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EXCLUSION COLUMN FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF OLIGONUCLEOTIDES

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SUMMARY

The I-125 Protein Analysis Column (Waters Assoc.), a size-exclusion column, gives separations of short oligodeoxyribonucleotides. The influence of the pH and molar concentration of the mobile phase on the resolution of the column has been studied. Volatile buffers such as aqueous triethylammonium acetate give good and rapid purifications of fully deprotected synthetic oligonucleotides.

INTRODUCTION

The chemical synthesis of oligodeoxyribonucleotides requires the preparation of completely protected fragments followed by the liberation of the amine functions of the nucleobases, the internucleotidic phosphates and the terminal hydroxy groups of the chain (see ref. 1 for a general review). This deprotection, which may be carried out by various procedures[2–4], is usually followed by purification of the oligomer obtained by high-performance liquid chromatography (HPLC).

The support used can be an ion-exchanger such as Partisil 10 SAX[5–7]. This method, however, requires the use of a non-volatile buffer (KH_2PO_4, KCl) for elution, followed by a desalting procedure using, for example, gel filtration on Sephadex; often the oligonucleotide obtained requires further purification by another method. Reversed-phase chromatography gives good results[8] but this method presents certain problems when employed alone as it is difficult to predict the elution properties of a given oligomer with respect to the other products in the mixture to be separated, and no information is afforded on the chain length of the oligomer purified.

We propose the use of the I-125 Protein Analysis Column which consists of silica gel bonded to a polyol (Waters Assoc., Milford, MA, U.S.A.) as a new chromatographic support particularly adapted to the separation of synthetic oligodeoxyribonucleotides.

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MATERIALS AND METHODS

The oligodeoxyribonucleotides studied were prepared by the phosphotriester method as reported elsewhere[9]. They were deprotected either by the procedures of Gait *et al.*[3], Stawinski *et al.*[4] or Chattopadhyaya and Reese[10].

The HPLC studies were carried out on an I-125 Protein Analysis Column (Waters Assoc.) and a column (300 × 4.7 mm) of LiChrosorb RP-8 (10 μm; E. Merck, Darmstadt, G.F.R.). The mobile phase was delivered to the system by a Milton-Roy minipump, and the products detected either at 254 nm with an Spectra-Physics SP 8200 detector or at other wavelengths using a Cecil Instruments CE 212 monitor. Products were introduced to the column using a Valco loop valve. Solvents were filtered before use through a Millipore 0.45-μm filter.

RESULTS

Table I lists the seventeen oligodeoxyribonucleotides, synthesized in this laboratory, which were studied.

TABLE I

LIST OF OLIGONUCLEOTIDES USED

No.	*Oligonucleotide*	*No.*	*Oligonucleotide*
1	d(TpC)	10	d(ApApTpTpTpApTpG)
2	d(CpT)	11	d(TpTpGpTpCpCpTpC)
3	d(ApG)	12	d(ApTpApTpApApCpA)
4	d(ApGpGpT)	13	d(ApApApTpTpGpTpCpCpTpC)
5	d(ApApGpC)	14	d(CpTpTpTpTpCpTpApGpCpC)
6	d(CpCpTpC)	15	d(CpApTpGpApApTpTpCpApTpG)
7	d(GpTpCpCpTpC)	16	d(TpCpApApCpCpApApGpApGpGpApCpA)
8	d(ApGpGpApGpGpT)	17	d(ApTpTpTpTpGpApApTpApTpApApCpA)
9	d(ApApTpTpCpApTpA)		

Influence of the oligodeoxyribonucleotide size on its retention volume, V_R

Fig. 1 shows the logarithms of the molecular weights of different oligodeoxyribonucleotides as a function of their retention volumes. The linearity of the plot would seem to indicate that the oligonucleotides studied interact with the chromatographic support by the phenomenon of exclusion*. This is in agreement with the characteristics of the column as given by the suppliers.

It may be noted that the selective permeation zone of the column corresponds to the retention volumes of synthetic oligonucleotides, whose chain length is typically between 2 and 20 nucleotide units.

We recently reported[9] the preparation of some oligodeoxyribonucleotides by the phosphotriester method using the condensation of a 5′-hydroxy terminal oligomer

* It has recently been found that oligomers $d(T_8)$ and $d(A_3TA_8)$ have respectively smaller and larger retention volumes than those predicted from Fig. 1. Nevertheless, during the separation of their crude deprotection mixtures, these products were eluted before the shorter oligomers produced by chain rupture.

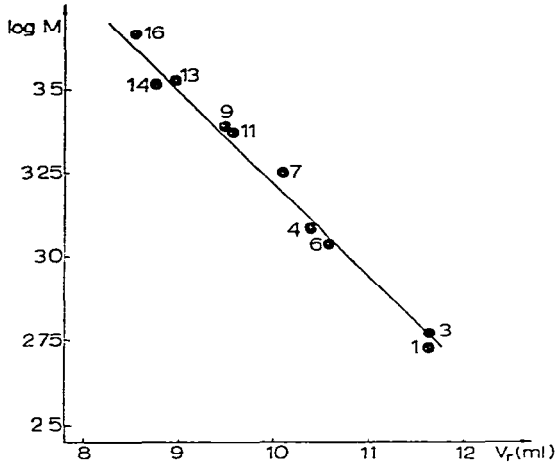

Fig. 1. Logarithms of molecular weights of oligodeoxyribonucleotides as a function of their retention volumes on the I-125 Protein Analysis Column. Solvent: 0.1 *M* triethylammonium acetate (TEAA), pH 7.0. flow-rate 1.04 ml min^{-1}.

with a 3'-phosphodiester terminal component (usually a di- or trinucleotide). After HPLC separation of the reaction mixture the product may still contain some unreacted 5'-hydroxy terminal oligomer, and during the final deprotection of the oligonucleotide chain some rupture of the internucleotidic bond may occur[11] leading to the formation of side products. Also the deprotection liberates salts of benzoic, anisoic and isobutyric acids, *p*-chlorophenol, etc. which is why the use of the I-125 column would appear to be of interest for the preparation of pure oligodeoxyribonucleotides.

Preparative use

Example 1. The protected pentadecanucleotide 16 (2.5 mg) was deprotected by the method of Chattopadhyaya and Reese[10]. The chromatogram corresponding to the preparative separation of the resulting mixture on the I-125 column is shown in Fig. 2A. It can be seen that the pentadecanucleotide 16 (peak 1) is well resolved from the nonamer remaining from the synthesis (peak 2). A further purification of the

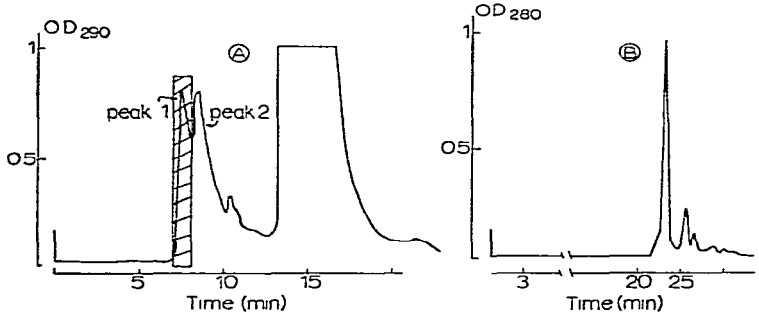

Fig. 2. A, Preparative separation of compound 16 on the I-125 column using 0.1 *M* TEAA, pH 6.4, at a flow-rate of 1.04 ml min^{-1}. B, Analysis of peak 1 on LiChrosorb RP-8 (300 × 4.7 mm). Gradient: 10–40% methanol in 0.1 *M* TEAA in 30 min. Flow-rate: 2 ml min^{-1}.

material collected in peak 1 was effected on a column of LiChrosorb RP-8 (Fig. 2B). A total of 1.40 A_{260} units of compound 16 were thus recovered.

Example 2. Higher purifications may be achieved by this method. After deprotection of the protected undecanucleotide 13 by ammonia and acetic acid[3], injection onto the I-125 column gave the result shown in fig. 3A, fractions 1 and 2 being collected. Fraction 1 was further purified on LiChrosorb RP-8, the chromatogram obtained (Fig. 3B) showing that recovery of the initial peak from the I-125 column corresponded to a relatively clean product. A chromatogram of fraction 2 (Fig. 3C) showed that this also contained some of the desired product.

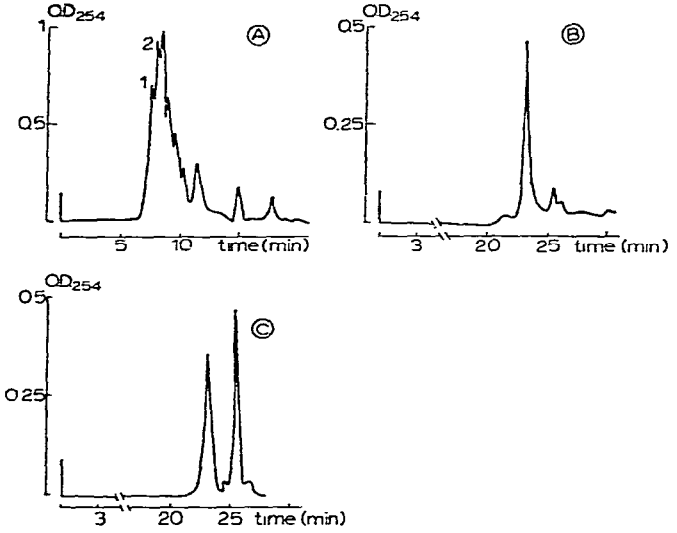

Fig. 3. A, Preparative separation of compound 13 on the I-125 column. Solvent: 0.1 *M* TEAA, pH 6.9; flow-rate 1.04 ml min^{-1}. B, Injection of fraction 1 on LiChrosorb RP-8 (300 × 4.7 mm). Gradient 10–40% methanol in 0.1 *M* TEAA in 30 min. Flow-rate: 1.4 ml min^{-1}. C, Injection of fraction 2 under the same conditions as B.

In every case the desired oligonucleotide (the longest) is the first to be eluted from the column and thus can readily be collected. The impurities arising from the deprotection (anisoates, benzoates, isobutyrates, *p*-chlorophenol) are eluted after a volume greater than the total permeation volume of the column. The reproducibility of the results obtained with analytical samples allows a rapid verification of the chain length of a given oligonucleotide by measurement of its V_R. The use of a volatile buffer for elution allows easy isolation of the products by lyophilization.

The fact that small molecules such as the different salts of organic acids and *p*-chlorophenol have retention volumes greater than the total permeation volume of the column indicates that phenomena other than exclusion operate during the elution, and so it was considered necessary to study the different factors influencing the separations obtained on the I-125 column.

Influence of the flow-rate

The retention volume of the tetranucleotide 4 measured at flow-rates from 0.7 to 1.5 ml min^{-1} showed no detectable variation.

Influence of the mobile phase pH on retention volume

The tetranucleotide 4 was analysed using 0.1 *M* triethylammonium acetate as eluent at pH values varying from 4.10 to 7.38. The results obtained are shown in Fig. 4.

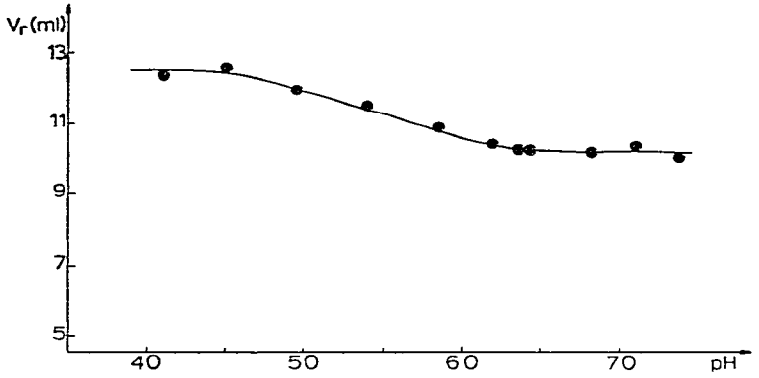

Fig. 4. Influence of pH on the retention volume of the tetranucleotide 4 using the I-125 column. Solvent: 0.1 *M* TEAA; flow-rate 1.04 ml min^{-1}.

The pH has a definite influence since the retention volume of a given component can vary by more than 2 ml, which represents a relatively large proportion of the intraparticle volume, 6.2 ml (total permeation volume, 11.6 ml; interparticle volume, 5.4 ml; measured by the retention volumes of water and dextran blue respectively). The retention volume of the tetranucleotide 4 is constant outside the range pH 4.5–6.5.

Influence of the mobile phase pH on resolution

This study was undertaken with the same solution of 0.1 *M* triethylammonium acetate whose pH was varied progressively from 9.24 to 5.55 by the addition of dilute acetic acid. The oligonucleotides used were the octamer 9 and the dimer 3.

The resolution, R_s, of two peaks 1 and 2 can be calculated by[12]

$$R_s = 1.176 \frac{(t_{R,2} - t_{R,1})}{(\delta_2 + \delta_1)}$$

where $t_{R,1}$, $t_{R,2}$ represent the retention times of peaks 1 and 2, and δ_1, δ_2 their widths at half peak height measured in the same units. Fig. 5A shows the value of R_s as a function of the pH for the dimer 3 and the octamer 9, and Fig. 5B and 5C show the chromatograms obtained in the extreme cases of resolution.

At approximately constant buffer molarity the pH has a relatively small effect on the resolution as the peaks are displaced almost equally in the same direction. However, when the concentration of the solution is significantly modified, as was necessary to obtain the extreme values of pH, the retention volumes undergo relatively large variations.

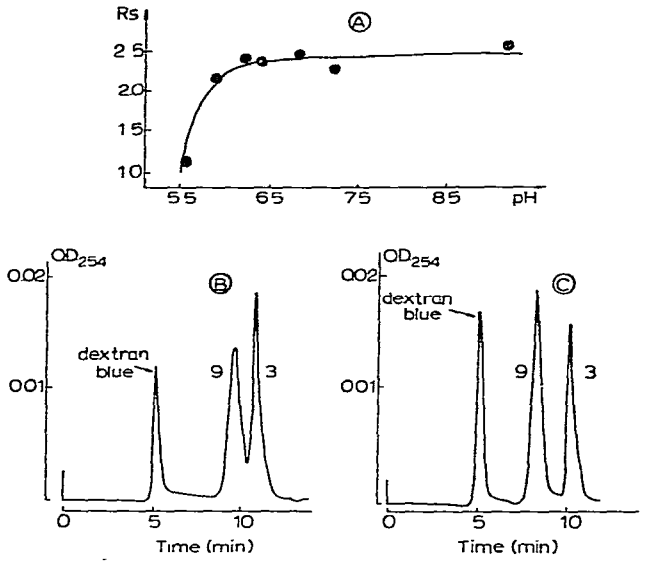

Fig. 5. A, Influence of pH on the resolution of the dimer 3 and the octamer 9 using the I-125 column. Conditions as in Fig. 4. B, Chromatogram at pH 5.55. Resolution between 3 and 9 is 1.12. C, As in B but at pH 7.24; resolution is 2.21.

It would seem that the molarity of the triethylammonium acetate in the mobile phase has a greater influence on the separation than its pH.

Influence of buffer molarity on R_s

For this study the solutions were prepared by successive dilution of the same stock solution. Fig. 6A–D show the separation of the dimer 3 and the octamer 9 using triethylammonium acetate concentrations of 1.0 *M*, 0.05 *M*, 0.01 *M* and 0.001 *M* respectively. Table II gives the retention volumes of components 3 and 9 and their resolution as a function of the molarity of the eluent.

As the concentration of triethylammonium acetate in the mobile phase is decreased the retention volume of the octamer approaches the interparticle volume, leading to a large increase in the resolution from the dimer, which then behaves similarly, being eluted as a very large molecule at a buffer concentration of 10^{-3} *M*. In water dimer was eluted at the interparticle volume of the column. This has the considerable advantage that, when using the I-125 column for the purification of synthetic oligonucleotides, whose chain lengths lie generally in the range of 8–15 nucleotide units, it is possible to optimize the separation by varying only the concentration of triethylammonium acetate in the mobile phase.

This sensitivity of the column to the concentration of the solvent would seem to indicate that phenomena other than exclusion also operate in the separation of oligonucleotides.

Influence of the nature of the solvent

Tests carried out using 0.05 *M* triethylammonium bicarbonate, pH 7.5, as the mobile phase gave similar separations as a function of the chain length. Any differ-

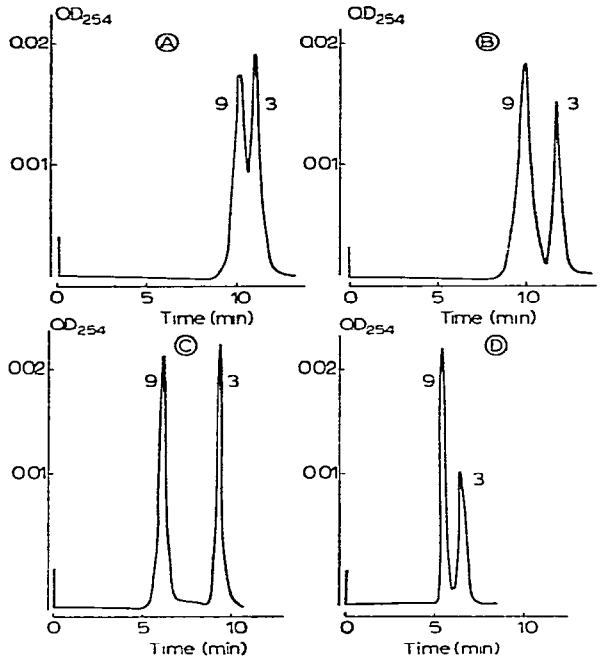

Fig. 6. Chromatograms for the separation of dimer 3 and octamer 9, obtained using the I-125 column at different concentrations of TEAA. Flow-rate: 1.04 ml min^{-1}. A, 1.0 *M* TEAA. $R_s = 0.77$; B, 0.05 *M* TEAA, $R_s = 1.70$; C, 0.01 *M* TEAA, $R_s = 4.81$; D, 0.001 *M* TEAA, $R_s = 1.43$.

TABLE II

RETENTION TIMES AND RESOLUTION OF COMPOUNDS 3 AND 9 AS A FUNCTION OF THE MOLARITY OF THE ELUENT

Triethylammonium acetate was delivered to the system at a flow-rate of 1.04 ml min^{-1}.

Parameter	*Concentration (M)*					
	1.0	*0.25*	*0.1*	*0.05*	*0.01*	*0.001*
$t_{R,9}$ (min)	10.1	10.6	10.55	9.85	5.95	5.3
$t_{R,3}$ (min)	11.0	11.45	11.6	11.6	9.1	6.3
R_s	0.77	0.81	0.99	1.70	4.81	1.43

ences in the results observed compared to those obtained using the acetate were probably due to the difficulty encountered in defining accurately the concentrations of volatile buffers, especially as before use these were filtered through a 0.45-μm filter which leads to some degassing of the solution.

CONCLUSION

Although the phenomena governing the behaviour of oligodeoxyribonucleotides on the I-125 Protein Analysis Column are not known, it is nevertheless possible to vary the experimental conditions to obtain good, rapid separations of deprotected

synthetic oligonucleotides. The use of this column allows a rapid initial control of the chain length of a prepared component and requires much less time in the purification and analysis of synthetic oligonucleotides.

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