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USE OF AN AMINO–SILICA COLUMN FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SYNTHETIC OLIGODEOXYNUCLEOTIDES

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SUMMARY

The use of an amino–silica column in the chromatographic analysis of synthetic oligodeoxyribonucleotides and their derivatives from different stages of oligonucleotide synthesis has been investigated. By eluting with 0.10 *M* potassium phosphate solution of pH 3.30, the nucleotide composition of oligonucleotides can be established within 15 min. In a linear gradient of phosphate buffer (0.10–0.75 *M*) at neutral pH, the separation of oligonucleotides by length and in an acidic medium (pH 3.30–4.30) by composition is possible; the oligonucleotides may be in the free form or modified by the various protecting groups used in synthetic oligonucleotide chemistry.

The analysis of some reaction mixtures from different stages of oligonucleotide synthesis and of a number of synthetic oligodeoxyribonucleotides and their derivatives has been performed.

INTRODUCTION

One of the problems in the synthetic chemistry of oligonucleotides is the rapid and effective analysis of these compounds at different stages of oligonucleotide synthesis. Paper and thin-layer chromatography are not very effective for this purpose[1–3].

The use of high-performance liquid chromatography (HPLC) has enabled the sensitivity of the analysis of simple mixtures of nucleotides, nucleosides and heterocyclic bases to be considerably improved[4–8], but with synthetic oligonucleotides the method has so far found very limited application, although the monitoring of syntheses is very important and a versatile technique is needed.

In HPLC a few types of ion-exchange sorbents have been used successfully for separating and analysing mixtures of nucleic acids with various structural components. In particular, pellicular[6–8] and conventional fine[5] resins have been widely applied to mononucleotides. Reversed-phase chromatographic systems have been used to separate oligonucleotides on the basis of the length of the nucleotide chain[9]. These

packing materials may also help in certain analytical problems with oligonucleotides. They are, however, far from being universal and require different temperature regimes and different mobile phases, etc. In addition, it is time consuming to have to change the columns and the operating conditions for the analysis of different synthetic oligonucleotides, and it is not known whether particular sorbents are suitable for the separation of oligonucleotides on the basis of both the length of the chain and the nucleotide composition. Further, it is not known whether the sorbents are adequate for oligonucleotides with modified functional groups.

We investigated the possibility of using, for the analysis of mixtures of oligonucleotides and their derivatives, a weak anion-exchange sorbent consisting of silica with covalently bound primary aminoalkyl functions. Such a sorbent was selected because it can be used if the solutions to be analysed contain a certain amount of organic solvent, which often occurs with synthetic oligonucleotide preparations; this is not possible, however, if reversed-phase systems are used as the column material. Also, we believed that the use of a weak ion exchanger may facilitate the elution of polyanions and may allow weaker eluents to be employed. In this study we used a Micro-Pak NH_2-10 column (Varian, Zug, Switzerland) for the analysis of polar organic compounds in organic media.

MATERIALS AND METHODS

Standard solutions of monodeoxyribonucleotides were prepared with products obtained from Calbiochem (Luzern, Switzerland). The oligodeoxyribonucleotides and reaction mixtures were synthesized by known techniques[10–12]. Oligonucleotides were previously purified by chromatography in a 7 *M* solution of urea by the method of Tomlinson and Tener[13].

Buffers were prepared from a monosubstituted potassium phosphate purified by the method of Kennedy and Lee[14].

Chromatographic analyses were carried out in a Varian Aerograph Model 8500 liquid chromatograph. Detection of the nucleotide material was performed at 254 nm.

The MicroPak NH_2-10 column (0.2 × 25 cm) was previously washed with 0.75 *M* KH_2PO_4 solution at pH 3.30 and then equilibrated with 0.10 *M* phosphate buffer.

Samples were injected into the column with a Hamilton Microliter 701 syringe.

RESULTS AND DISCUSSION

Analysis of the nucleotide composition of oligodeoxyribonucleotides

The nucleotide composition of oligodeoxyribonucleotides is usually determined by analysing a mixture of mononucleotides obtained by digestion of the oligonucleotide chain to the nucleotide level with snake venom or spleen phosphodiesterase[12,15]. The separation of monodeoxyribonucleotides by means of HPLC was first carried out on a pellicular anion-exchange support at 80° in a linear gradient of phosphate buffer by Burtis *et al.*[5]. The duration of the analysis, including column regeneration, was about 30 min. Anion-exchange sorbents of the Zipax SAX type with controlled surface porosity can be used to separate a mixture of four common deoxyribonucleotides at room temperature without gradient elution. Under these conditions, however, the time of analysis is doubled[16].

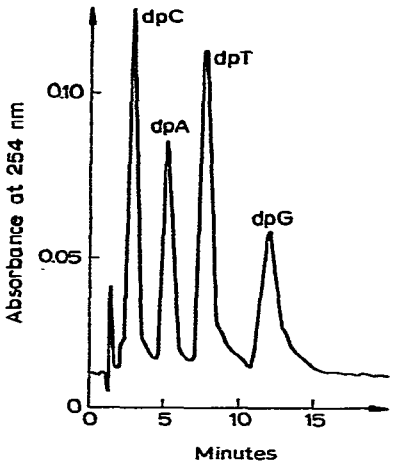

Fig. 1. Separation of a standard mixture of four deoxyribonucleotides (20 n*M*) at room temperature on a MicroPak NH_2-10 column (0.2 × 25 cm) using 0.10 *M* KH_2PO_4, pH 3.30, at 60 ml/h. Inlet pressure, 3500 p.s.i.

The use of an amino–silica column for this purpose allowed the time of analysis to be greatly reduced, as shown in Fig. 1. The elution of nucleotides is carried out at room temperature, the column needs no regeneration owing to the isocratic conditions of the elution and the time of analysis is less than 15 min.

It is interesting that the sequence of elution of the nucleotides dpA and dpT in an amino–silica column is the reverse of that from pellicular and conventional polymerized anion-exchange resins at similar pH values of the eluent[5]. It is known that the order of the elution of nucleotides is determined not only by ionic but also by hydrophobic and other interactions and, consequently, purine nucleotides are usually more strongly adsorbed on resins that contain organic polymers. On silica gel with bonded aminoalkyl radicals, the interactions of a non-ionic nature, although still present, seem to be less pronounced and deoxyriboadenylic acid is therefore eluted earlier than thymidylic acid.

The results of the resolution of the mixture of deoxyribonucleotides were used for the characterization of the nucleotide composition of a large number of synthetic oligodeoxyribonucleotides.

Separation of oligonucleotides and their derivatives

The chromatography of oligonucleotides and their derivatives on an amino–silica column is carried out by gradient elution with a phosphate buffer. As illustrated in Fig. 2, at neutral pH of the buffer, the elution of oligonucleotides is performed in accordance with the total value of the negative charge of the dissociated phosphate groups. A slight drift of the baseline as the concentration of the elution buffer increases is probably caused by trace amounts of polyphosphates formed during the storage of the buffer.

Under the conditions indicated in Fig. 2, the mixture prepared by polymerization of the trinucleotide d(pT–T–T) in the presence of mesitylene sulphochloride was analysed[11].

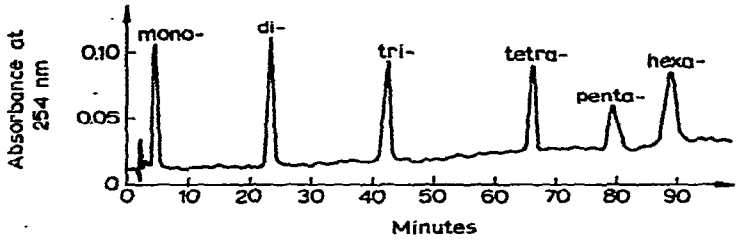

Fig. 2. Chromatography of oligothymidylic acids on a MicroPak NH_2-10 column at pH 7.0 in a linear gradient of phosphate buffer at room temperature. Starting eluent, 0.10 *M* KH_2PO_4. Gradient eluent, 0.75 *M* KH_2PO_4. Gradient slope, 0.32. Flow-rate, 60 ml/h. Inlet pressure, 3500 p.s.i.

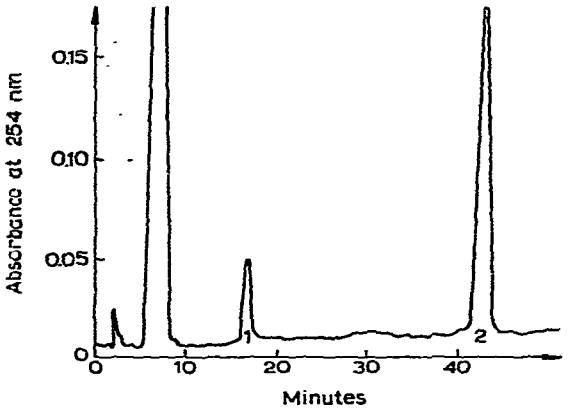

Fig. 3. Separation of a reaction mixture after phosphorylation of d(MeOTrT–$\overset{\text{An}}{\underset{|}{\text{C}}}$–$\overset{\text{iBu}}{\underset{|}{\text{G}}}$) and removal of protecting groups. The conditions of separation are shown in Fig. 2. Peak 1, d(T–C–G) (20%); peak 2, d(T–C–Gp) (80%).

Under the same conditions, oligonucleotides with and without phosphate terminal groups can be separated. Fig. 3 shows a chromatogram of the reaction mixture prepared by phosphorylation of the trinucleotide d(MeOTrT–$\overset{\text{An}}{\underset{|}{\text{C}}}$–$\overset{\text{iBu}}{\underset{|}{\text{G}}}$)* by phosphoryl chloride imidazolide[12].

In order to separate oligonucleotides on the basis of their nucleotide composition, it is necessary to shift the pH of the eluent to the acidic region where heterocyclic bases are protonated. We carried out the chromatography of different oligonucleotides at pH 3.30, which is a suitable value for separating monodeoxyribonucleotides. From Fig. 4, it follows that at this pH value, by using gradient elution, small oligonucleotides can be separated simultaneously according to their chain length and composition. These conditions were applied to the analysis of the homogeneity of a number of synthetic oligonucleotides (Table I).

* MeOTr = monomethoxytrityl; An = anisoyl; iBu = isobutyl.

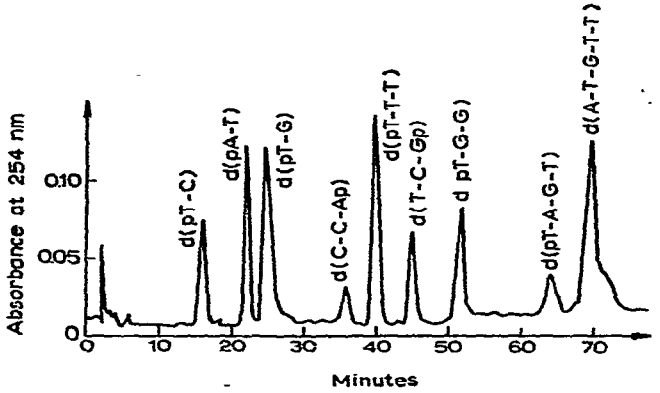

Fig. 4. Separation of oligodeoxyribonucleotides on a MicroPak NH_2-10 column at pH 3.30 in a linear gradient of phosphate buffer at room temperature. Starting eluent, 0.10 *M* KH_2PO_4. Gradient eluent, 0.75 *M* KH_2PO_4. Gradient slope, 0.26. Flow-rate, 60 ml/h. Inlet pressure, 3500 p.s.i.

The introduction of protecting groups into the residues of deoxyribose and heterocyclic bases in order to perform a certain oligonucleotide synthesis affects the ability for protonation to occur, the hydrophobic properties and, hence, the chromatographic behaviour of the compounds concerned. Chromatography of oligonucleotides containing different acyl groups (N-benzoyl, -anisoyl, -isobutyl and 3'-O-

TABLE I

RETENTION TIMES OF OLIGONUCLEOTIDES AND THEIR DERIVATIVES ON A MICROPAK NH_2-10 COLUMN UNDER CONDITIONS OF GRADIENT ELUTION AT pH 3.30

The conditions of separation are shown in Fig. 4.

No.	*Oligonucleotide*	*Retention time (min)*
1	d(pT–C)	16.5
2	d(pA–T)	22
3	d(pT–G)	24.5
4	d(pT–T)	23
5	d(C–C–Ap)	36
6	d(pT–T–T)	39.5
7	d(T–C–Gp)	45
8	d(pT–G–G)	52
9	d(pT–A–G–T)	63
10	d(A–T–G–T–T)	69.5
11	d(pT–TAc)*	25.5
12	d(pT–T–TAc)*	42.5
13	An An Bz d(C – C–Ap)* Bz iBu	57.5
14	d(A –T– G –T–TAc)*	112.5

* Ac = acetyl; An = anisoyl; iBu = isobutyl; Bz = benzoyl.

acetyl) on an amino–silica column shows that they are eluted much later than unprotected oligonucleotides (Table I).

The presence of N-substituents in the heterocyclic residues prevents their protonation in an acidic medium. The increase in the retention times of these derivatives may, however, be associated not only with the increased total negative charge but also with some hydrophobic interactions of the alkyl radicals of a sorbent with the large organophilic groups being now present in the molecules of oligonucleotides. A similar effect arises from etherification of the 3′-hydroxyl group of deoxyribose with an acetyl group.

The possibility of separating free oligonucleotides and those with protected functional groups makes possible their effective purification and the introduction of protecting groups controls almost at any step of oligonucleotide synthesis.

The results indicate that the application of HPLC enables one to carry out comparatively rapid analyses of nanomole amounts of individual compounds and various reaction mixtures obtained by the condensation of oligonucleotide blocks, phosphorylation and acylation of oligonucleotides. The amino–silica columns are commercially available, are durable and give fairly reproducible results. The conditions used in this work for separating mono- and oligonucleotides can be used successfully for analysing the structures of native nucleic acid fragments.

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