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Note

Use of a volatile buffer at ambient temperature

Versatile approach to the purification of self-complementary synthetic deoxyoligonucleotides by reversed-phase high-performance liquid chromatography*

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High-performance liquid chromatography (HPLC) is an efficient technique for the analytical and preparative separation of synthetic oligonucleotides. Different standard columns containing normal-phase silica gel[1], ion-exchange[2,3] and reversed-phase[1,3–6] materials and special columns such as RPC-5 (ref. 7) have been used extensively for this purpose. However, despite being a late entrant into the field of oligonucleotide purification, reversed-phase (C_{18}) columns have become more popular than the others. This is probably due to the fact that they allow the use of volatile buffers and lipophilicity-based separations. The lipophilicity has been exploited in the selective purification of 5′-dimethoxytrityl-bearing oligomer, which in a synthetic mixture is present only in the final sequence of the synthesis[4].

In a typical reversed-phase HPLC method, elution is carried out at elevated temperatures with a mixture of acetonitrile and a volatile buffer such as ammonium acetate, ethyldiammonium diacetate or triethylammonium acetate (TEAA). Interestingly, triethylammonium bicarbonate (TEAB), a buffer of higher volatility, has not been employed for such HPLC purification of synthetic oligonucleotides, presumably because of its instability at higher temperatures, heating being essential to keep the self-complementary sequences in the denatured form during analysis.

We have developed a simple method for purification of the synthetic self-complementary deoxynucleotides on a reversed-phase C_{18} column using TEAB as buffer at ambient temperature. This method has been found to be efficient for both analytical and semi-preparative uses. Additionally, the use of such a buffer at room temperature results in a considerable reduction in the time and cost of analysis.

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MATERIALS AND METHODS

Apparatus

The Model 342 gradient HPLC system (Beckman Instruments, Berkeley, CA, U.S.A.) comprising two 112 pumps, a 340 organizer, 420 system controller, 210 sample injector and 165 variable wavelength detector was used for all studies. Signals at two different wavelengths, *viz.*, 260 and 280 nm for the analytical and 260 and 295 nm for the preparative mode, were simultaneously recorded using a double channel BD 41 chart recorder (Kipp and Zonen, Delft, The Netherlands). A C_{18} reversed-phase Ultrasphere ODS column (250 mm × 4.6 mm I.D.), particle size 5 μm, was used in conjunction with a precolumn (Beckman). Column heating was achieved by circulating hot water through a fabricated glass jacket. A constant temperature was maintained by using a circulating bath (Colora Messtechnik, Lorch, Württ, F.R.G.).

Reagents

Formamide was from Fluka. Reagent grade acetic acid, acetonitrile, diethyl ether and triethylamine (TEA) were from BDH, E. Merck and SM Chemicals (all India). Both acetonitrile and TEA were distilled before use. A 0.1 *M* TEAA solution was obtained by suitable dilution of a 2 *M* stock solution of TEAA (pH 6.8) prepared by adding TEA to a chilled solution of acetic acid in water[8]. TEAB (1 *M* stock solution, pH 7.2) was made by bubbling carbon dioxide through an aqueous solution of TEA at 4°C[9]. Since TEAB buffer tends to change its pH on storage, carbon dioxide was bubbled through it just before use. Removal of TEAA and TEAB from eluted materials was achieved by freeze drying. The last traces of TEAA were removed by repeated co-evaporation with ethanol.

Nucleotide preparation

The deoxyoligonucleotides d(CCGG) and d(AATT) were synthesized by the solid-phase method on a silica gel matrix using a column fitted with a sintered disc. The phosphotriester and phosphoramidite routes were employed for d(AATT) and d(CCGG), respectively. The reaction intermediates such as the mononucleoside-phosphodiester and phosphoramidites were prepared by suitable modifications of the existing methods[2,10]. The coupling reactions were carried out according to a new approach developed in our laboratory. In this method, the coupling was performed in two or three steps, each time employing a 3–5 fold excess of the monomer over the dimethoxytrityl (DMTr) loading of the silica gel* (Table I). Thus, typically, the total amount of the monomer used is only in 4–6 fold excess. In the conventional approach, a 10–20 fold excess of the monomer is used.

The removal of the nucleotide chains from the silica support and their subsequent deacylation were carried out by treatment with ammonia. Removal of the DMTr group from the HPLC-purified oligomer was carried out by treating the dried material with 80% aqueous acetic acid for 45 min at room temperature. Water was then added to the reaction mixture, extracted thrice with diethyl ether and the

* Since it is essential to maintain a minimum concentration of the monomer of 0.1 *M*, the repeat coupling approach is very useful for large scale synthesis of smaller oligomers. It is also essential to avoid any detritylation while washing the silica gel for repeat couplings, as otherwise undesired sequences of greater length will ensue.

aqueous layer was lyophilized. Oligomers were dissolved in the elution buffer or formamide. Denaturation of the oligonucleotide solution containing 80% formamide was effected by heating to 90–95°C for 5–10 min.

Radioactive labelling and electrophoresis

The tetranucleotides were end-labelled with ^{32}P and analyzed by polyacrylamide gel electrophoresis according to the method of Maniatis *et al.*[11].

RESULTS AND DISCUSSION

Since the introduction of reversed-phase HPLC for the purification of deoxyoligonucleotides, TEAA has been the buffer of choice. However, due to its low volatility, removal of the last traces of TEAA from the purified oligonucleotide sample is a very time-consuming process. A number of volatile buffers have also been employed. Surprisingly, TEAB, a buffer of excellent volatility which finds extensive use in DEAE-cellulose column chromatography, has to our knowledge not been introduced to HPLC. We have found that a solvent system comprising acetonitrile and 0.1 *M* TEAB is equally efficient as the conventionally used systems such as acetonitrile–0.1 *M* TEAA, for oligonucleotide separation. This seems logical as the oligonucleotides form ion pairs with the triethylammonium cation of the buffer, which is common to both TEAA and TEAB.

The purification of the deoxyoligonucleotides, d(AATT) and d(CCGG), from the crude deacylated product was achieved by a two-step procedure. Since in the desired sequence DMTr acts as the 5′-protecting group, initial purification of the product could easily be achieved by taking advantage of the higher lipophilicity of the DMTr group. Thus, in a typical experiment, the DMTr-bearing group was the most strongly retained species and was easily separated from the failure sequences (*i.e.* the intermediate sequences obtained during the chemical synthesis due to the failure to obtain 100% coupling yield in each step) which were totally unprotected.

Fig. 1A shows the chromatogram obtained upon semi-preparative purification of d(DMTrAATT) using TEAB as buffer. The fractions containing the desired product (shown by asterisks in Fig. 1) were pooled and evaporated to dryness. The residue was then detritylated and injected into the reversed-phase column. Due to its reduced hydrophobicity, a lower concentration of acetonitrile was sufficient for this step. Fig. 1B shows the HPLC profile of d(AATT). The fractions containing the tetramer were freeze-dried to obtain the desired product as the triethylammonium salt. This material showed a single peak upon reversed-phase HPLC (Fig. 1C). Further, the purity of the product was tested by polyacrylamide gel electrophoresis of the end-labelled d(AATT). On subjecting the gel to autoradiography, a single spot corresponding to the tetramer was obtained (not shown). This material could be converted into its sodium or ammonium form by passing through a suitable Dowex 50W column. The ^1H NMR (270 MHz) spectrum of the sodium form of the tetramer (not shown) confirmed the absence of any impurity. Thus Fig. 1 shows that the TEAB buffer is equally efficient in the purification of d(AATT) as the frequently used buffer systems. Due to its higher volatility, the time taken for the total purification of d(AATT) was far less than that with the conventional methods.

The purification of deoxyoligonucleotides is customarily carried out at elevated

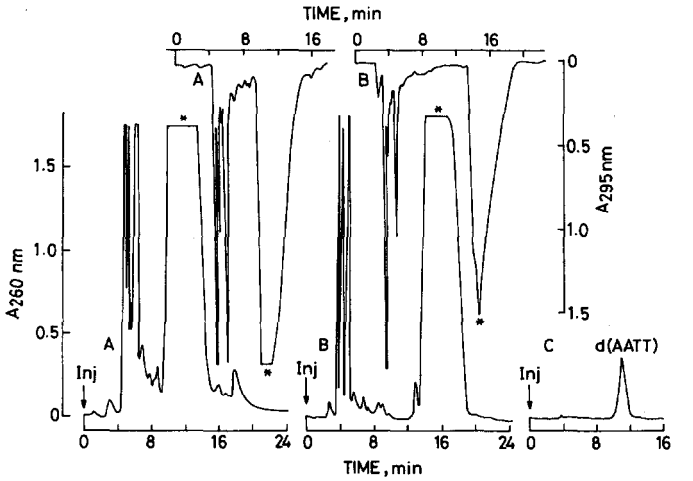

Fig. 1. Semi-preparative HPLC purification of an AT-containing tetramer sample: (A) d(DMTrAATT); (B) d(AATT); (C) purified d(AATT). Column: Ultrasphere ODS. Detection: UV at 260 (bottom) and 295 (top) nm. Range: 2 a.u.f.s. Flow-rate: 1 ml/min. Temperature: 23°C (ambient). Eluent: x% of acetonitrile in 0.1 M TEAB; (A) $x = 10$; (B) $x = 7.5$; (C) $x = 8.5$. Desired products are indicated by asterisks.

temperatures in order to keep the sample in the single-stranded state. However, such temperatures were not necessary in the present method since d(AATT) does not become double stranded even at room temperature, as evidenced by the UV and NMR studies. This property of the d(AATT) molecule alone enabled us to employ TEAB as the buffer for its purification. On the other hand, when the same procedure was applied to the semi-preparative purification of d(DMTrCCGG), multiple peaks were obtained for the desired product (not shown). This is not unlikely as d(CCGG) can undergo intermolecular association in highly concentrated solutions. The formation of these aggregates is usually prevented by injecting the sample onto a column

TABLE I

SYNTHESIS OF DEOXYOLIGONUCLEOTIDES BY THE REPEAT COUPLING METHOD

Method of synthesis	*Sequence (base Nos. 4321)*	*Specific base addition step*	*Amount of monomer added in excess of the support-bound nucleotide**	*Average coupling yield per step (%)*
Phospho-triester	d(AATT)	T2 to T1	10–12	91–92
		A3 to T2	5–7	85–87
		A4 to A3 (in two steps)	(5 + 2) = 7**	90–92
Phosphor-amidite	d(CCGG)	G2 to G1	10–12	95–97
		C3 to G2	3–5	90–91
		C4 to C3 (in two steps)	(3 + 1) = 4**	94–96

* DMTr-bearing sequence.

** See footnote to p.

maintained at high temperature. However, an elevated column temperature (50°C) alone was found to be insufficient as the product still gave rise to multiple peaks (Fig. 2A). (As TEAB is unstable at elevated temperatures, TEAA was used for this analysis.) Since the multiple peaks originate from the aggregated species, it is expected that denaturation of the sample prior to injection will resolve this problem. Indeed this was found to be the case for d(DMTrCCGG). The denaturation was effected by heating the sample in the presence of a denaturant such as formamide. Fig. 2B shows the HPLC profile of a concentrated solution of the sample denatured in 80% formamide. The column temperature was maintained at 50°C. It may be noted that a single peak was obtained for the desired product under these conditions (Fig. 2B).

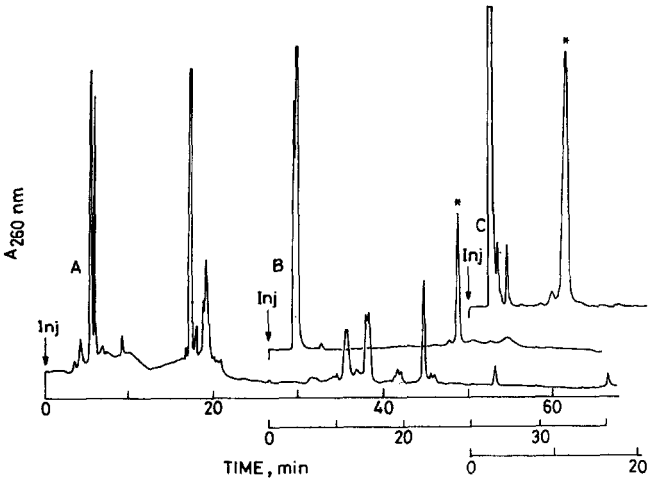

Fig. 2. Effect of denaturation of a concentrated solution of the sample prior to injection. Sample: d(DMTrCCGG); (A) not denatured; (B) and (C) denatured. Column: Ultrasphere ODS. Detection: UV at 260 nm. Range: 0.2 a.u.f.s. Flow-rate: 1 ml/min. Temperatures: 50°C for (A) and (B); 23°C for (C). Eluent: gradient of acetonitrile in 0.1 *M* TEAA; for (A), 5–20% in 7.5 min, 20–100% in 60 min; for (B), 15–25% in 20 min and then 25% isocratic (no peak was seen above 25%) for (C), 26% acetonitrile in 0.1 *M* TEAB, isocratic. The desired product is indicated by an asterisk.

Once loaded on the column, the denatured oligomer is unlikely to form double-stranded or any other aggregated species. Therefore, injection of the denatured sample onto a column at room temperature should also give rise to a single peak for the product. Additionally, this enables the usage of TEAB as buffer even for CG-containing sequences. Fig. 2C shows the single peak of d(DMTrCCGG) obtained at room temperature using TEAB as buffer.

The purification of d(DMTrCCGG) was carried out using TEAB as buffer (Fig 3A). The purified material was detritylated and repurified on the same column. Fig. 3B is a representative chromatogram of the semi-preparative purification of d(CCGG) carried out at ambient temperature using TEAB as buffer. As expected, this doubly purified material showed a single peak on further analysis (Fig. 3C). The homogeneity was confirmed by 270-MHz NMR spectroscopy. Other spectroscopic properties of the product were found to be the same as reported previously for d(CCGG)[12]. Detailed structural studies will be carried out on the purified material.

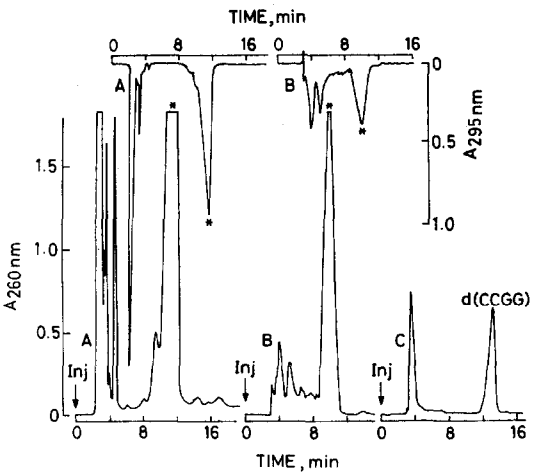

Fig. 3. Purification of d(CCGG). Column: Ultrasphere ODS. Detection: UV at 260 (bottom) and 295 (top) nm. Range: 2 a.u.f.s. Flow-rate: 1 ml/min. Temperature: 23°C (ambient). Eluent: x% of acetonitrile in 0.1 *M* TEAB; (A) $x = 25$; (B) $x = 5.5$; (C) $x = 5$.

In this report we have presented the separation of both 100% AT- and 100% GC-containing sequences in order to show the suitability of this approach for sequences of various base compositions. As the separation is carried out at ambient temperature, the scope for using other highly volatile buffers which may be unstable at elevated temperatures is practically unlimited. Additionally, carrying out analysis at room temperature is operationally more convenient. While this work was in progress, Mahoney and Yount[13] reported the use of a TEAB–ethanol system for the reversed-phase HPLC purification of micromolar quantities of mononucleotide analogues. Subsequently, Greenhut and Rudolph[14] used TEAB as buffer for the preparative separation of mono-, di- and triphosphates of mononucleosides by ion-exchange HPLC.

The versatility of the present approach lies in its applicability to other columns. Thus, we have been successful in developing similar procedures for oligonucleotide purification using ion-exchange columns[15].

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