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High-performance affinity chromatography of oligonucleotides on nucleic acid analogue immobilized silica gel columns

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ABSTRACT

The nucleic acid analogues poly(9-vinyladenine) (PVAd), poly(9-adenylethyl methacrylate) and poly(thymylethyl methacrylate) (PTM) were chemically bonded to porous silica gel, which had been pretreated with 3-trimethoxysilylpropyl methacrylate, by free radical copolymerization to produce novel packing materials for affinity chromatographic columns. The columns separated nucleosides and nucleotide dimers on the basis of hydrophobic interaction using an aqueous buffer and complementary hydrogen bonding interaction in methanol as an eluent. The PVAd– and PTM–silica gel columns gave a nucleobase-selective separation of oligonucleotides differing in length from mixtures of oligoadenylic and oligouridylic acids. On the PVAd–silica gel column terminal phosphate isomers of oligouridylic acid up to seven mer were resolved and the elution order of the isomers was different from that on an ODS column.

INTRODUCTION

During the past decade, the separation of nucleic acids has been increasingly performed by high-performance liquid chromatography (HPLC) in the fields of molecular biology and genetics [1–4], because the availability of numerous separation columns makes HPLC an attractive alternative to conventional separating techniques for nucleic acids and oligonucleotides. Polynucleotides with a wide range of chain lengths have easily been separated with high resolution using ion-exchange [5–7], reversed-phase [8–11], and mixed-mode [12–15] stationary phases, with separation based on differences in the size, charge, hydrophobicity, shape, base sequence and base composition of the nucleic acids. In ion-exchange chromatography, the elution order of nucleic acids relies on the number of phosphodiester residues and is largely independent of sequence. Reversed-phase chromatography successfully separates oligonucleotides on the basis of hydrophobicity. Mixed-mode chromatography allows the separation of nucleic acids by size and, in part, by nucleobase sequence [15]. However, these present certain problems as it is difficult to predict the chromatographic behaviour and to control the elution order.

As polynucleotides naturally form double-stranded complexes by the specific interactions, *i.e.* complementary hydrogen bonding and stacking interactions, the development of a stationary phase in which one strand of the polynucleotide is immobilized on a support such as cellulose, agarose and silica gel allows the specific separation of the complementary strand in mixtures of polynucleotides. Chromatographic behaviour is easily predictable in HPLC using such stationary phases and the elution order of polynucleotides is simply controlled by designing an appropriate sequence of immobilized

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polynucleotides because of the highly specific formation of duplexes between two strands of complementary polynucleotides.

Conceptually, this is affinity chromatography, which has been available for over 25 years [16–18]. Affinity chromatography is a powerful technique for the specific base recognition of polynucleotides, but its use in HPLC has developed slowly due to the low durability of packing materials for affinity chromatography. Recently, Goss *et al.* [19] prepared the octadecamer of thymidylic acid [$(dT)_{18}$] covalently coupled to macroporous silica gel and separated oligoadenylic acid (A_{12-18}) by high-performance affinity chromatography (HPAC). The high resolution of separation shows that HPAC has potentially significant advantages in the purification and separation of nucleic acids. Most packing materials for affinity chromatography and HPAC will resolve nucleic acids with high specificity, but there is a problem of the stability for the nucleic acids attached to the stationary phases. Nucleic acids are decomposed by enzyme-catalysed hydrolysis. To overcome the defect and to apply the HPAC system easily, the immobilization of nucleic acid bases [20–24] and their polymeric analogues, which are stable against chemical and enzymatic hydrolysis, to silica gel [25–27] has been performed, and nucleic acid bases, nucleosides and oligonucleotides were separated on the columns.

We have found that poly(9-vinyladenine) (PVAd)-immobilized silica gel possesses a potent ability for the nucleobase-selective separation of nucleosides and sequence isomers of oligonucleotide dimers for HPLC [28]. Moreover, an agarose–PVAd-conjugated gel showed nucleobase-selective separation of RNA and could discriminate between single- and double-stranded DNA in affinity electrophoresis [29].

In this paper, we report the immobilization of PVAd, poly(9-adenylethyl methacrylate) (PAM) and poly(thymylethyl methacrylate) (PTM) to silica gel and the separation of nucleosides, nucleotide dimers and oligoadenylic [oligo(A)] and oligouridylic [oligo(U)]acids based on complementary hydrogen bonding interactions between the stationary phases and oligonucleotides for HPAC.

EXPERIMENTAL

Materials

Nucleosides (A, G, C, U, dA, dG, dC, dT), nucleotide dimers [ApA; adenylyl(3′→5′)adenosine, ApG, ApC, ApU, UpU], polyadenylic acid [poly (A)] and polyuridylic acid [poly(U)] were purchased from Sigma (St. Louis, MO, USA) and Yamasa Shoyu (Chiba, Japan). Nuclease S1 and micrococcal nuclease, and tris(hydroxymethyl)aminomethane (Tris) were also obtained from Sigma. Adenine and thymine were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Ethylenediaminetetraacetate (EDTA) and other chemicals were of analytical-reagent grade from Nakarai Tesque (Kyoto, Japan). Azobisisobutyronitrile and benzoyl peroxide (BPO) were recrystallized from ethanol. Triethylamine was distilled and then stored on NaOH pellets. Benzene was purified in the usual manner and distilled over sodium metal. N,N,-Dimethylformamide was distilled with a small amount of dried benzene and distilled under reduced pressure and then stored over molecular sieves. HPLC-grade acetonitrile was used for HPLC.

The preparation of 9-vinyladenine was previously reported [30]. 9-Adenylethyl methacrylate and thymylethyl methacrylate were prepared according to the previously reported method [31]. IR and NMR spectra and melting points were in reasonable agreement with reported values.

Preparation of oligo(A) and oligo(U)

Freshly autoclaved distilled water was used in all experiments. Oligo(A) was prepared by alkaline hydrolysis of poly(A) (5 mg in 2 ml of 0.15 *M* KOH solution) at 37°C. A portion (150 μl) of the reaction mixture was withdrawn at an appropriate time and the reaction was stopped by the addition of 3.8 μl of 6 *M* acetic acid; the resulting oligomer was analyzed on an ODS column [Shim-pack CLC-ODS (M), 150 × 4.6 mm I.D. or Tosoh, TSK-Gel ODS-120T, 150 × 4.6 mm I.D.] as soon as possible. It was found that the hydrolyzed oligo(A) had 2′- and 3′-terminal phosphates and the 2′,3′-cyclic terminal phosphate of oligo(A) could not be detected under these conditions.

Oligo(U) with 5′-terminal phosphate was prepared by enzymatic hydrolysis of poly(U) with nuclease S1. To a solution of poly(U) (1 mg) in an

acetate buffer (0.5 ml) with 0.3 *M* NaCl and 3 m*M* $ZnCl_2$ (pH 4.5; S1 buffer) was added an aliquot (33 μl) of nuclease S1 (1 U/μl in S1 buffer) and the solution was allowed to react at 37°C for 120 min. The reaction was stopped by the addition of 0.25 ml of 0.1 *M* Tris–HCl buffer with 20 m*M* EDTA (pH 8.8).

Oligo(U) with 3′-terminal phosphate was prepared by micrococcal enzyme hydrolysis. To a solution of poly(U) (1 mg) in 0.1 *M* Tris buffer (0.50 ml) with 10 m*M* $CaCl_2$ (pH 8.8) was added an aliquot (7.5 μl) of micrococcal nuclease (1 U/μl in Tris buffer) and the solution was allowed to react at 37°C for 10 min. The reaction was stopped by the addition of 0.75 ml of 0.1 *M* Tris buffer with 20 m*M* EDTA (pH 8.8).

Alkaline hydrolyzed oligo(U) was prepared in a similar manner as oligo(A) [14,32] and the resulting oligomer was analyzed on an ODS column as soon as possible. A portion of the alkaline-hydrolyzed oligo(U) was incubated at a pH less than 2 by addition of $HClO_4$ to eliminate 2′,3′-cyclic phosphates (60°C for 10 min) [8]. This sample [a mixture of 2′- and 3′-oligo(U)] and 3′-oligo(U), 5′-oligo(U) and 2′-, 3′-, and 2′,3′-cyclic phosphate unimers of uridine were used as authentic samples to assign the peaks in a chromatogram of the alkaline hydrolyzed oligo(U). The terminal phosphates of the alkaline hydrolyzed oligo(U) were a mixture of 2′-, 3′- and 2′,3′-cyclic phosphates. All samples were stored at −20°C until use.

Preparation of stationary phase

Four stationary phases with nucleic acids bases were prepared and the structures are schematically shown in Fig. 1. The preparation of adenine-immobilized silica gel (Si-A) and poly(9-vinyladenine) (PVAd)-immobilized silica gel (Si-PVAd) were previously reported [28].

PAM and PTM were chemically bonded to silica gel (Wako gel LC-10K, 100 Å pore size, 10-μm, beads) pretreated with 3-trimethoxysilylpropyl

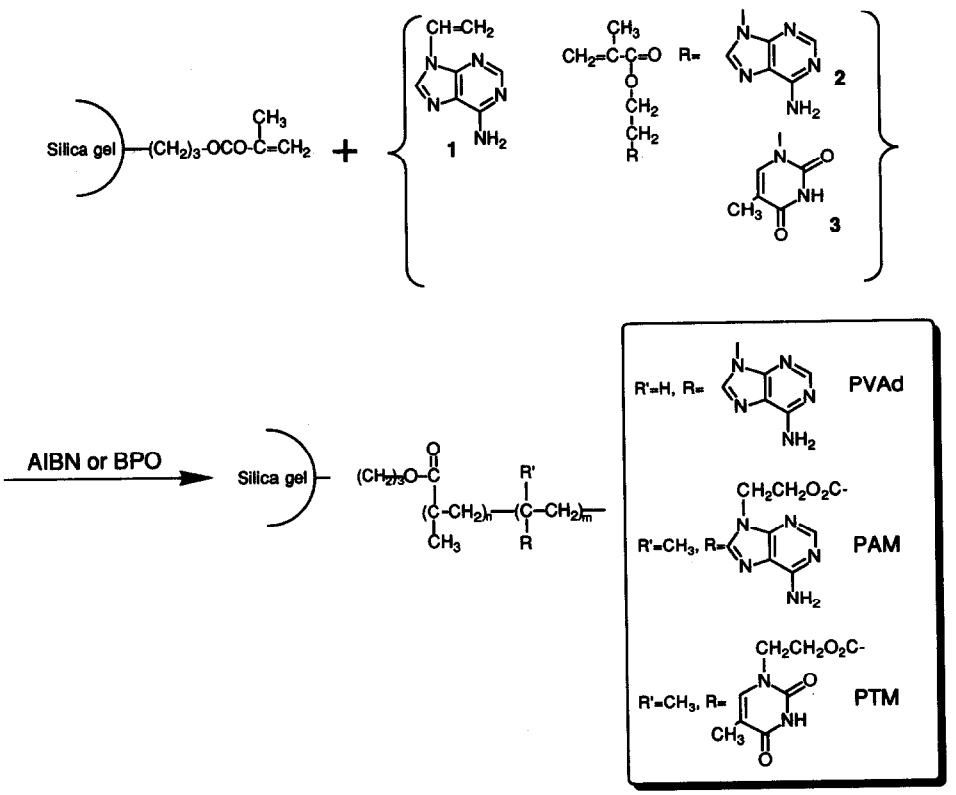

Fig. 1. Structures of the nucleic acid analogue immobilized silica gels.

methacrylate by free radical copolymerization. The typical copolymerization method is as follows: silica gel (1.5 g) was dried at 200°C for 6 h *in vacuo* and then treated with 3-trimethoxysilylpropyl methacrylate (4 ml) in toluene containing 80 μl of triethylamine; the suspension mixture was refluxed for 4 h under nitrogen atmosphere. The silanized silica gel was filtered, washed with toluene, acetone and diethyl ether, and then dried *in vacuo*.

To a silanized silica gel (1.5 g) suspended in dioxane (25 ml) was added a solution of 9-adenylethyl methacrylate (0.75 g) in dioxane (40 ml) and azobisisobutyronitrile (0.04 g). The mixture was refluxed for 6 h under nitrogen and the resulting PAM–immobilized silica gel (Si-PAM) was filtered, washed with dioxane, dimethylsulfoxide, ethanol, diethyl ether and acetone, and then dried at 60°C for 12 h *in vacuo*. The amount of the adenyl group coupled to the silica gel was estimated on the basis of the content of the nitrogen determined by the micro-Kjeldahl method (the amounts of the adenyl group were 0,49, 0.49 and 0.47 mmol/g for Si–PAM, Si–PVAd and Si–A, respectively). PTM was immobilized to silica gel (Si-PTM) by the same method. The amount of the thymyl group coupled to silica gel was 0.62 mmol/g.

The packing materials thus obtained were packed in a stainless-steel tube (125 × 4.6 mm I.D.) at 350 kg/cm^2 by a slurry method as reported previously [33]. The plate numbers of these columns were 2000–5000 for acetone with methanol (0.5 ml/min) as an eluent at 25°C.

Apparatus

Melting points were determined on a Yamato melting point apparatus Model MP-21 and not corrected. ^{1}H NMR spectra were recorded on a JEOL GSX-400 (400 MHz) instrument and tetramethylsilane (TMS) was used as the internal standard. IR spectra were measured on a Jasco A-3 infrared spectrometer.

Chromatographic analysis was performed on a Shimadzu LC-6A chromatograph equipped with a gradient controller (Shimadzu SLC-6B) and UV detector (Shimadzu SPD-6A, 260 nm) at a flow-rate of 0.5 or 1.0 ml/min and a temperature of 25 or 30°C unless stated otherwise. A Shimadzu CR-3A instrument was used as a data processor for HPLC. All gradients were performed with a binary gradient elution technique.

RESULTS AND DISCUSSION

Separation of nucleosides and nucleotide dimers

Table I shows the results of separation of various ribo- and deoxyribonucleosides on Si–A, Si–PVAd,

TABLE I

RETENTION TIMES OF NUCLEOSIDES

Conditions: column, 125 × 4.6 mm I.D.; flow-rate, 0.5 ml/min; temperature, 20°C.

Column	Eluent[a]	Retention time (min)							
		Ribonucleoside				Deoxyribonucleoside			
		A	G	C	U	dA	dG	dC	dT
ODS	A	6.0	4.2	3.6	3.8	6.5	4.5	3.8	5.2
	B	3.6	3.6	3.6	3.6	3.7	3.7	3.7	3.7
Si–A	C	16.9	11.5	4.7	4.6	18.8	12.5	5.2	6.7
	B	4.5	4.5	3.4	5.1	3.4	3.7	3.1	4.0
Si–PVAd	C	9.6	8.0	3.5	4.0	11.8	8.5	3.9	5.8
	B	4.0	5.6	5.6	7.3	3.2	4.1	3.8	4.1
Si–PAM	C	15.2	9.1	4.0	4.6	17.8	10.2	4.2	7.4
	B	4.4	4.5	4.2	7.7	3.7	3.9	3.4	4.7
Si–PTM	C	11.1	5.4	3.4	3.3	14.2	6.4	3.8	4.6
	B	4.0	3.3	3.3	2.9	3.9	3.2	3.1	2.8

[a] Eluent A, water–methanol (3:1, v/v); B, methanol; C, 1/15 *M* phosphate buffer (pH 7.0).

Si–PAM, Si–PTM and commercially available ODS columns. An aqueous phosphate buffer and methanol were used as an eluent. The retention times of nucleosides in an aqueous eluent revealed that purine nucleosides were retained more than pyrimidine nucleosides and deoxyribonucleosides were retarded more than the corresponding ribonucleosides (dA > A, dG > G, dT > U and dC > C). These indicate that the hydrophobic base–base interaction between the stationary phases and nucleic acid bases of nucleosides plays an important part in the separation of nucleosides in an aqueous eluent, as seen in reversed-phase chromatography. On the other hand, when methanol was used as an eluent, the elution order was altered. Uridine was adsorbed most strongly on Si–A, Si–PVAd and Si–PAM, and thymidine was more retained than the other deoxyribonucleosides. In non-aqueous eluent such as methanol or chloroform, the hydrogen bonding interaction between complementary nucleic acid bases seems to be favoured rather than the hydrophobic interaction [34]. Therefore, U and T were more retained on the columns with adenyl groups. The Si–PVAd column is superior to the Si–A column on a rapid separation of nucleosides. On the Si–PTM column, A and dA were found to have longer retention times than the other nucleosides. In this instance, both hydrophobic and complementary hydrogen bonding interactions may take part in the separation of nucleosides. We have reported similar results previously [28], as have Nagae *et al.* [24], who prepared nucleic acid bases containing resins or silica gel and nucleoside-immobilized silica gel columns and separated nucleic acid bases and nucleosides on these columns.

There was a similar tendency in the separation of nucleotide dimers. In an aqueous eluent, five dimers were almost completely separated on all columns and purine–purine dimers such as ApA and ApG were more retained than purine–pyrimidine dimers (ApU, ApC); however, ApU and UpU dimers were adsorbed most strongly on the columns except Si–PTM when 0.5 *M* triethylammonium acetate (TEAA) in methanol was used as an eluent. The results of the separation of nucleotide dimers on Si–A, Si–PVAd, Si–PAM and Si–PTM using TEAA are summarized in Table II. It was evident that the retardation of ApU and UpU on the columns packed with silica gel containing adenine was due to the hydrogen bonding interaction between the adenyl group and bases, whereas in an aqueous eluent reversed-phase mode separation proceeded. Therefore, the elution order of nucleosides and nucleotide dimers could be controlled on the nucleic acid analogue immobilized silica gel columns by an alteration of the eluents.

Separation of oligo(A) and oligo(U)

In these results, it was found that in an aqueous eluent, nucleosides and nucleotide dimers interact

TABLE II

RETENTION TIMES OF DINUCLEOTIDES

Conditions: column, 125 × 4.6 mm I.D.; flow-rate, 1.0 ml/min; temperature, 25°C.

Column	Eluent[a]	Retention time (min)					
		CpA	ApC	ApG	ApU	ApA	UpU
Si–A	A	17.9	20.5	50.9	21.7	[b]	6.6
	B	2.8	2.7	3.1	3.4	3.3	4.0
Si–PVAd	A	2.4	2.6	4.6	3.2	4.9	2.4
	B	2.0	2.2	2.3	3.2	2.7	5.7
Si–PAM	A	4.1	4.7	10.1	6.8	12.7	2.6
	B	2.0	2.2	2.3	4.1	2.9	6.4
Si–PTM	A	2.4	3.0	4.1	2.8	5.8	1.5
	B	2.0	2.2	2.3	2.2	3.4	1.9

[a] Eluent A, 0.1 *M* TEAA in water; B, 0.5 *M* TEAA in methanol.
[b] Not detected.

with the adsorbents with nucleic acid bases not via a hydrogen bonding interaction, but via a hydrophobic interaction. However, we have already found that PVAd can form a complex with poly(U) by complementary base-pairing (hydrogen bonding) in water based on UV and imino-proton NMR spectroscopic analysis of the PVAd–poly(U) complex [35]. This may indicate that oligo(U) with a particular chain length will interact with the PVAd on silica gel by hydrogen bonding in water. The complementary base-pairing ability of nucleic acid analogues might become a powerful technique for the separation and isolation of DNA and RNA [36]. To examine such an ability, oligo(A) and oligo(U) were applied to the columns.

The retention times of oligo(A) and oligo(U) on ODS, Si–A, Si–PVAd, Si–PAM and Si–PTM columns were plotted against the degree of polymerization (DP) of the oligomers (Fig. 2). The ODS column separated oligo(A) and oligo(U) (DP = 1–10) with high resolution and the elution order depended on the chain length and hydrophobicity; oligo(A) with a particular chain length was more retained than the corresponding oligo(U). A similar tendency was observed with Si–A, although the differences in the elution times between oligo(A) and oligo(U) decreased. On the Si–PVAd column, the elution time of oligo(U) drastically increased with an increase in the DP (Fig. 2C). This strongly suggests that the oligo(U) with DP > 4–5 can interact with PVAd by complementary hydrogen bonding.

On the Si–PAM column the chromatographic behaviour of the oligomers was different from that on the Si–PVAd and was similar to that on the ODS column. Si–A and Si–PAM columns may be classified as mixed-mode columns (base-pairing and reversed-phase). In an aqueous system, only the reversed-phase mode seems to appear. The reason for the different separation properties between Si–PVAd and Si–PAM was not clear, but the structures of the polymers and the spacer effect for using PAM might influence the separation behaviour.

On the other hand, oligo(A) with DP = 3–8 was adsorbed strongly on the Si–PTM column, but the oligo(U) (DP = 1–10) was eluted within about 10 min under these conditions. The Si–PTM column seems to be the closest to an ideal base-pairing column. Both hydrogen and hydrophobic (stacking) interactions between oligo(A) and PTM must have

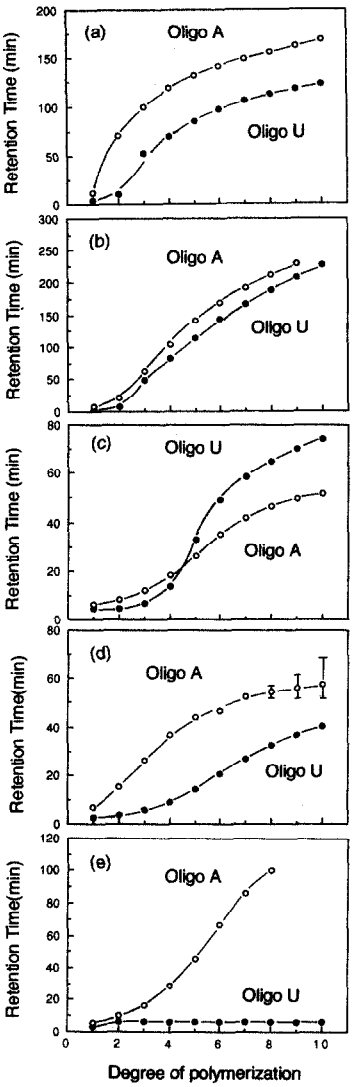

Fig. 2. Relationships between the retention times of oligo(A) and oligo(U) and the degree of polymerization of the oligonucleotides on (a) ODS, (b) Si–A, (c) Si–PVAd, (d) Si–PAM and (e) Si–PTM. The terminal phosphates of oligo(U) and oligo(A) were 3′- and a mixture of 2′- and 3′-phosphates, respectively. Column: ODS, 150 × 4.6 mm I.D.; other modified columns, 125 × 4.6 mm I.D. Eluents and gradient programme: (a) linear gradient from A [0.1 *M* ammonium acetate (pH 7.0) (100% at 0 min)]to 15% acetonitrile in 0.1 *M* ammonium acetate (100% at 270 min); (b) linear gradient from A (100% at 0 min) to B [water (100% at 270 min)]; (c) 0–15 min from 3.0 *M* ammonium acetate (pH 7.0) (100% 0 min) to 8% B with linear gradient, 15–120 min from 8 to 100% B with convex type gradient; (d) 0–120 min from 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to 100% B with convex type gradient; (e) 0–120 min from 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to 100% B with linear gradient. Flow-rate, 0.5 ml/min; temperature, 30°C.

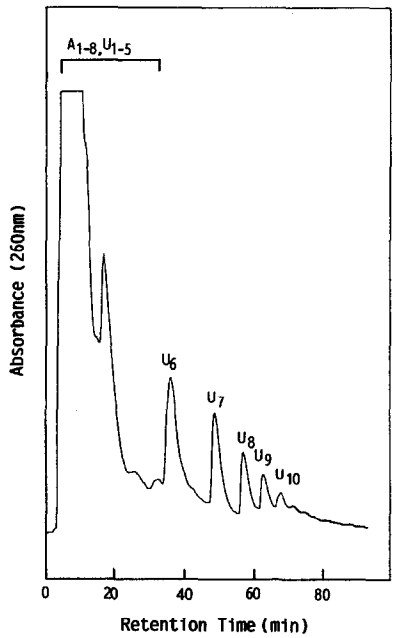

Fig. 3. Separation of mixtures of oligo(U) (DP = 1–11) and oligo(A) (DP = 1–13) on Si–PVAd column (125 × 4.6 mm I.D.). The terminal phosphates of oligo(U) and oligo(A) were 3′- and a mixture of 2′- and 3′-phosphates, respectively. Eluents and gradient programme: 0–15 min from (A) 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to (B) water (8% at 15 min) with linear gradient, 15–120 min from 8 to 100% B with convex type gradient. Flow-rate, 0.5 ml/min; temperature, 30°C.

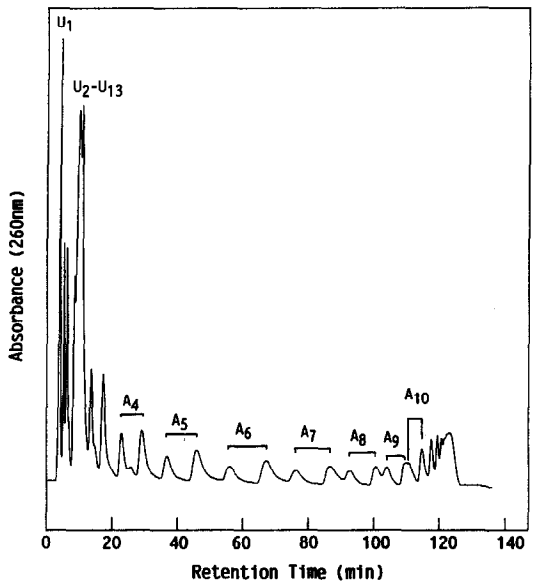

Fig. 4. Separation of mixtures of oligo(A) (DP = 1–12) and oligo(U) (DP = 1–13) on Si–PTM column (125 × 4.6 mm I.D.). The terminal phosphates of oligo(U) and oligo(A) were 3′- and a mixture of 2′- and 3′-phosphates, respectively. Eluents and gradient programme: 0–120 min from 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to 100% water with linear gradient. Flow-rate, 0.5 ml/min; temperature, 30°C.

a significant effect, as hydrophobic interaction between nucleic acid bases in an aqueous system decreases in the order purine–purine > purine–pyrimidine > pyrimidine–pyrimidine.

The chromatographic conditions described in Fig. 2 were not optimized. The optimization of the salt gradient gave a rapid and nucleobase specific HPAC. Fig. 3 shows the separation of a mixture of oligo(A) and oligo(U) on an Si–PVAd column. A mixture of oligo(A) (DP = 1–10; mixture of 2′- and 3′-terminal phosphates) and oligo(U) (DP = 1–12; 3′-terminal phosphate) was loaded and the column temperature was kept at 30°C. Most of the oligo(A) (DP = 1–8) was eluted, together with oligo(U) (DP = 1–5), within 30 min, and the remaining oligo(U) (DP >5) was eluted slowly with high resolution. Fig. 4 shows the separation of a mixture of oligo(A) (DP = 1–15) and oligo(U) (DP = 1–10) on the Si–PTM column at 30°C using the salt gradient technique. As is expected from Fig. 2e, oligo(A) with DP 4–15 was separated from the mixture of the oligonucleotides. Si–PVAd and Si–PTM can separate and isolate a desired oligomer (A or U) by the choice of the column. The [$(dT)_{18}$] covalently coupled silica gel prepared by Goss *et al.* [19] will also separate oligo(A) specifically in a similar manner. The present columns seem to be superior to the nucleic acid-immobilized columns in regard to the stability against nuclease and ease of preparation. Moreover, our system should also be applicable to affinity electrophoresis and HPAC of polynucleotides, RNA and DNA. Si–PTM especially could be used to isolate m-RNA.

The Si–PAM column was not effective for the nucleobase-specific separation of oligo(U) from a mixture of oligo(A) and oligo(U). However, the Si–PAM column was found to separate oligo(U) rapidly with high resolution (Fig. 5).

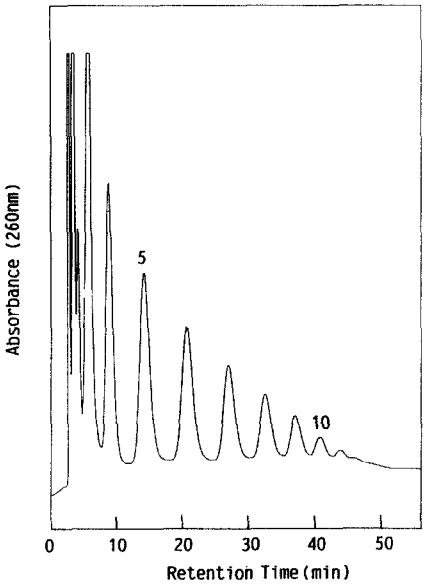

Fig. 5. Separation of 3′oligo(U) (DP = 1–13) on Si–PAM column (125 × 4.6 mm I.D.). Eluents and gradient programme: 0–120 min from 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to 100% water with convex type gradient. Flow-rate, 0.5 ml/min; temperature, 30°C.

Separation of terminal phosphate isomers of oligo (U)

The reversed-phase ODS column separates terminal phosphate isomers of oligonucleotides in a wide range of chain lengths (at least DP = 1–7 from our experiments); however, as the oligonucleotides increase in length, the resolving power of ODS slowly decreases. Therefore, the separation of terminal phosphate isomers of oligonucleotides is difficult by ODS and ion exchange chromatography. To examine the ability of the terminal phosphate recognition on the Si–PVAd column, alkaline hydrolysed oligo (U) was loaded onto the column. Fig. 6 shows the separation of oligo(U) (DP = 1–7) prepared by alkaline hydrolysis, the terminal end of which was a mixture of 2′-, 3′- and 2′,3′-cyclic phosphates. The peak assignment in Fig. 6 was carried out carefully using authentic samples (see under Experimental). The conditions of the alkaline hydrolysis were similar to those reported by Bischoff and McLaughlin [14] except for the concentration of KOH. Bischoff and McLaughlin stated that most of the hydrolyzed oligo(U) contained a 2′,3′-cyclic phosphate and the

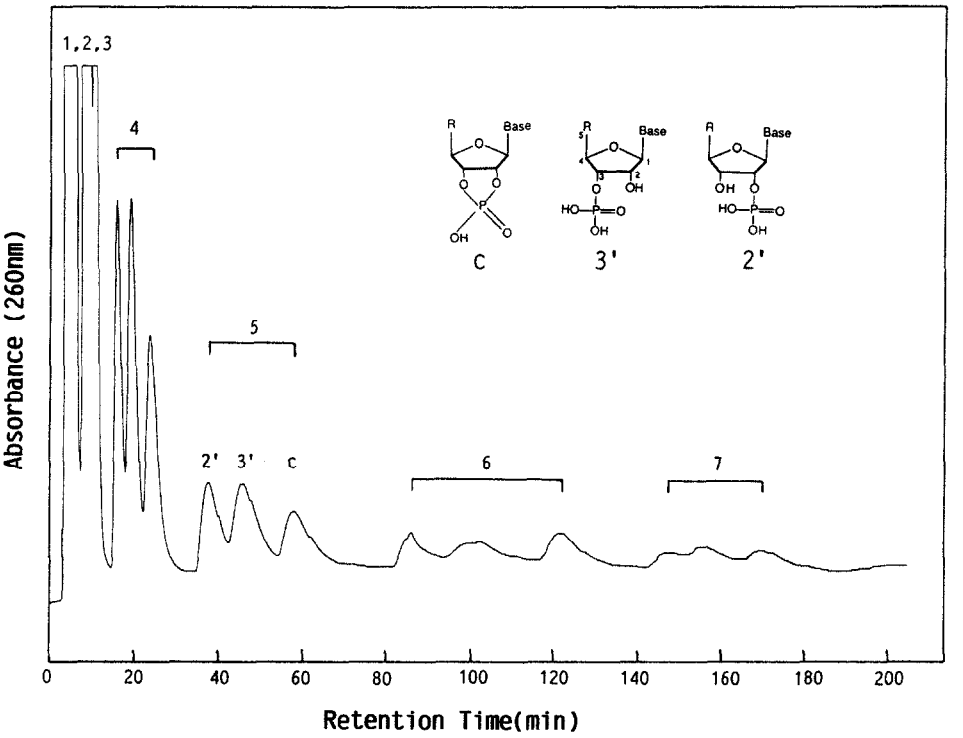

Fig. 6. Separation of terminal phosphate isomers of oligo(U) (DP = 1–7) on Si–PVAd column (125 × 4.6 mm I.D.). Eluents and gradient programme: linear gradient from 1.5 *M* ammonium acetate (pH 7.0) (100% at 0 min) to water (100% at 240 min). Flow-rate, 0.5 ml/min; temperature, 30°C.

amount of 2′- or 3′-terminal phosphate was roughly 5%. The ratio of the terminal phosphates of the oligo(U) in Fig. 6 varied much less with the DP and was rougly estimated to be 2′-U/3′-U/cyclicU = 1:1:1.

The terminal phosphate isomers of oligo(U) were well resolved on the Si–PVAd column and the resolution was only slightly dependent on the DP of oligo(U), although the peaks broadened as the oligonucleotides increased in length. The elution order was cyclicU > 3′-U > 2′-U. This was different from the elution order on the ODS column (2′-U > cyclic U > 3′-U).

In conclusion, nucleic acid analogues, especially polymer analogues immobilized on silica gels, are effective for the nucleobase-selective separation of nucleosides, nucleotides and oligonucleotides. The elution order of the nucleosides and nucleotide dimers can be controlled by eluents and the desired oligomers (A or U) could be separated by using Si–PVAd or Si–PTM columns. Moreover, these stationary phases have significant advantages in stability against enzyme-catalysed hydrolysis and ease of preparation compared with other nucleic acid immobilized resins and will be applicable to the separation of longer polynucleotides with nucleobase-selectivity.

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