Journal of Chromatography, 283 (1984) 462–467
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,263

Note

Use of reversed-phase high-performance liquid chromatography for the purification of 5'-phosphorylated oligonucleotides

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(First received May 25th, 1983; revised manuscript received September 3rd, 1983)

The separation of oligonucleotides phosphorylated at the 5'-position from those having a 5'-hydroxy group is an important practical problem that has not been well studied. Usually, oligonucleotides are phosphorylated in the 5'-position by [γ^{32}P]ATP* in the presence of polynucleotide kinase. If the ^{32}P-labelled compounds are used as probes to detect a part of the genomic DNA, it is of great interest to have a ^{32}P oligonucleotide free of 5'-hydroxy-oligonucleotide, in order to avoid the competitive hybridization of the unlabelled molecules and so obtain better sensitivity.

Recently, Conner *et al.*[1] separated a nonadecanucleotide from the ^{32}P-labelled nonadecamer by homochromatography on a DEAE-cellulose chromatographic plate in a homomix at 75°C.

Reversed-phase high-performance liquid chromatography (HPLC) is frequently used for the separation of oligodeoxyribonucleotides[2–9]. As far as we know, most examples reported in the literature do not involve the separation of an oligonucleotide from its phosphorylated derivative. Crowther *et al.*[10] and Aukaty *et al.*[11] have reported the separation of dp(CG)$_3$ from d(CG)$_3$ and dp(TCG) from d(TCG), respectively.

However, these examples are of limited application as these fragments contain only three or six bases and therefore cannot be used as molecular probes. Further, the use of a phosphate buffer as the eluting solvent requires a further time-consuming desalting step.

We report here a general method for the separation of oligonucleotides up to nineteen bases from their phosphorylated homologues on a Nucleosil C_{18} column with a volatile buffer which can be eliminated by lyophilization.

EXPERIMENTAL

The oligonucleotides were prepared by the phosphotriester approach using

* Abbreviations used: A = deoxyadenosine; G = deoxyguanosine; C = deoxycytidine; T = thymidine; P = O-5'-phosphate; d = deoxy-; TEAA = triethylammonium acetate; BOP = benzotriazol-1-yloxytris(dimethylaminophosphonium) hexafluorophosphate; ATP = adenosine triphosphate; HPLC = high-performance liquid chromatography; A_{254} = absorbance units (254 nm).

0021-9673/84/$03.00

benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the condensing agent[12–15].

^{32}P labelling of the free oligonucleotide by T_4 polynucleotide kinase[16]

An amount of 100 μmol of oligonucleotide (*ca.* $2.5 \cdot 10^{-3}$ A_{254}) in 2 μl of distilled water and 2 μl of a commercial solution of [γ^{32}P]ATP (Amersham International; >2000 Ci/mol, 10 mCi/ml) were mixed with 1 μl of a buffered solution (0.5 *M* glycine, 8% sodium hydroxide, 0.1 *M* magnesium chloride, pH 9.5). The mixture was heated at 90°C for 2 min, then cooled in an ice-bath. A 0.5-μl volume of enzyme solution (Boehringer, T_4 polynucleotide kinase, E.C. 2.7.1.78, 2 units) and 1 μl of 20 m*M* dithiothreitol were added. Incubation at 37°C for 30 min was followed by heating at 90°C for 2 min to stop the reaction.

Chemical phosphorylation of the protected oligonucleotide

Protected oligonucleotide (0.5 μmol, *ca.* 1 mg) having a free 5'-hydroxy group was added to an anhydrous solutions of pyridinium cyanoethylphosphate (2 μmol in 200 μl of pyridine). This solution was mixed with BOP (3 mg, 100 μl of pyridine) and co-evaporated twice with dry pyridine, leaving a final volume of 100 μl. The mixture was left for 4 h at 50°C. Water (25 μl) was then added, the mixture evaporated to dryness and the residue taken up in 1 ml of chloroform. The chloroform solution was washed with 5% sodium hydrogen carbonate solution (2 × 1 ml) and then with distilled water (3 × 1 ml). The chloroform phase was evaporated to dryness. The residue was disolved in 100 μl of pyridine, treated with 500 μl of concentrated ammonia at room temperature for 16 h and then at 50°C for 5 h in sealed vials. Prior to HPLC the crude product was lyophilized[16].

High-performance liquid chromatography

Apparatus. HPLC was performed with a Milton-Roy pump. The solvents were delivered by a Gilson-type Mixograd gradient former. A Nucleosil C-18 (Whatman, 10 μm) column (240 × 4.7 mm I.D.) was prepared in the laboratory. The reversed-phase slurry was suspended in *n*-butanol and packed in the column in methanol–water (1:1, v/v) with an Askell pump[17].

Solvents. Stock 1 *M* solutions of triethylammonium acetate were prepared by dissolving the calculated amount of triethylamine and slightly less than the equivalent amount of acetic acid in deionized water. The mixture was passed through a 0.45-μm Millipore filter and adjusted to a pH of 7.0. Eluents were prepared by diluting the 1 *M* stock buffers in distilled water.

Procedure. The crude enzymatic reaction mixture (10 μl) was injected directly into the column. A linear methanol gradient (Table I) in TEAA over a period of 30 min at a flow-rate of 1.5 ml/min was used. The eluted fractions were collected at regular intervals of 30 sec and counted with a Packard Tricarb 2405 (Cerenkov effect). The values obtained from each fraction were compared with the 254-nm UV absorption profile.

In the chemical phosphorylation (see above) the deprotected crude oligonucleotide mixture was lyophilized and injected in 100 μl of distilled water. Both products A and B (Fig. 2)) were detected by UV absorption at 254 nm with a Waters Model 440 detector. The yields were calculated by the measurement of peak areas.

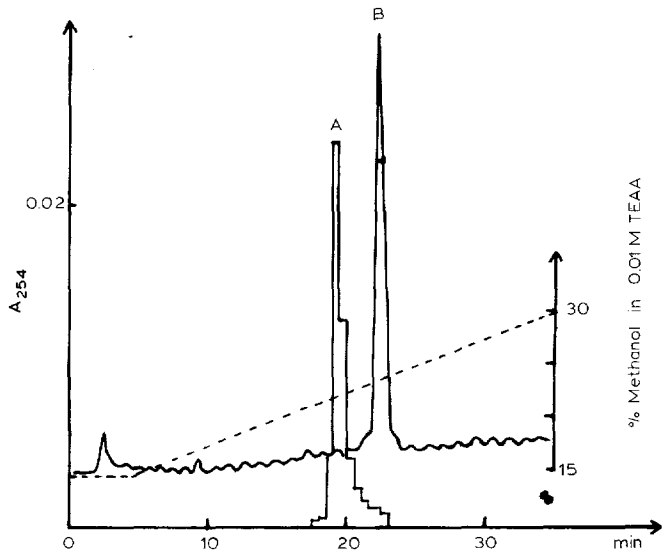

Fig. 1. UV absorption curve (254 nm) of dA_3TA_8 (B) and scintillation counting curve of its ^{32}P-labelled 5'-phosphorylated derivative (A). Nucleosil C_{18} column (240 × 4.7 mm I.D.). Gradient of methanol in 0.01 *M* TEAA from 15 to 30% in 30 min. Flow-rate, 1.5 ml/min.

RESULTS

Fig. 1 shows an example of the performance that can be achieved with a Nucleosil C_{18} column using a 15–30% methanol gradient in 0.01 *M* TEAA (pH 7) for a dodecanucleoside undecaphosphate, $d(A_3TA_8)$, and its enzymatically phosphorylated derivative, $d(pA_3TA_8)$. The comparison of the 254-nm UV absorption curve of the 5'-hydroxylated oligodeoxyribonucleotide with the scintillation counting plot of its ^{32}P-labelled 5'-phosphorylated derivative shows that the latter compound can be obtained in a very high degree of purity. The TEAA solution was eliminated by lyophilization and the desired compound was obtained free of any contamination (salt, polymer, etc.) and was then ready for further use in biological reactions.

Table I gives retention times relating to the purification of various 5'-phosphorylated oligonucleotides obtained by reaction of ATP with polynucleotide kinase. For all the oligonucleotides tested, the phosphorylated derivative was the first to be eluted. The use of a 10–40% methanol gradient provided a convenient separation for various oligonucleotides 8–19 units long. However, the separation was improved with a 15–30% gradient of methanol in 0.01 *M* TEAA.

The method has been applied successfully to the purification of chemically phosphorylated oligonucleotides, which were prepared as follows. The hydroxy function at the 5'-end of the completely protected oligonucleotide was liberated by acid treatment of the 5'-dimethoxytritylated derivative. It was further treated with pyridinium cyanoethylphosphate in the presence of BOP[12,18]. After condensation the protecting groups were removed by alkali. The crude product mixture was injected on to the reversed-phase column.

Fig. 2 illustrates how various products resulting from the initially protected

TABLE I

RETENTION TIMES FOR OLIGONUCLEOTIDES AND THEIR 5'-PHOSPHORYLATED DERIVATIVES PREPARED WITH T_4 POLYNUCLEOTIDE KINASE

HPLC was performed with 10^{-2} *M* TEAA (pH 7) with a linear 30-min methanol gradient on a 240 × 4.7 mm I.D. column. Flow-rate, 1.5 ml/min. Gradient started at $t = 0$ min.

Oligonucleotide	*Methanol*		*Retention time (min)*
	Initial concentration (%)	*Final concentration (%)*	
d(AAATAAAAAAAA)	15	30	22.5
d(pAAATAAAAAAAA)			19.25
d(AAATAAAAAAAA)	20	40	17.5
d(pAAATAAAAAAAA)			15.5
d(ATTTTGAATATAACA)	20	40	20.
d(pATTTTGAATATAACA)			18.5
d(AAATTCTCCTC)	20	40	16.75
d(pAAATAAGTCCTC))			15.5
d(TTTATCGATGC)	20	40	17.5
d(pTTTATCGATGC)			16.5
d(GTACGTAC)	10	30	23.
d(pGTACGTAC)			18.5
d(TTGA)	20	40	17.5
d(pTTGA)			15.5
d(CCTTGGACCCAGAGGTTCT)	20	40	13*
d(pCCTTGGACCCAGAGGTTCT)			11*
d(CACACACACA)	20	40	13*
d(pCACACACACA)			10.1*

* Performed with a shorter column (200 × 4.7 mm I.D.) and a Varian LC 5000 instrument.

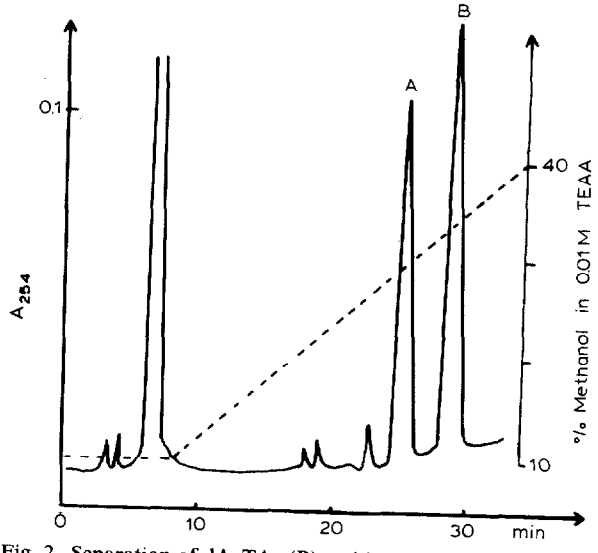

Fig. 2. Separation of dA_3TA_8 (B) and its chemically phosphorylated derivative (A): 254-nm UV absorption curve. Nucleosil C_{18} column (240 × 4.7 mm I.D.). Gradient of methanol in 0.01 *M* TEAA from 10 to 40% in 30 min. Flow-rate, 1.5 ml/min.

oligomer (benzoate and isobutyrate ions, chlorophenol, etc.) were eluted from the column. The 5'-phosphorylated oligonucleotide A, $d(pA_3TA_8)$, was conveniently separated from the 5'-hydroxylated oligonucleotide B, $d(A_3TA_{88})$, without any contamination by the above-mentioned side-products. The resolution, R_S, of the two peaks A and B could be calculated by

$$R_S = 1.176 \cdot \frac{t_{RB} - t_{RA}}{\delta_A + \delta_B}$$

where t_{RA} and t_{RB} represent the retention times of peaks A and B and $\delta_A\delta_B$ the peak widths at half-height, and was found to be 1.58. This result demonstrates the practical interest of the phosphorylation method. The measurement of the areas of the UV absorption peaks A and B at 254 nm showed a satisfactory yield of 45%. The chromatographic method described here thus allowed an accurate determination of the yield of phosphorylated oligonucleotide.

DISCUSSION

A variety of HPLC techniques have been developed for the separation of oligonucleotides[9]. They are mainly concerned with the separation of oligonucleotides that are different from their neighbours by one base unit. Either $d(N)_n$ is separated from $d(N)_{n-1}$ or $dp(N)_n$ from $dp(N)_{n-1}$, where $(N)_n$ represents an oligonucleotide with n bases, p a phosphate group at the 5'-end and d the deoxyriboside series. The chain length limits for complete separation within 30 min by reversed-phase and reversed-phase–ion-pair HPLC have been calculated to be 10 and 15 nucleotides, respectively[9].

The method described here is aimed at the separation of the 5'-phosphorylated oligonucleotides from their parent 5'-hydroxylated oligonucleotides; $dp(N)_n$ is separated from $d(N)_n$. They have the same heterocyclic bases and differ only by a 5'-phosphate terminal group. Crowther *et al.*[10] described the separation of $dp(CG)_3$ from $d(cG)_3$. With our experimental conditions, we have been able to separate phosphorylated oligonucleotides from unphosphorylated derivatives up to 19 bases.

Chemically synthesized specific oligonucleotide probes (19-mers) have been used to develop a direct and sensitive test for the presence or absence of mutant genes in human cells. Short oligonucleotides (< 10) cannot be used as molecular DNA or RNA probes because they lack specificity. To improve the sensitivity, free carrier [^{32}P]ATP with the highest specific activity available (> 7000 Ci/mmol) is used to label the oligonucleotide. To limit the radio-induced degradation, the highly ^{32}P-labelled oligonucleotide needs to be utilized very rapidly. It is of practical interest to avoid phosphate buffer, which requires a subsequent time-consuming desalting step. The volatile TEAA buffer that is used in our experiments can be easily and rapidly removed by lyophilization.

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