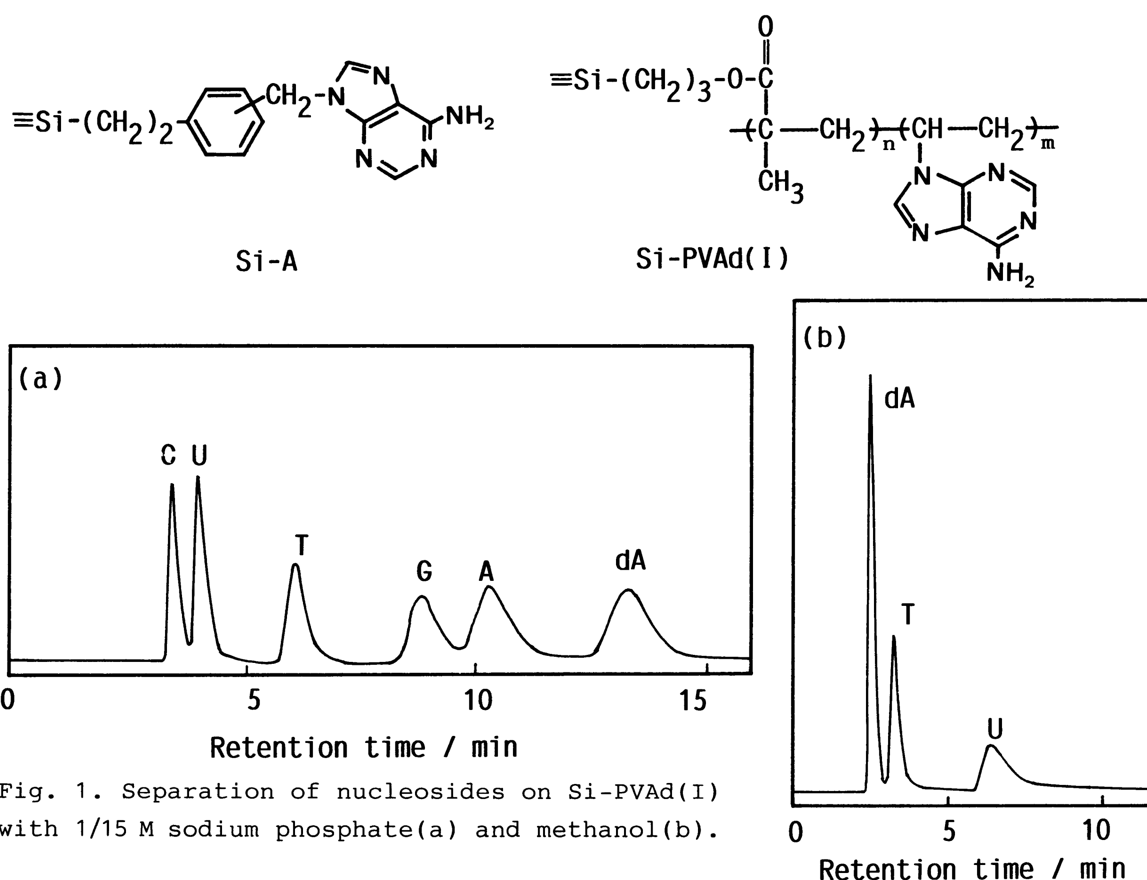

Fig. 1. Separation of nucleosides on Si-PVAd(I) with 1/15 M sodium phosphate(a) and methanol(b).

treated with 3-trimethoxysilylpropyl methacrylate in 65 ml of acetonitrile, 0.05 g (0.2 mmol) of benzoyl peroxide was added and then refluxed for 6 h under nitrogen atmosphere. The resulting PVAd-immobilized silica gel (Si-PVAd(I)) was filtered and washed with acetonitrile, water, ethanol, and acetone. Finally, the silica gel thus obtained was dried under vacuum at 60 °C for 12 h. Immobilization of PVAd on silica gel was confirmed by IR. Si-PVAd(II) was prepared by adsorption of PVAd on silica gel[7] which had been treated with m,p-chloromethylphenylethyltrimethoxysilane (CPS). PVAd was obtained by the polymerization of VAd with ammonium persulfate in H_2O in a manner similar to that reported.[8] The content of adenyl group was estimated on the basis of the content of nitrogen which was obtained by micro Kjeldahl method. The columns thus obtained were packed in a stainless steel tube (12.5 cm X 0.4 (i.d.) cm) at 350 kg cm^{-2} by slurry method using methanol.[9]

Chromatographic analysis was performed on a Shimadzu LC-6A equipped with UV detector (Shimadzu SPD-6A, 265 nm) at 20 °C, 1/15 M sodium phosphate (pH 7.0), 1/10 M triethylammonium acetate (pH 4.9) (TEAA), and methanol or methanol-$CHCl_3$ mixture (1/3(v/v)) being used as eluents at flow rate of 0.5 ml min^{-1}. A Shimadzu CR-3A was used as a data processor for HPLAC.

Figure 1 shows the chromatograms of the separation of nucleosides on an Si-PVAd(I) column. Six nucleosides were completely separated in a short time using isocratic elution mode of dilute sodium phosphate buffer (Fig. 1(a)). The retention times of nucleosides revealed that purine nucleosides were more retained than pyrimidine nucleosides, indicating that the hydrophobic base-base stacking inter-

Table 1. Retention time (min) of nucleosides[a)]

Column	1/15 M sodium phosphate						Methanol-$CHCl_3$(1/3)			Methanol		
	C	U	T	G	A	dA	dA	T	U	dA	T	U
Si-ODS	3.6[b)]	4.0[b)]	6.1[b)]	4.8[b)]	7.9[b)]	8.6[b)]	3.0	3.0	3.0	3.4	3.4	3.4
Si-CPS	3.6	3.3	5.9	4.5	8.4	12.0	3.4	2.3	16.0	4.2	2.5	2.5
Si-A	4.1	3.9	6.1	10.1	14.4	17.3	3.9	4.6	18.4	3.2	3.2	4.0
Si-PVAd(I)	3.6	4.1	6.2	8.9	10.3	13.3	2.7	7.0	51.0	2.4	4.4	8.4
Si-PVAd(II)	-	-	-	-	-	-	11.8	7.4	55.0	8.6	4.0	11.0

a) Flow rate: 0.50 ml/min, temperature: 20 °C. The contents of adenyl group of Si-A, Si-PVAd(I), and Si-PVAd(II) were 0.37, 0.49, and 0.26 mmol/g, respectively. b) Eluent, H_2O/methanol=3/1(v/v).

action between the PVAd, probably purine rings, and bases of nucleosides, plays an important role for separation of nucleosides in aqueous eluent. On the other hand, when methanol was employed as an eluent, the elution order was reversed; pyrimidine nucleosides (T and U) were more retained than dA (Fig. 1(b)). In nonaqueous eluent such as methanol and chloroform, the hydrogen bonding interaction between complementary nucleic acid bases may be much favored than the stacking interaction.[10,11)] Therefore, U and T which have complementary bases to adenine are more retained.

The results of separation of nucleosides with aqueous and nonaqueous eluents on Si-CPS, Si-A, Si-PVAd(I), Si-PVAd(II) and commercially available ODS column (Si-ODS; Shim-pack CLC-ODS(M), 15 cm X 0.46 (i.d.) cm) are summarized in Table 1. Almost complete separation of nucleosides was attained using the reversed phase partition mode of HPLC (Si-ODS and Si-CPS). However, the nucleobase-selective separation of nucleosides could not be achieved on these columns. This could be achieved first on Si-A and Si-PVAd(I) columns although Si-A did not resolve C and U. The retention times of G, A, and dA on Si-PVAd(I) with aqueous eluent were decreased compared to that on Si-A. On the other hand, when methanol or methanol-$CHCl_3$ mixture was used as the eluent, Si-PVAd(I) interacted with T and U more strongly than Si-A. These results gave us a useful information on the structure of PVAd. We assume that the adjacent adenyl groups in PVAd interact to each other by base-base stacking interaction in part and 6-amino groups of adenyl rings in PVAd may be aligned along the polymer chain. The hypochromic effect of PVAd in an aqueous solution also supported this structure.[8)] These will lead to a less retention of purine nucleosides in aqueous eluent but will delay the elution of pyrimidine nucleosides in nonaqueous eluent.

Si-PVAd(II) column showed a different recognition ability compared to Si-PVAd(I) column. The former exhibited a particularly strong interaction with dA as shown in Table 1.

The Si-PVAd(I) was found to be also effective for the separation of oligonucleotides. Figure 2 shows the chromatogram of separation of dinucleoside monophosphates on the column. In order to separate nucleotides, it has been suggested that a use of buffer like TEAA as the eluent is effective.[2)] Five nucleotides including sequence isomers of oligonucleotides[12)] (ApC and CpA) were separated in a short time and the elution order was similar to that of nucleosides. Nucleotides with two purine bases (ApA and ApG) were more retained than ApC and ApU.[13)] In

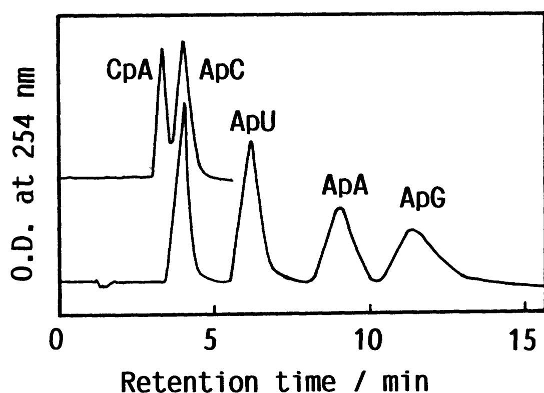

Fig. 2. Separation of dinucleoside monophosphates on Si-PVAd(I) column. (Eluent:0.1 M TEAA, flow rate: 1.0ml/min)

this case, the driving force for the separation of nucleotides is considered to be due to the hydrophobic base-base stacking interaction between PVAd on silica gel and solutes. The Si-PVAd(I) column reported here, will be expected to be applicable for the analysis of DNA adducts.[14)]

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6) The abbreviations used are as follows: A, adenine nucleoside; dA, adenine deoxynucleoside; G, guanine nucleoside; C, cytosine nucleoside; T, thymine nucleoside; U, uracil nucleoside; ApA, adenylyl(3'->5')adenosine; ApG, adenylyl(3'->5')guanosine; ApC, adenylyl(3'->5')cytidine; ApU, adenylyl-(3'->5')uridine, CpA, cytidylyl(3'->5')adenosine. These compounds were purchased from Sigma Chemical Co.

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