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USE OF A C_4 COLUMN FOR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES

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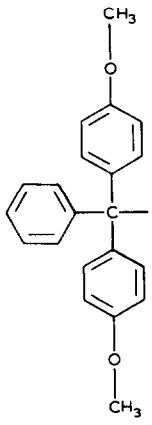
SUMMARY

The small-pore C_{18} column materials commonly used for reversed-phase high-performance liquid chromatographic purification of synthetic oligodeoxyribonucleotides do not yield DNA sufficiently pure for some applications. Separation and purification on large-pore C_4 columns was investigated. Model studies presented demonstrate excellent resolution of synthetic DNAs of similar sequence. Also presented are the results of purification of larger compounds.

INTRODUCTION

Recent advances in the chemistry[1,2] and automated synthesis of oligodeoxyribonucleotides have enabled the production of multiple compounds daily and of sequences over 100 nucleotides in length. Many techniques have been used for the purification of chemically synthesized oligodeoxyribonucleotides. Among these are thin-layer[3], paper[4], gravity-fed column[5], and high-performance liquid chromatography (HPLC) by ion-exchange[6–8], normal[3] and reversed-phase[9–11] modes. Polyacrylamide gel electrophoresis (PAGE) is also a commonly used method of purifi-



DMT Group

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cation[12,13]. PAGE has the advantages of requiring inexpensive apparatus and yielding relatively pure DNA; however, there are also disadvantages, including poor recovery and time-consuming and labor-intensive procedures.

The reversed-phase HPLC (RP-HPLC) separations take advantage of the fact that the 5′ protecting group, the dimethoxytrityl (DMT) group, is a large hydrophobic moiety. The DMT group is covalently bound to the 5′-position of each derivatized nucleotide added to the chain and must be cleaved to yield the 5′-hydroxyl prior to addition of the next DMT nucleotide. In oligonucleotide synthesis procedures, this addition step is not always quantitative and, therefore, a capping reaction (5′-O-acetylation) is required to terminate any chains left with a free 5′-hydroxyl group[2,3]. Ultimately, the desired product DNA bears the DMT on the final nucleotide at completion of the synthesis. The other DNA species produced, the "failure sequences", bear the base-labile 5′-O-acetyl cap which is subsequently hydrolyzed during the work-up leaving a mixture of components that differ significantly in hydrophobicity. Rather than resolving oligomers differing by a single base, the separation involves resolving 5′-DMT DNA from 5′-hydroxylated DNA.

This separation technique does have limitations. For instance, often a small percentage of other DMT-bearing species is produced in addition to the desired DMT product. With the RP-HPLC methodologies commonly employed, little if any separation of different DMT-bearing species is achieved. Often a second RP-HPLC separation is carried out after cleavage of the DMT group from the collected product. Even after this second separation, the product DNA is not sufficiently pure for some purposes. There are also size limitations: it is generally accepted that this chromatographic technique is only adequate for oligonucleotides up to 30 nucleotides in length[14].

Here we describe a fast, efficient method for the separation of not only DMT-bearing DNA from non-DMT-bearing DNA, but also the separation of similar tritylated DNAs on a 300-Å pore, C_4 functionalized RP-HPLC column.

EXPERIMENTAL

Chemicals

The aqueous buffer for HPLC was 0.10 *M* triethylammonium acetate (TEAAc) (pH 7.0). This was prepared by dilution from a 2.0 *M* stock solution which was obtained in the following manner: 557 ml (4.0 moles) of triethylamine (Kodak) distilled from tosyl chloride (Baker) were slowly added with stirring to an aqueous solution containing 229 ml (4.0 moles) of glacial acetic acid (Baker HPLC grade acetic acid) at 4°C. After addition was complete, the solution was made up to 2.0 l and the pH adjusted to 7.0 by the addition of acetic acid or triethylamine as needed. The water used was obtained from a Milli-Q® water purification system. The organic component of the mobile phase was acetonitrile (Baker HPLC grade).

Columns

The columns used in this study were 250 × 4.6 mm for analytical purposes and 250 × 10 mm for preparative purposes and were packed with a silica based 5 μm C_4 matrix of 300 Å pore size [Vydac C_4; The Separations Group (Hesperia, CA, U.S.A.)].

Oligodeoxyribonucleotides

All oligonucleotides were produced on an Applied Biosystems (Foster City, CA, U.S.A.) Model 380A DNA synthesizer using Applied Biosystems reagents and wash solvents from Baker.

Apparatus

The chromatography was performed with a Waters (Milford, MA, U.S.A.) system consisting of two Model 6000 pumps, a Model 440 detector (254 nm), a Model 720 system controller, a Model M730 data module, and a UK6 injector or a WISP Model 710B auto-injector.

RESULTS

A typical chromatogram of a synthetic DMT-bearing oligonucleotide is shown in Fig. 1. The first peak at 2.83 min contains the more polar unretained 5'-hydroxylated failure sequences. Benzamide, a side product from a deprotection step is eluted at 4.45 min. The third peak at 15.10 min contains the DMT-bearing product DNA. The trailing shoulder on the product peak and subsequent minor peaks are DMT-bearing failure sequences.

In our experience, the main impurities obtained from reversed-phase separations of synthetic oligonucleotides are the few percent of DMT-bearing failure sequences that are usually 1–3 nucleotides shorter than the desired product. These failure sequences seem to be produced by a depurination reaction occurring during the acid treatment used to cleave the DMT following each nucleotide addition. The DNA backbone is then cleaved at these sites by a subsequent 55°C concentrated ammonium hydroxide treatment for removal of other protecting groups. This chain

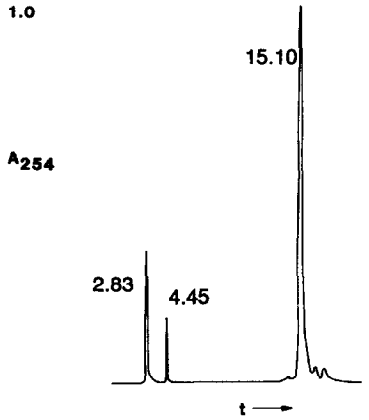

Fig. 1. Typical chromatogram of an oligodeoxyribonucleotide, 15 nucleotides in length. The peak at 2.83 min consists of unretained 5'-hydroxyl failure sequences which failed to couple and were subsequently capped. The peak at 4.45 min is due to benzamide, a product of deprotection. The peak at 15.10 min is due to the 5'-dimethoxytritylated product DNA. The trailing shoulder on the product peak and subsequent peaks at 16.33 and 17.04 min are due to 5'-DMT failure sequences. Chromatography was carried out with a linear gradient ranging from 16 to 22% acetonitrile against 0.1 *M* triethylammonium acetate over 10 min at 1.5 ml/min on a 250 × 4.6 mm Vydac C_4 column.

TABLE I

RETENTION TIMES (t_R) OF DMT DNA FRAGMENTS OF SIMILAR SEQUENCE

Chromatography was carried out at 1.5 ml/min using the following linear gradient conditions: (A) 20–30% acetonitrile over 10 min; (B) 15–21% acetonitrile over 10 min; (C) 10–16% acetonitrile over 10 min.

Compound	t_R *(min)*	t_R *compound 1 − t_R compound 2–5 (min)*
(A) 1 18-mer	8.72	
2 19-mer	7.94	0.78
3 19-mer	7.61	1.11
4 19-mer	6.70	2.02
5 19-mer	9.81	−1.09
(B) 1 18-mer	18.93	
2 19-mer	17.26	1.67
5 19-mer	22.07	−3.14
		Difference (min)
(C) 6 28-mer	28.28	
7 29-mer	21.27	7.01

cleavage is analogous to the piperidine treatment used in Maxam-Gilbert[14] sequencing. Therefore, we investigated the separation of DMT compounds from similar but shorter DMT compounds.

Compounds 1–5 were synthesized:

(1) DMT TCA CAG TCT GGT CTC ACC
(2) DMT A TCA CAG TCT GGT CTC ACC
(3) DMT C TCA CAG TCT GGT CTC ACC
(4) DMT G TCA CAG TCT GGT CTC ACC
(5) DMT T TCA CAG TCT GGT CTC ACC

The sequences are identical through 18 bases; only the 5′ base differs on compounds 2–5 and is absent on compound 1. Compound 1 could be a likely DMT failure sequence generated in the synthesis of compounds 2–5.

Each of the five oligomers was run under the conditions shown in Table I. As shown, each oligomer eluted at a characteristic retention time (Table IA) and the separations could be optimized on gradients using less acetonitrile and with slower changes in acetonitrile concentrations as a function of time (Table IB). A chromatogram illustrating the separation of compounds 1 and 2 is shown in Fig. 2.

In a similar study, compounds 6 and 7, a 28-mer and 29-mer with identical sequences through 28 bases were synthesized and separated. (Table IC).

(6) DMT CGG TCT CAC CTC ACA GTC TGG TCT CACC
(7) DMT A CGG TCT CAC CTC ACA GTC TGG TCT CACC

Again, compound 6 would be a likely failure and contaminant in the synthesis of compound 7.

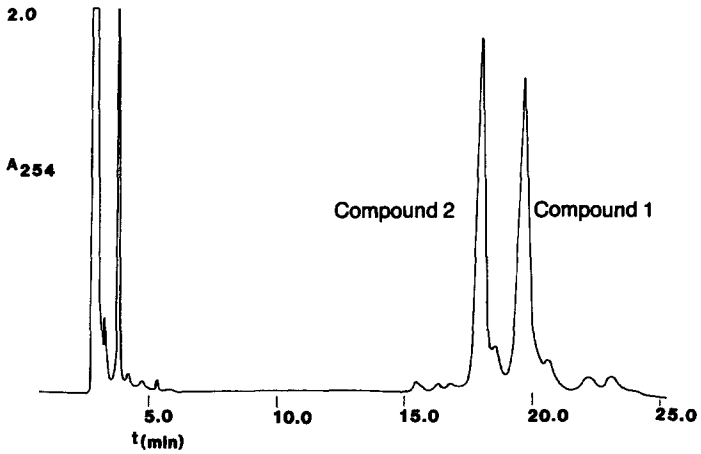

Fig. 2. Separation of compound 1, a DMT 18-mer, and compound 2, a DMT 19-mer of similar sequence; compound 1 being a typical failure sequence in compound 2. Chromatography was carried out using a linear gradient ranging from 15 to 21% acetonitrile against 0.1 *M* triethylammonium acetate over 10 min at 5.0 ml/min on a 250 × 10 mm Vydac C_4 column.

Despite the successful separations described above, most product peaks possess a shoulder on the trailing edge which is due to depurination. We assume that depurination is responsible for this shoulder as it does not occur with compounds containing only pyrimidines and no purines. It is only through judicious collection of the leading portion of the peak that a pure compound can be obtained. This is illustrated by the chromatographic results of a 51-mer shown in Fig. 3. The 51-mer was chromatographed twice. In the first separation, the entire trityl peak was collected (pool a). Analysis by autoradiography of the radio-labeled collected product (lane 2) shows the desired product is contaminated with some smaller oligonucleotides. In the second separation, the trityl peak was collected more conservatively (pool b). Autoradiography of this fraction (lane 3) shows a single band. The yield of 1.25 OD units (approximately 40 μg) of pure pool b from this single chromatogram is sufficient recovery for most biological applications.

DISCUSSION

The use of a high-quality, large-pore C_4 column matrix enables fast and efficient purification of oligodeoxyribonucleotides up to 104 nucleotides in length[15]. Both analytical and preparative chromatograms can be obtained in less than 1 h. Four to ten OD units of purified DMT oligonucleotide can be obtained from a single preparative separation. As the final step in preparation the DMT group must be hydrolyzed and removed by desalting, extraction or a another reversed phase separation with lower amounts of acetonitrile.

Numerous synthetic oligodeoxyribonucleotides have been purified by this method. Most compounds in the size range of 15 to 30 nucleotides can be purified successfully under the conditions shown in Table IB. The acetonitrile concentration may need to be increased for smaller compounds and decreased for larger compounds.

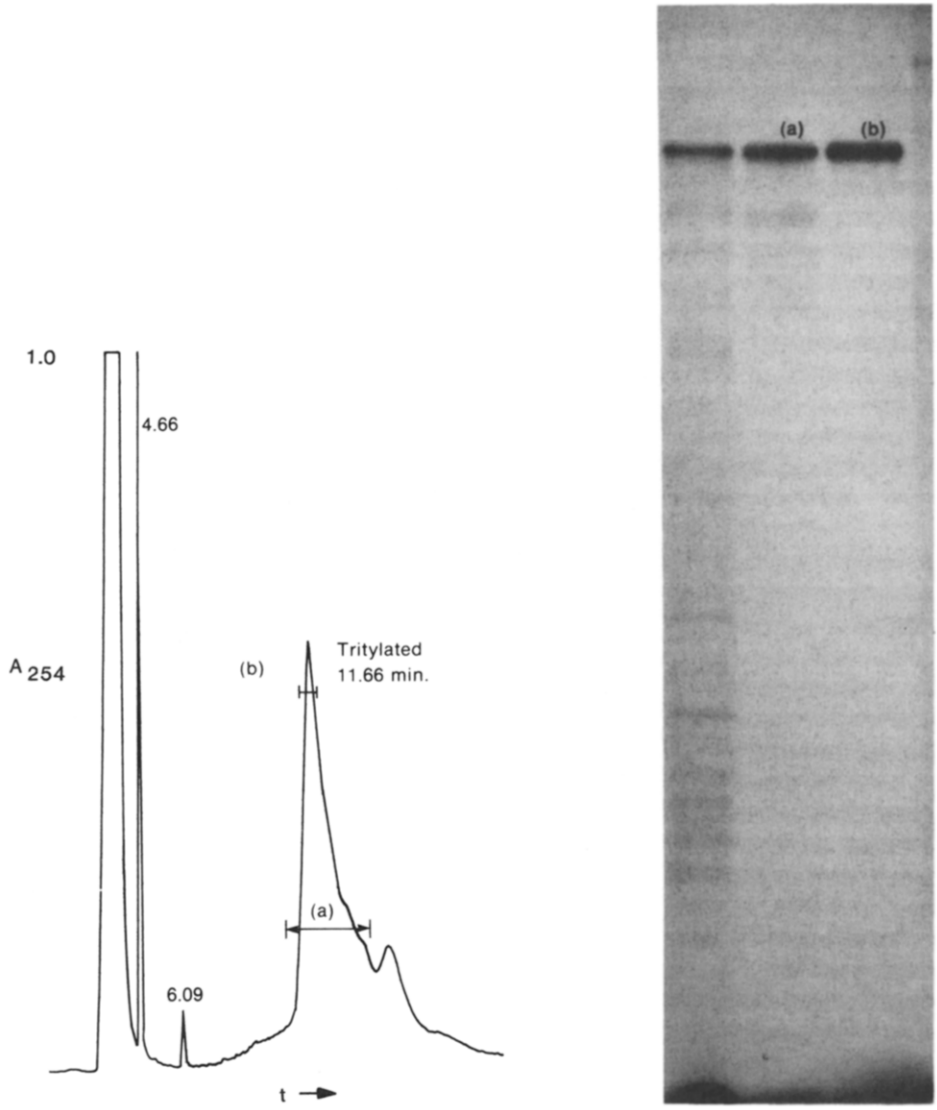

Fig. 3. Chromatogram of the unpurified material from the synthesis of a 51-mer. From separate preparative chromatograms, two different cuts were collected. In the autoradiogram, lane 1 is the unpurified 51-mer, lane 2 is the fraction (a) collected from 10.50 to 14.00 min while lane 3 is the fraction (b) collected between 11.35 and 11.95 min. Chromatography was carried out isocratically using 19% acetonitrile against 0.1 *M* triethylammonium acetate at 5.0 ml/min on a 250 × 10 mm Vydac C_4 column.

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