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REVERSED-PHASE ION-PAIR CHROMATOGRAPHY OF OLIGODEOXYRIBONUCLEOTIDES

KEISUKE MAKINO*, HIROAKI OZAKI, TETSUFUMI MATSUMOTO, HIROMASA IMAISHI and TAMIO TAKEUCHI

Department of Polymer Science and Engineering, Faculty of Textile Science, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606 (Japan)

and

TOSHIKAZU FUKUI

Faculty of Pharmaceutical Science, Osaka University Yamadaoka, Suita, Osaka 565 (Japan)

SUMMARY

The separation of large oligodeoxyribonucleotides, synthesized chemically and subsequently deblocked, was carried out by reversed-phase ion-pair chromatography (RP-IPC) with a linear gradient of acetonitrile concentration. It was found that tetrabutylammonium phosphate is suitable as an ion-pairing reagent and produces a linear relationship between the base numbers of the samples and their elution volumes. It was also verified that various factors effective in reversed-phase chromatography, such as temperature, end-capping of the columns, differences in the type of C_{18} alkylating reagents and in the base silica, and the pore size of the base silica have little effect on the resulting separation by RP-IPC.

INTRODUCTION

The chemical synthesis of large single-stranded oligodeoxyribonucleotides (oligo-DNAs) has become possible by the phosphite method[1,2]. The recent introduction of automated DNA synthesizers has increased the attainable molecular size. However, the purification of the crude samples produced is difficult with larger sample sizes and improved purification procedures are essential. Particularly when oligo-DNAs are used as DNA probes[3,4], which have increasing molecular size, highly purified samples are required in order to avoid misleading conclusions.

Gel electrophoresis, conventionally utilized to purify oligo-DNA fragments resulting from enzymic digestion of DNA, cannot be applied in this situation, because it is suitable only for small amounts of samples, whereas samples prepared chemically are obtained in amounts of the order of milligrams.

In order to overcome this problem, applications of high-performance liquid chromatography (HPLC) have been studied[5,6]. We have investigated the application of anion-exchange chromatography (AEC) to oligo-DNA mixtures and demonstrated that single-stranded oligo-DNAs ranging up to 26b could be eluted as a function

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of the base number of the samples, in a short time and under simple conditions, and that this mode can be used for preliminary purification[7]. For further purification of this roughly purified oligo-DNA, we have studied the application of reversed-phase liquid chromatography (RP-LC) on silica-based columns and reported that well designed RP-LC ODS columns are useful for the final purification[8]. However, in this series of separations, two different chromatographic systems are needed. It would be more convenient if both systems operated in similar modes. From this point of view, our attention was drawn to reversed-phase ion-pair chromatography (RP-IPC)[9,10] in which columns and eluent systems common to RP-LC could be used. In this study, we applied this method for the preliminary purification of oligo-DNAs and studied the effect of the chromatographic conditions and column properties on the resulting separations.

EXPERIMENTAL

Oligo-DNAs used as samples were synthesized chemically by the liquid-phase triester method described previously[6,11,12]. The base sequences of the samples were dTCAAATC (7b), dCGGATTTGA (9b), dCCIAAITCCATCCAICCITAIGC (23b) and dCCIAAITCCATCCAICCCATITAITC (26b). The ion-pairing reagents used were tetramethylammonium acetate (TMAA) (Kanto Kagaku, Tokyo, Japan), tetraethylammonium acetate (TEAA) (Kanto Kagaku), tetrabutylammonium sulphate (TBAS) (Tokyo Kasei, Tokyo, Japan) and tetrabutylammonium phosphate (TBAP) (Eastman Kodak, Rochester, NY, U.S.A.). Acetonitrile of HPLC grade was purchased from Nakarai Chemicals (Kyoto, Japan). Water was purified with a Milli R/Q water purifier (Millipore, Bedford, MA, U.S.A.).

The HPLC system used was a Model HLC-803D (Toyo Soda, Tokyo, Japan), equipped with a GE-4 gradient system (Toyo Soda) and a thermostatically controlled column oven. The column effluents were monitored at 260 nm with a UV-8 Model II variable-wavelength UV detector (Toyo Soda). The RP-LC columns used were LiChrospher 100RP-18e (5 μm) (250 mm $\times$ 4 mm I.D.) and LiChrospher 100RP-18 (5 μm) (250 mm $\times$ 4 mm I.D.) (both from E. Merck, Darmstadt, F.R.G.), ODS-7 (A) (5 μm, 150 Å) (250 mm $\times$ 4.6 mm I.D.), ODS-7 (B) (5 μm, 200 Å) (250 mm $\times$ 4.6 mm I.D.) and ODS-7 (C) (5 μm, 300 Å) (250 mm $\times$ 4.6 mm I.D.) (all three from Nomura Kagaku, Seto, Japan), and MCODS (5 μm) (250 mm $\times$ 4.6 mm I.D.) and TCODS (5 μm) (150 mm $\times$ 4.6 mm I.D.) (both prepared from Toyo Soda silica by the conventional procedure[13]). An AEC column of DEAE-5PW (10 μm) (75 mm $\times$ 7.5 mm I.D.) (Toyo Soda) was used for comparison.

Eluents were prepared by adding fixed amounts of ion-pairing reagents to phosphate buffer (0.1 *M*, pH 7.0), containing acetonitrile, and were degassed for 10 min immediately prior to use by evacuation in a Bransonic 52 sonication bath (Branson Instruments, Shelton, CT, U.S.A.).

RESULTS AND DISCUSSION

Four quaternary alkylammonium salts were used as ion-pairing reagents. In order to see how ion-pairing reagents change the retention behaviour of oligo-DNAs on ODS columns, oligo-DNAs were chromatographed by two modes, RP-LC and

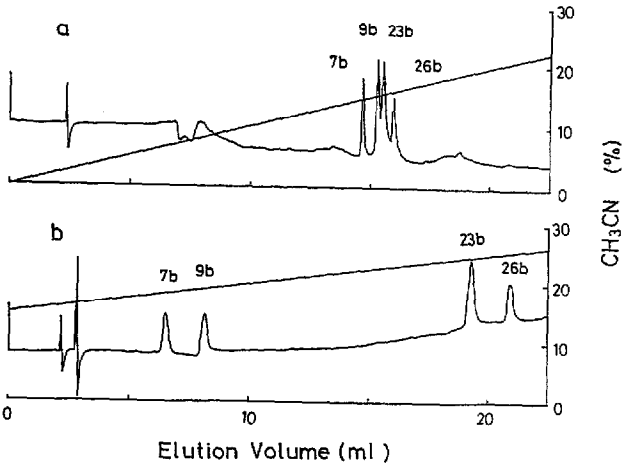

Fig. 1. Separation of oligo-DNAs by (a) RP-LC and (b) RP-IPC. Samples: 7b, 9b, 23b and 26b. Conditions: column, LiChrospher 100RP-18e; flow-rate, 0.5 ml/min; temperature, 25°C; injection volume, 5 μl; detection, 260 nm; eluent, (a) (A) phosphate buffer (0.1 *M*, pH 7.0), (B) phosphate buffer (0.1 *M*, pH 7.0) containing 30% acetonitrile; (b) (A) phosphate buffer (0.1 *M*, pH 7.0) containing 2 m*M* TBAP and 15% acetonitrile, (B) phosphate buffer (0.1 *M*, pH 7.0) containing 2 m*M* TBAP and 30% acetonitrile; gradient, linear for 60 min; temperature, 20°C.

RP-IPC, using the same column, LiChrospher 100RP-18e. TBAP (2 m*M*) was used in RP-IPC as an ion-pairing reagent. The gradient of acetonitrile concentration is depicted in Fig. 1 and it can be seen that RP-IPC separated the samples in well resolved peaks as a function of molecular size, whereas all the samples were eluted closely together by RP-LC. This implies a strong association of the reagent with the ionic moieties of the samples, which increases the interaction of the samples with the stationary phase. Broadening of peaks of the larger samples, which was observed previously in AEC[7], was not produced in this instance.

The effects of differences in the ion-pairing reagents on the retention behaviour of oligo-DNA were studied using the same column and TMAA, TEAA and TBAP. The concentration of each reagent was 2 m*M* and a linear acetonitrile concentration gradient from 10 to 30% was applied for 120 min. The resulting elution volumes of the four oligo-DNA are plotted against their base numbers in Fig. 2. With TBAP, an almost linear relationship was obtained between the parameters, whereas the other ion-pairing reagent gave a steeper linear relationship. This desirable effect of TBAP may be due to the stronger interaction of its complex with the samples, caused by the longer hydrophobic alkyl chains. The effect of counter ions of the tetrabutylammonium salts was also investigated, using TBAP and TBAS, in which only the anions differ. TBAS broadened the peaks, although the peaks appeared at similar retention volumes. Therefore, TBAP was utilized exclusively as the ion-pairing reagent in the subsequent experiments.

The concentration effect of TBAP on the retention behaviour was studied using the same column. The concentrations of TBAP were varied from 0.5 to 2 m*M*. The linear gradient of acetonitrile concentration increased from 10 to 30% in 120 min.

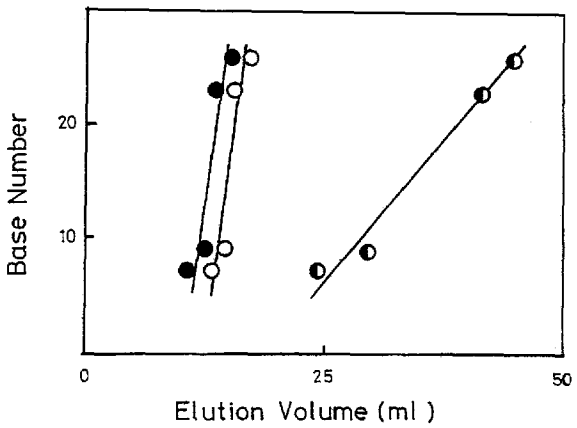

Fig. 2. Relationship between the base numbers of oligo-DNAs and the elution volumes in RP-IPC. Ion-pairing reagents: (●) TMAA; (○) TEAA; (◑) TBAP. Conditions: eluent, (A) phosphate buffer (0.1 *M*, pH 7.0) containing the ion-pairing reagents (2 mM) and 10% acetonitrile, (B) phosphate buffer (0.1 *M*, pH 7.0) containing the reagents (2 mM) and 30% acetonitrile; gradient, linear for 120 min. Other conditions and samples as in Fig. 1b.

In this experiment, the retention volumes of the larger oligo-DNAs were increased with increasing concentration of the ion-pairing reagent. For instance, the value of 23b was altered from 6.34 ml at 0.5 m*M* to 43.5 ml at 2 m*M*. This increase in retention was not accompanied by peak broadening. A TBAP concentration of 2 m*M* was used in the subsequent experiments.

The effect of column temperature on the separation of oligo-DNAs was also studied under the conditions mentioned above. Elevation of the temperature caused little change in the elution volumes of samples in the range 20–50°C, as shown in Fig. 3. A small effect of temperature on the peak shape was also observed. For instance, on increasing the temperature from 20 to 50°C, the peak width of 26b was changed from 14.3 to 11.8 ml. These results indicate that in RP-IPC the temperature effect is insignificant, in contrast to RP-LC.

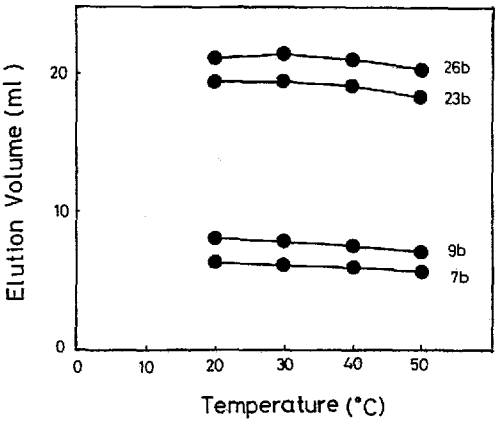

Fig. 3. Relationship between the elution volume of oligo-DNAs and temperature in the range 20–50°C. Other conditions and samples as in Fig. 1b.

Consequently, it was concluded that RP-IPC with 2 m*M* TBAP with an acetonitrile concentration gradient is useful for separating oligo-DNAs as a function of the base number of the samples.

As a good linear relationship between the base number of the samples and the elution volumes could be obtained by RP-IPC with TBAP, further experiments were carried out with a mixture of oligo-DNAs having the same base number but different base sequences, to see whether this chromatographic mode can compete with AEC. The results were compared with those obtained by AEC. The sample used was dGTCWAXYTAZTCCAT (W = A, G, C and T; C = A and G; Y = A and G; Z = T and C). This mixture contains 32 oligo-DNAs differing in their base sequences. The column used was LiChrospher 100RP-18e. Chromatograms obtained by RP-IPC under the conditions described above and by AEC on a DEAE-5PW column are shown in Fig. 4a and b, respectively. In both modes, the isomeric oligo-DNAs were eluted in a single peak without separation. This implies that both RP-IPC and AEC can be used to separate oligo-DNAs according to their base number.

In a previous paper[8], we demonstrated that in the RP-LC of oligo-DNAs, columns closely tailored to such samples are needed. Therefore, the effect of the column properties on the RP-IPC of oligo-DNAs was also investighated here. First, the effect of remaining silanol groups, which always have a large influence on the retention behaviour in RP-LC, was studied in the RP-IPC of oligo-DNAs. The columns used were end-capped (LiChrospher 100RP-18e) and non-end-caspped (LiChrospher 100RP-18). The chromatographic conditions applied were the same as in Fig. 1b. The major chromatographic features, including peak shapes and retention order, were unchanged by this factor, although with the non-capped column a marked increase in the retention volumes was produced. Second, the effect of differ-

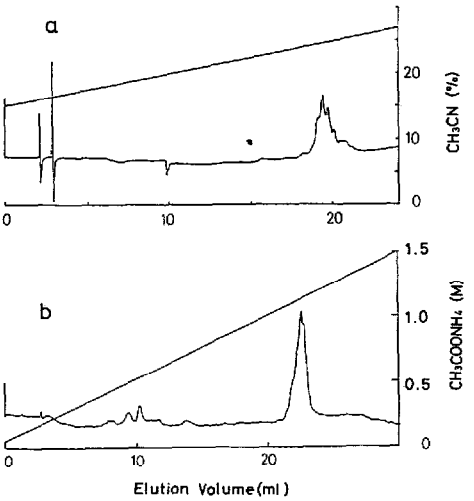

Fig. 4. Elution profiles of sequentially isomeric 15-mers, dGTCWAXYTAZTCCAT (W = A, G, C and T; X = A and G; Y = A and G; Z = T and C) by (a) RP-IPC and (b) AEC. Conditions: column, (a) LiChrospher 100RP-18e, (b) DEAE-5PW; eluent, (a) as in Fig. 1b, (b) (A) 0.05 *M* ammonium acetate, (B) 1.5 *M* ammonium acetate; gradient, (a) in Fig. 1b, (b) linear for 60 min. Other conditions as in Fig. 1b.

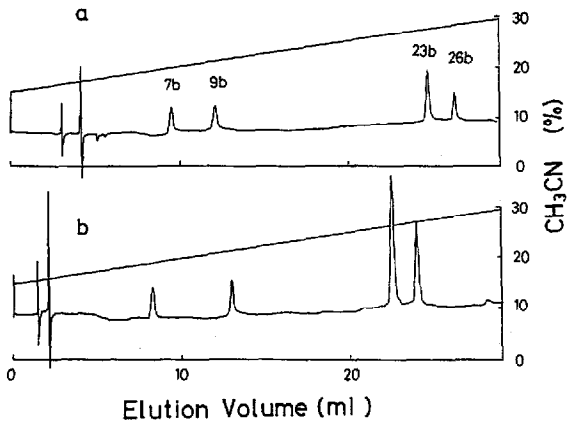

Fig. 5. Effect of the difference in the modifying reagents of ODS columns on the separation of oligo-DNAs. Conditions: (a) MCODS column (modifying reagent, monochlorodimethyloctadecylsilane); (b) TCODS column (modifying reagent, trichlorooctadecylsilane). Other conditions as in Fig. 1b, except the concentrations of 23b and 26b.

ences in chemical modifications of the silica support on the retention behaviour was studied. Two different test columns, MCODS and TCODS, prepared by a conventional procedure, were used. The modifying reagents used were monochlorodimethyloctadecylsilane (MCODS) and trichlorooctadecylsilane (TCODS). The chromatograms obtained with these columns under the same conditions are presented in Fig. 5. Equally good resolutions were obtained, although with TCODS the separation between 7b and 9b increased. As a consequence, it was found that the retention behaviour of oligo-DNAs in RP-IPC was hardly influenced by differences in the chemical modifiers, compared with RP-LC of the same samples. Third, the pore size of the silica was varied. The columns used were ODS-7 (A) (150 Å), ODS-7 (B) (200 Å) and ODS-7 (C) (300 Å), all being end-capped. The chromatographic conditions used were the same as above. The resulting peak shapes represented by the peak widths of the long-chain samples 23b and 26b are summarized in Table I. There was no marked effect, although the values obtained with columns having pore sizes of 150 and 200 Å were relatively smaller than those obtained with the column of pore size 300 Å.

TABLE I

EFFECT OF PORE SIZE ON THE PEAK WIDTH OF OLIGO-DNA

Columns: ODS-7 (A) (150 Å), ODS-7 (B) (200 Å) and ODS-7 (C) (300 Å). For other conditions, see Fig. 1b.

Column	*Peak width (ml)*	
	23b	*26b*
ODS-7 (A)	0.27	0.25
ODS-7 (B)	0.20	0.19
ODS-7 (C)	0.45	0.42

The results led to the following conclusions: (1) in RP-IPC with TBAP, oligo-DNAs are eluted as a function of the base number; (2) the elution volumes and the peak shapes are hardly influenced by elevating the temperature; (3) samples with the same base number but different base sequences are eluted closely together in RP-IPC; (4) the separation of oligo-DNAs by this method is hardly influenced by differences in the column properties, in contrast to RP-LC.

In summary, it has been found that RP-IPC with TBAP is easy to apply and useful for separating oligo-DNAs according to their base numbers, and can compete with AEC.

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