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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE OLIGONUCLEOTIDES

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SUMMARY

Improved separations of the protected oligonucleotides are reported using a polar bonded CN phase with gradient elution (methylene chloride–methanol mobile phase). This packing yielded high recoveries, and overcame some of the problems associated with the use of highly polar silica columns. In addition, the application of reversed-phase and ion-pair high-performance liquid chromatography is demonstrated for the oligonucleotides and for the nucleosides and mononucleotides, respectively. Ion-pair chromatography was used to accomplish the simultaneous separation of the ribo- and deoxyribonucleosides and their mononucleotides. A novel method for the determination of oligomer chain length is also reported, using diesterase cleavage and ion-pair high-performance liquid chromatography.

INTRODUCTION

The synthesis and purification of moderately long chain sequences of the ribo- and deoxyribonucleotides are of fundamental importance to emerging research on recombinant DNA[1], in the mapping of ribonucleic acid materials[2] and in general biomedical studies involving these nucleotide polymers. Recent advances in sequencing techniques for DNA[3] have made possible the rapid, accurate determination of exact chain sequences. The synthesis of base-specific sequences of DNA and RNA have generally not kept pace with these advances, although enormous strides have been made in recent years in both solid-phase[4–9] and solution[10–12] oligomer synthesis, utilizing the phosphodiester and triester intermediates.

The recent explosion of interest in recombinant DNA studies has created an increasingly urgent need for efficient and sensitive separative techniques that can be used to purify and identify key intermediates and final products during the synthesis of desired chain segments. In general, the separation and identification of synthetic intermediates have proven to be the most time-consuming aspect of the overall synthetic procedure[11]. Classical methods, such as counter-current distribution, open-column chromatography, and thin-layer chromatography, are time consuming, and require large sample sizes.

High-performance liquid chromatography (HPLC) has been used with increasing frequency for separations of the oligonucleotides, although its enormous poten-

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tial for separations of these compounds has only recently been exploited to any large degree. A variety of modes of HPLC are potentially useful, depending upon the chemical state of the oligonucleotide.

In terms of their chromatography, the intermediates and final products of the oligonucleotides can be grouped into two general polarity categories, *i.e.* the protected and deprotected oligomers. The protected chains may have a wide variety of hydrophobic protecting groups reversibly bonded to the polar hydroxyl, phosphate and amino groups. These protecting groups (*e.g.* dimethoxytrityl) make the oligomer quite lipophilic. Conversely, with the protecting groups removed, the oligonucleotides are polar and water-soluble. The deprotected oligomers may or may not have terminal phosphates at the various hydroxyl positions, thus potentially making the oligonucleotides ionic as well. This wide range of polarities provides a diverse array of chromatographic methodologies that can be successfully applied to specific separation problems associated with oligonucleotides.

The strong anion-exchange (SAX) mode of HPLC has met with great success for separations of the ionic oligomers[5,6,13–16], although it is less useful for longer chain lengths where hydrophobic forces predominate. Also, ion exchangers tend to exhibit lower efficiencies and shorter lifetimes than reversed-phase bonded phases.

Reversed-phase HPLC has proven to be remarkably versatile for separations of the deprotected oligonucleotides with both ribosyl and deoxyribosyl sugars[10,11,17]. Jones *et al.*[11] and others[18] have extended the utility of the reversed-phase mode by adding lipophilic groups of various polarities to the oligonucleotide fragments, thus rendering them more strongly retained on reversed phase, and enhancing their purification from polar and ionic reaction products.

RPC-5 phases (which exhibit apparent mixed-mode reversed-phase–ion-exchange behavior) have also proven to be very powerful[19–24]. Size exclusion chromatography has been infrequently utilized in HPLC of the oligonucleotides, despite its obvious advantages for chain-length separations. Recent advances in silica-based polyglycol-bonded phases should prove beneficial in this regard[25–27].

For the fully protected intermediates, reversed-phase cannot always be used owing to the limited solubility of many of the oligomers in the presence of even a few percent of water. Silica has been used for the normal-phase chromatography of these compounds. However, problems have been encountered with normal-phase separations involving silica, including poor recoveries[28] and occasional rearrangements due to the high activity of silica. Guanine bases are particularly sensitive in this regard.

Owing to the above limitations of both the normal- and reversed-phase separations, the suitability of the more polar bonded phases was investigated. It was found that a bonded cyano–amino phase gave excellent separations of the fully protected oligomers, without any apparent reactivity problems.

In addition to bonded normal phases, the reversed-phase mode was investigated as a complement to the normal phase, and was utilized to provide further purification and identification of the unprotected oligomers. In addition, a variety of ancillary enzymatic, chemical and physical techniques were investigated as aids in the identification of the oligonucleotides.

A novel method of chain-length determination was devised, based on the diesterase-catalyzed cleavage of the oligomers, with subsequent ion-pair reversed-phase HPLC. Preliminary data from this technique are reported.

MATERIALS AND METHODS

Instrumentation

A Waters ALC 204 gradient HPLC was used for all gradient separations, while either Waters M6000A, M45 or Kratos LC250 pumps were used individually for isocratic separations (Waters Assoc., Milford, MA, U.S.A. and Kratos, Westwood, NJ, U.S.A.). Rheodyne 7125 (Rheodyne, Cocati, CA, U.S.A.) and Waters U6K injectors were used throughout the studies. Detection was via either a Waters 440 dual-wavelength absorbance monitor, or with a Kratos SF770 scanning UV–VIS detector. All UV absorbance scans were performed on-line using the Kratos 770.

The data were electronically integrated using either a Waters 660 data module, or a Hewlett-Packard 3390A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

Columns

A variety of column lengths and stationary phases were investigated over the course of the work. Partisil 10 ODS-3 and Partisil 5 ODS-3 were used for the bulk of the reversed-phase separations (Whatman, Clifton, NJ, U.S.A.). Whatman Partisil 10 C_8 and Partisil 5 C_8 were used for the ion-pairing studies. Partisil 10 PAC was used for all the normal-mode separations of the protected oligomers. All the above columns were 25 cm in length.

For certain applications, columns were packed in the laboratory using a Shandon-Southern slurry reservoir and blank columns (Shandon-Southern, Selwickey, PA, U.S.A.). The reversed-phase columns were packed using *ca.* 1.7 g of material (10 cm column) slurried in 10 cm^3 of isopropyl alcohol, and packed upwards at *ca.* 4000 p.s.i., using methanol as the packing solvent[29,30]. A Haskel Model DSTV 122 pneumatic pump was used for packing, purchased as a system (Alltech, Avondale, PA, U.S.A.). Blank columns of 25 cm were also packed using the above procedure.

Pre-columns of the appropriate pellicular materials were utilized to protect the analytical columns. Columns were run at ambient temperature.

Chemicals and chromatographic standards

Oligonucleotide standards and the various enzymes were obtained from Sigma Biochemicals (St. Louis, MO, U.S.A.), P-L Biochemicals (Milwaukie, WI, U.S.A.) or Worthington Biochemicals (Freehold, NJ, U.S.A.). Several of the protected and deprotected dimers and tetramers of cytidine and guanine were synthesized in our laboratory. Other protected and deprotected oligomers were purchased from P–L Biochemicals.

Tetrabutylammonium iodide used for ion-pairing was obtained from Kodak Chemicals (Rochester, NY, U.S.A.). Solvents were of HPLC grade (Burdick & Jackson, Muskegon, MI, U.S.A.).

Preparation of the samples and mobile phases

The various mobile phases were prepared on a volume percent basis, and were degassed using a flowing stream of helium for 3–5 min. Aqueous solvents were filtered through 0.45-μm filters prior to use. Protected standards were dissolved in 100% dichloromethane.

Chromatographic conditions

Normal phase. After extensive investigations of a wide variety of mobile phases, the following conditions were adopted for routine separations of the various $(GC)_n$ oligomers, and other fully protected oligonucleotides; gradient elution, from 0% methanol–dichloromethane (5:95) (solvent A) to 100% methanol–dichloromethane (50:50) (solvent B) in 20 min, using a Waters 660 solvent programmer, (linear curve 6).

It is essential that both solvent reservoirs be thoroughly degassed, and continuously mixed during use, owing to the poor miscibility and density differences of the two solvents.

For the semi-preparative separations using a Magnum-9 PAC column (Whatman) isocratic elution was used, with a mobile phase of methanol–dichloromethane (7.5:92.5), at a flow-rate of 6.0 cm^3 min^{-1}.

Reversed phase. Both gradient and isocratic elution were used for the reversed-phase separations. For gradient elution, the conditions were: solvent A, 0.1 mol dm^{-3} in KH_2PO_4 (pH 5.0) containing 1.2% methanol; solvent B, 0.1 mol dm^{-3} in KH_2PO_4 (pH 5.0) containing 50% methanol. Gradient curves: curve 6, 0–100% in 20 min. Flow-rate, 1.0 cm^3 min^{-1}. Other gradient slopes and various isocratic delay times were used for certain specific applications, as described in the text. The above conditions however proved to be quite versatile, and offer a convenient starting point for developing new separations.

Ion pairing

Tetrabutylammonium iodide was used as the hetaeron ($3 \cdot 10^{-3}$ mol dm^{-3}) in an aqueous mobile phase containing 7.5% methanol. The buffering salt was 0.05 mol dm^{-3} KH_2PO_4, pH 4.9. Either 5- or 10-μm C_8-bonded phases were used for all ion-pairing separations, with isocratic elution. Columns were equilibrated with at least 100 cm^3 of eluent before use, in order to coat the stationary phase thoroughly and to equilibrate the system[31].

Deprotection of the deoxy oligomers

Detritylation. The dimethoxytrityl protecting groups were removed by mixing equal volumes of a dichloromethane solution of the oligomer and a solution of 2% benzenesulfonic acid in dichloromethane–methanol (70:30) at 0°C. After 5 min reaction time, an equal volume of 10% HCO_3 was added in order to quench the reaction. The oligomer product is found in the (lower) dichloromethane phase, which was removed and evaporated.

Side chain. The residue from the above procedure was treated with 60% NH_4OH in methanol for 6 h at 50°C. Upon evaporation, the residue was dissolved in methanol–water (30:70), and immediately frozen and stored at −20°C.

Dephosphorylation. Terminal phosphates were removed using alkaline phosphatase, Type VII (Sigma) (*ca.* two units of enzyme activity to one absorbance unit of oligonucleotide). The phosphatase solution was heated to *ca.* 80°C for 4 min prior to use in order to remove contaminating diesterase activity. The enzyme and the oligomer were incubated in 0.05 mol dm^{-3} Tris buffer and reacted for 3 h, or until all terminal phosphates were removed.

Chain-length determination

Terminal phosphates were removed (if present) using the alkaline phosphatase procedure outlined above. The phosphatase activity was then reduced to negligible levels by the addition of sufficient phosphate to make the solution $5 \cdot 10^{-3}$ mol dm^{-3} in phosphate, and by lowering the pH to 7.3.

About 0.5 mg of phosphodiesterase (Type VII, Sigma) was then added to the above solution, and it was incubated for 1.5 h at 50°C. The reaction was terminated by heating to 70°C for 5 min. Residual phosphatase activity did not present a problem when solutions were stored at −20°C for periods of several months.

RESULTS AND DISCUSSION

Normal-phase chromatography of the protected oligonucleotides

Fig. 1 shows the structure of a fully protected cytidylyl(3′–5′)guanosine (CG) dimer. The structure is hydrophobic, being insoluble in methanol solutions containing more than *ca.* 15% water. This hydrophobicity limits the use of reversed-phase HPLC, since the minimum organic concentration required for solubility elutes the protected CG oligomers with very low capacity ratios (k'). Therefore the normal-phase mode of HPLC was investigated utilizing a polar (cyano-amino) bonded phase. This phase proved to be highly efficient and versatile, as shown in Fig. 2, where the protected dimer CG is found to be separated into two distinct peaks. The identities of the peaks labeled I and II were unknown, but it was suspected that they arose from incomplete side-chain reactions. Another possibility, however, was either that some rearrangement of the bases had occurred, or that peaks I and II represented diastereomers.

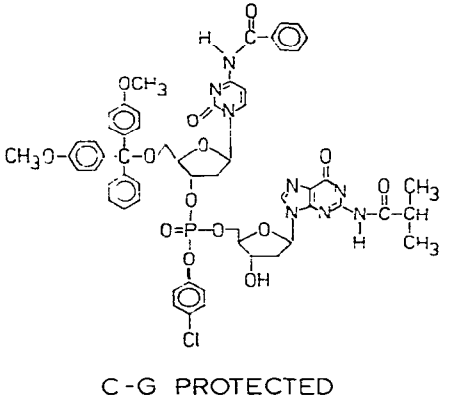


Fig. 1. Structure of the dimer cytidylyl(3′–5′)guanosine (CG) showing the presence of various protecting groups routinely used during the synthesis of the higher oligonucleotides.

In order to characterize further the peaks labeled I and II in Fig. 2, UV scans were obtained on-line using the stop-flow techniques. These scans are shown in Fig. 3. Although not definitive in a positive sense, these data nevertheless indicate that peaks

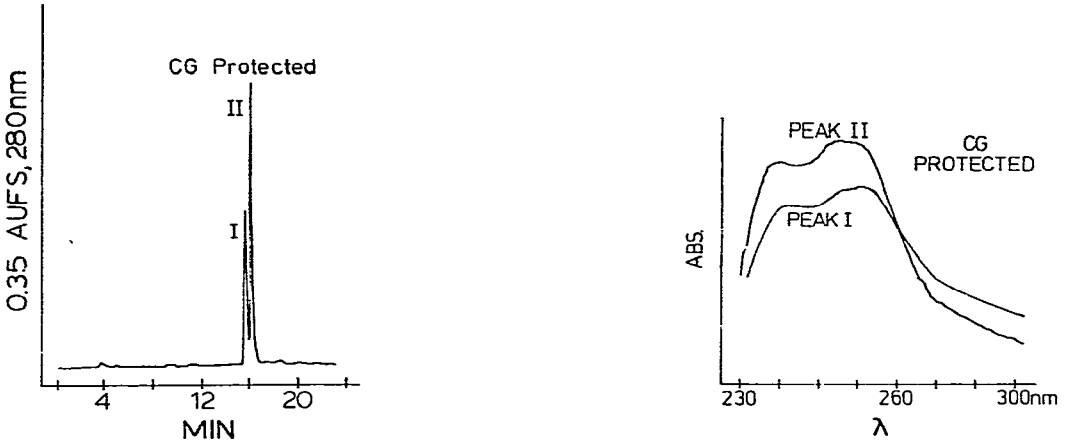


Fig. 2. Normal-phase HPLC of the protected dimer CG, using a cyano–amino-bonded phase (Whatman PAC) and a dichloromethane–methanol mobile phase. (See Materials and methods section for exact conditions of mobile phases and gradient elution.)

Fig. 3. UV scans of peaks I and II of Fig. 2, obtained on-line using the stop-flow technique. (Chromatographic conditions: same as Fig. 2, detector: Kratos-Schoeffel SF770.)

I or II were not due to excess starting material or protecting groups, but that both peaks possessed similar chromophores.

Fig. 4 shows a chromatogram of the protected tetramer, CGCG, which again had been extensively purified prior to chromatography. The multiple peaks indicated either reaction mixture impurities, or perhaps again some side-chain loss or possibly diastereomers. Co-injection of the tetramer with the dimer GC indicated that the first two peaks of Fig. 4 co-eluted with the dimer peaks I and II of Fig. 2, thus suggesting that these peaks were the same dimer reaction intermediates.

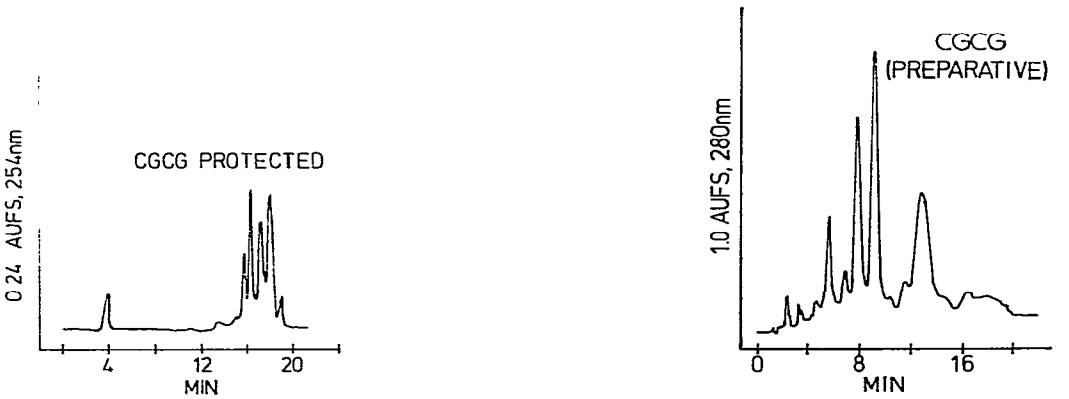


Fig. 4. Normal-phase separation of the protected tetramer CGCG (same conditions as in Fig. 2).

Fig. 5. Semi-preparative normal-phase separation of the tetramer of Fig. 4, using a Whatman Magnum-9 column. Flow-rate, 6.0 cm^3 min^{-1} (see Materials and methods section for other details). Injection size, *ca.* 2 mg of total solute.

In order to collect the various fractions of Figs. 2 and 4 for purification and further analysis, a semi-preparative separation was developed using a Magnum-9 column, packed with the same 10-μm bonded CN phase as used in the above studies. Fig. 5 shows the separation of *ca.* 2 mg of the protected tetramer CGCG, using mobile phase conditions slightly different from those used in Fig. 4. Owing to the excellent selectivities afforded by this stationary phase, much higher loadings could be used successfully.

The cyano-bonded phase was found to be useful for the separation of a variety of other protected oligomers, with good reproducibility, and column lifetimes. Over the course of *ca.* 3 months, retention times generally varied by less than 10%, provided that proper care was taken of the mobile phase preparation (see Materials and methods), and that a pre-column was used. Although no apparent rearrangements were found to occur, further studies are currently underway to confirm this. However, it appears that the bonded CN phase is milder than underivatized silica. Quantitative spectrophotometric studies indicated recoveries in excess of 98% for the protected dimer CG.

Reversed-phase separations for the deprotected oligonucleotides

The reversed-phase mode of HPLC has been demonstrated to be well suited for separations of the deprotected (water-soluble) oligonucleotides, with both ribo and deoxyribo sugars[10,11,17,18]. Fig. 6 shows the separation of base-hydrolysis products of mRNA obtained during a time study. Nearly 40 discrete peaks are seen to be emerging from the molecular envelope of the parent RNA peak, and these serve to illustrate the selectivity and efficiency of modern bonded reversed-phase materials. The sensitivity of HPLC allows studies such as the above hydrolysis to be conducted using only nanomoles or even picomoles of total solute.

Strong anion exchange has also been successfully used for separating those oligomers with terminal phosphate groups[5,16]. Ion exchange, however, generally suffers from shorter lifetimes and overall poorer efficiency than reversed-phase chromatography. Since the chain length of the oligomer increases, the ionic contribution becomes less important relative to the hydrophobic forces involved, as would be expected from solvophobic theory[31]. The utility of reversed-phase chromatography for separations of ionic oligomers is illustrated in Fig. 7, in which the deprotected deoxy-oligomers of CG are separated using a reversed-phase (C_{18}) column with gradient elution. The presence of the terminal phosphate on the hexamer $(CG)_3$ has only a moderate effect on the k' value relative to the dephosphoylated hexamer. Thus the reversed-phase mode is well suited to separations of the longer (tetramers and higher) deprotected oligomers, even in the presence of ionic phosphate groups.

One limitation of reversed-phase chromatography is that no absolute relation exists between k' values and chain length or structure[17], although certain trends are evident, such as that longer chains of the same repeating monomer will have longer retentions, etc. Further studies will be necessary to determine even empirical trends to relate structure with retention.

Ion-pair chromatography of the nucleosides and nucleotides

In the course of base determination and structural studies of the oligonucleotides, it is often necessary to be able to quantitate and identify the individual

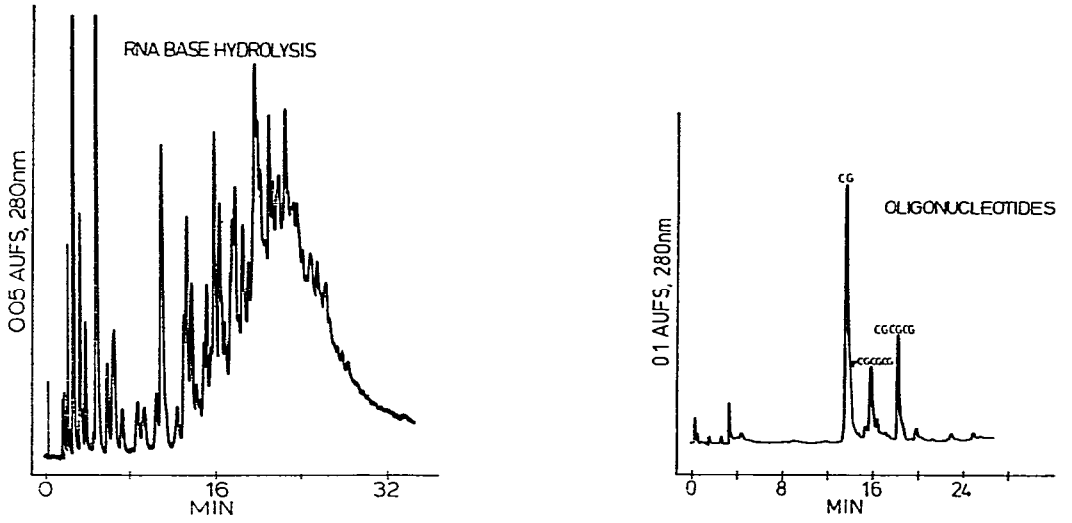


Fig. 6. Reversed-phase separation of the hydrolysis products of mRNA, after 8 h at pH 8.9. Gradient elution, from 0 to 100% of pump B in 40 min, after an initial 10-min gradient delay; flow-rate, 1.0 cm^3 min^{-1} (see Materials and methods section).

Fig. 7. Reversed-phase separation of the dimer and hexamer of cytidine and guanosine, showing the effect both of chain length and of terminal ionic groups on the capacity factors of the deprotected oligomers in reversed-phase HPLC. (Same chromatographic conditions as Fig. 6.)

monomers at the base, nucleoside or nucleotide level of structure. The bases and nucleosides have been successfully separated using reversed-phase HPLC[32,33], while the nucleotides (mono-, di- and tri-) have been separated on reversed-phase using hetaeric (ion-pairing) agents[34]. It was found necessary in our work with the oligonucleotides to separate both the nucleosides and mononucleotides simultaneously. Therefore, a reversed-phase, ion-pairing system was investigated.

Fig. 8 shows a separation of the major ribonucleosides and -nucleotides found in RNA (except tRNA). Ion-pairing proved to be remarkably effective for the simultaneous separation of neutral and charged species. It was found that the retention of the nucleotides could be varied almost independently of the nucleosides by increasing the ionic strength or pairing-ion concentration, while changing the percentage of organic modifier reduced the k' of both the neutral and charged species. This is similar to the behavior of other neutral and charged solutes observed when using alkyl sulfate hetaerons[35].

APPLICATIONS

Identification of the oligonucleotide chains

In order to characterize further the two major peaks of the CG dimer separated in Fig. 2, the dimer was chromatographed on the semi-preparative separation discussed above, and deprotected according to the procedures outlined in the Materials and methods section. Combination of the two collected peaks and subsequent reversed-phase separation (after deprotection) yielded a single peak labeled CG in Fig. 9. This peak had a k' value identical to pure CG standard. UV scans and spectral ratio (254/280) gave further confirmatory evidence that the same basic CG structure

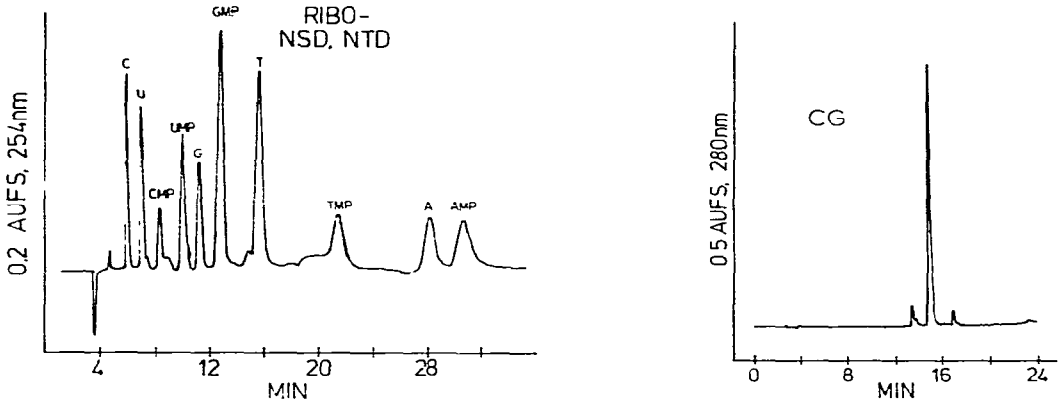


Fig. 8. Ion-pair separation of the eight major ribonucleosides and monophosphate nucleotides. Column: Whatman Partisil 10 C_8, 250 × 4.6 mm I.D. Hetaeron, 15 m*M* tetrabutylammonium iodide. Isocratic elution, 9% methanol, 0.05 *M* KH_2PO_4. (See Materials and methods section for details.)

Fig. 9. Chromatography of peaks I and II from Fig. 2, after collection and deprotection. The total collected fraction of I and II yielded a single peak on the reversed phase, indicating a single CG dimer. (Chromatographic conditions, same as Fig. 7, but 0–100% of pump B in 30 min.)

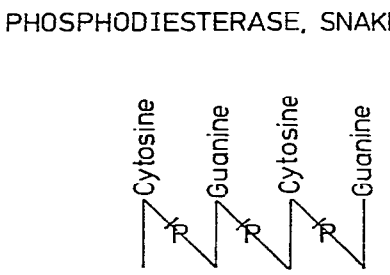
produced both peaks I and II in Fig. 2, and that either diastereomers or incomplete protection was responsible for these two species. Similar work-up confirmed the presence of CG dimer along with the CGCG tetramer shown in Figs. 4 and 5.

Determination of chain length

During the course of a separation or synthetic scheme, it would often be of great value to know the chain length and base composition of an oligonucleotide, in lieu of determining its exact sequence by an absolute sequencing method[3]. Fig. 10 shows a schematic diagram of the tetramer CGCG. Any terminal phosphates are first removed using alkaline phosphatase. The enzyme snake venom diesterase (I), will hydrolyze the phosphate linkages on the 3′ side, thus producing a single nucleoside (terminal monomer), and 5′-monophosphate nucleotides. When chromatographed under conditions separating the nucleosides and monophosphate nucleotides, the mole fraction of nucleotides to nucleosides will give the chain length, absolute base composition and leading nucleotide of the oligomer. Thus the nucleoside cytidine and the nucleotides CMP and GMP should be produced from the structure of Fig. 10.

A separation of the deoxynucleoside and -nucleotide standards using ion-pair chromatography is shown in Fig. 11. These are the same basic conditions as described above (see *Ion pairing*), but with a shorter column length and different particle size. The single peak labeled CG in Fig. 9 was collected and subjected to diesterase-catalyzed hydrolysis. The reaction products are shown in Fig. 12, where the nucleoside Cyd and the nucleotide GMP are separated. The normalized mole percent confirmed the anticipated 1:1 ratio, while the absence of CMP precluded the possibility of $(CG)_2$, $(CG)_3$, etc.

It was found possible to determine the lengths of longer chains, as illustrated in Fig. 13, where the chain length of the hexamer ATGCAT was determined. The appearance of the nucleoside Ado, along with the correct mole ratio of TMP, GMP,



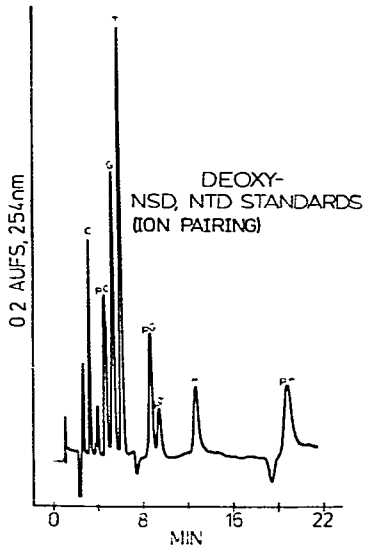
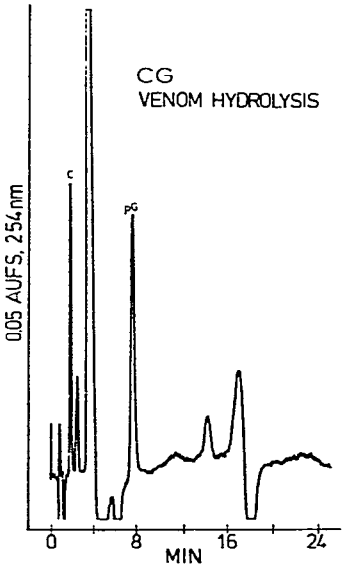


Fig. 10. Schematic diagram of the oligonucleotide CGCG, indicating where phosphate cleavage should occur with diesterase (I). Hydrolysis for chain-length determination should yield one nucleoside (Cyd), one CMP, and two GMP monomers.

Fig. 11. The deoxyribonucleoside standards separated by reversed-phase ion-pairing chromatographic conditions identical to Fig. 8, but using a 100 × 4.6 mm I.D. column (Shandon-Southern), packed with Partisil 5 C_8 (Whatman).



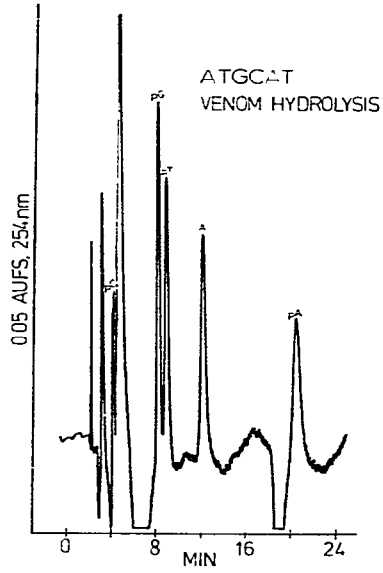


Fig. 12. Snake-venom hydrolysis products of the major peak collected in Fig. 9, indicating the presence of a single nucleoside (C = Cyd) and a monophosphate nucleotide (pG = GMP). Conditions the same as Fig. 11.

Fig. 13. Chain-length and base-composition determination of the hexamer ATGCAT, utilizing diesterase (I) and ion-pair HPLC. Same conditions as in Fig. 11. Peaks: pC = CMP; pG = GMP; pT = TMP; A = adenosine; pA = AMP.

CMP and AMP, strongly supported the probability that the hexamer peaks (which had been collected from a reversed-phase separation) did indeed contain the desired hexamer.

UV scans, absorbance ratios and further enzymatic reactions (such as phosphorylations) were used to obtain further evidence of the structures.

Since pure synthetic standards are rarely available for the longer oligonucleotides, absolute data, such as chain length and base compositon, are essential for full utilization of the chromatography.

CONCLUSIONS

The potential applications of the various modes of HPLC to several important aspects of the purification and identification of both the ribo- and deoxyribonucleotides have been illustrated in the above studies. The polar cyano–amino-bonded phase investigated is well suited for selective separations of the protected oligomers, and from preliminary investigations, does not appear to degrade or irreversibly bind the guanine residues, as does silica[18]. By using both the normal-phase and the reversed-phase modes of HPLC, highly effective purification and identification procedures can be developed, especially when coupled with various enzymatic, chemical and spectrophotometric techniques.

A novel method of chain-length determination has been presented based upon the enzymatic cleavage of the 3′–5′ phosphate linkages, followed by ion-pair chromatography. Further studies are currently underway to determine the optimal chemical and chromatographic conditions for the routine determination of chain lengths. The maximum chain length that can be determined in this manner is a function of the accuracy of the quantitation; however, it appears that with the present system, chains of 5–10 monomers can be determined rapidly and accurately using only nanomoles of total solute. Further investigations are currently underway to optimize the enzymatic and chromatographic conditions.

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