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Reversed-Phase Ion-Pair Chromatography of Oligodeoxyribonucleotides

A. Sokolowski, N. Balgobin, S. Josephson, J. B. Chattopadhyaya and G. Schill

Department of Analytical Pharmaceutical Chemistry, University of Uppsala, Biomedical Center, Uppsala, Sweden and Department of Microbiology, University of Uppsala, Biomedical Center, Uppsala, Sweden

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Abstract

Reversed-phase ion-pair chromatography of oligodeoxyribonudeoticles. Sokolowski, A. [1]; Balgobin, N. [2]; Josephson, S. [2]; Chattopadhyaya, J. B. [2]; Scill, G. [1] (Department of Analytical Pharmaceutical Chemistry,[1] University of Uppsala, Biomedical Center, P.O. Box 574, S-751 23 Uppsala, Sweden and Department of Microbiology,[2] University of Uppsala, Biomedical Center, P.O. Box 581, S-571 23 Uppsala, Sweden).

DNA fragments ranging from one to four nucleotidyl units have been chromatographed in a reversed-phase ion-pair chromatographic system with tetraalkylammonium ions of different kind as counter ions. It has been possible in the present work to show that the regulation of retention of the DNA fragments can be controlled by changing the concentration of the counter ions. Thus a DNA fragment with a higher phosphate charge can have higher retention time and, therefore, requires a lower concentration of counter ions to elute them from the column.

Introduction

The unambiguous chemical synthesis of DNA segments of defined sequences constitute an important step in the approaches toward the reconstruction of a plasmid with a specific gene to obtain a desired polypeptide (hormones or proteins) [1–3]. Such a chemical synthesis not only involves regiospecific formation of internucleotide linkage ($3' \rightarrow 5'$) between the nucleotides along the chain length of DNA but it also seriously depends on the separation techniques for isolation of the desired oligo-DNA fragments of right chain length from the shorter ones.

The ion exchange chromatography [4–8], both at low and high pressure, have been widely used for such separations. Separations of oligonucleotides by reversed-phase chromatography on octadecyl bonded silica with aqueous ammonium acetate buffer as mobile phase have also been exploited [9, 10]. These two modes of separations have been clearly based on different structural properties: (i) number of phosphate residues (anionic content; $pK_a \sim 3$ for internucleotidyl phosphate group and $pK_a \sim 3.8$ for the first dissociation constant of 3′-terminal phosphomonoester, whereas the second dissociation constant lies within the range pK_a 6–7 [11, 12]). (ii) the hydrophobicity of the molecule in question.

In the present study of separation by reversed-phase ion-pair chromatography, both kinds of properties are exploited. The anionic oligonucleotides are distributed to the hydrophobic stationary phase as ion pairs with hydrophobic quaternary ammonium ions present in the mobile phase. The retention increases with the hydrophobicity and with the number of quaternary ammonium ions in the ion pair, i.e. with the number of negative charges in oligonucleotides.

Jost [13] has separated oligoribonucleotides by ion-pair chromatography with LiChrosorb RP-18 as stationary phase and unbuffered aqueous solutions of tetraethylammonium chloride with a low content of methanol as mobile phases.

In the present report we have applied the ion-pair technique for oligodeoxyribonucleotides of different charge and hydrophobicity using buffered mobile phases of constant ionic strength containing highly hydrophobic counter ions in low concentrations.

Experimental

Apparatus

The pump was a LDC Model 711-47 solvent delivery system (Milton-Roy Minipump with pulsdampener) and the detector was a LDC Model 1205 UV Monitor used at 254 nm wavelength. A Valco CV-6-HPax sample injection valve with a 10.6-μl loop was used.

Both the eluent reservoir and the column were thermostated at 25.0 °C with circulating water.

The pH was measured with an Orion Research Model 801 A/digital pH meter equipped with an Ingold combined electrode type 401.

Eluent

The eluent was prepared from equal volumes of methanol and phosphate buffer pH 7.0. The phosphate buffer had an ionic strength of 0.032 and were prepared from phosphoric acid and sodium hydroxide. When preparing the eluents containing tetraalkylammonium ions an equivalent amount of the sodium hydroxide was exchanged for tetraalkylammonium hydroxide to keep the ionic strength constant.

Samples

The oligonucleotides have been prepared following a phosphotriester methodology [8].

The abbreviations used are as follows. For example TpCpApGp, where T is the 5′-end and Gp is the 3′-end of the oligonucleotide. T, C, A, G, denote thymidine, 2′-deoxycytidine, 2′-deoxyadenosine and 2′-deoxyguanosine respectively. Internucleotidyl phosphodiester linkage is denoted by p and 3′-terminal p denotes a phosphomonoester.

All oligonucleotide samples were dissolved in methanol + phosphate buffer pH 7 of ionic strength 0.032 (1:1). The exact amount of nucleotide in the injected sample was not known.

Characterization of the peaks

The structural assignments of deoxyoligonucleotide residues eluted under each main peak has been carried out by ^{32}P labelling at the 5′-OH of the deoxyoligonucleotide with γ-^{32}P-ATP and kinase, followed by electrophoresis on 20 % polyacrylamide gel (pH 8.3) along with the authentic markers and then visualisation by autoradiography.

Determination of the hold-up volume, V_m

The hold-up volume of the column was determined by injection of water. The same hold-up volumes was obtained by injecting sodium nitrate when no tetraalkylammonium ion was present in the eluent.

Results and discussion

Retention of oligodeoxyribonucleotides as ion pairs

The retention of an anionic compound in a reversed phase system increases with increasing concentration and hydrophobicity of the counter ion present in the mobile phase. An illustration of this fundamental principle is given in Fig. 1, which shows a separation of a mononucleotide, Ap, and a dinucleotide, ApAp, using methanol + phosphate buffer pH 7.0 as solvent in the mobile phase. At concentration of 0.012 M or lower, tetrapropylammonium (TPrA) and tetrabutylammonium (TBA) give very low retention while tetrapentylammonium (TPeA) is the most suitable counter ion in the present case. The trivalent ApAp has a higher retention than the divalent Ap at TPeA concentrations of 0.001 M or higher and the separation factor (k'_{ApAp}/k'_{Ap}) increases with increasing concentration of TPeA.

The choice of counter ion must be adapted to the hydrophobicity and charge of the oligonucleotide. The retention behaviour of a series of oligodeoxyribonucleotides with different charges and content of base is demonstrated in Fig. 2. The retention of compounds containing the same base increases with increasing charge as seen from the series TpT – TpTp – TpTpTpTp. The curves indicate that TPeA is suitable as counter ion for the trivalent TpTp only. The pentavalent TpTpTpTp is strongly retained even by 0.0016 M TPeA and a counter ion with a lower content of alkyl carbon is preferable (TBA 0.0105 M gives $k' = 6.2$). The monovalent TpT is, on the other hand, very slightly retained even at the highest concentration of TPeA. A more suitable retention can be obtained by use of a more hydrophobic counter ion or a lower content of methanol in the mobile phase.

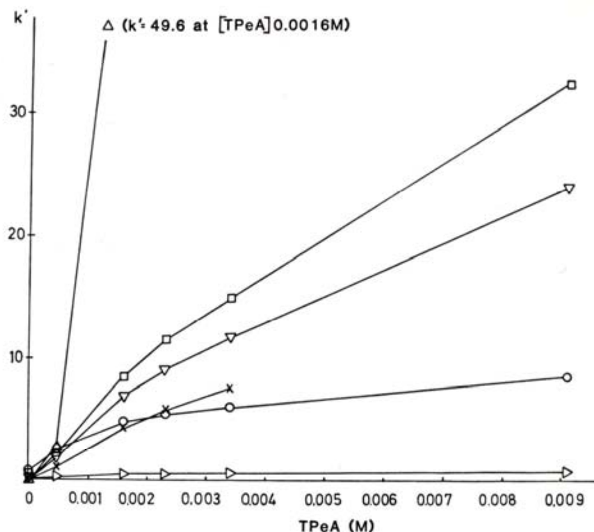


Fig. 2. Effect of the concentration of tetrapentylammonium (TPeA) on retention.
Eluent: TPeA in methanol + phosphate buffer pH 7 (1 + 1)
Solid phase: LiChrosorb RP-18, 5 μm (column 100 × 4.5 mm)
Samples: ▷ = TpT
○ = Ap
× = CpGp
▽ = ApAp
□ = TpTp
△ = TpTpTpTp

Fig. 2 also demonstrates the good possibilities to separation of compounds of the same charge containing different bases (TpTp – ApAp – CpGp) when a counter ion of suitable kind and concentration is used. However, if the sample contains oligonucleotides with widely different charge and composition it might be difficult to make the separation under isocratic conditions. Gradient elution with change of the concentration of the counter ion might be preferable in such cases.

The oligonucleotides, as a rule, show a very good chromatographic behaviour with narrow and symmetrical peaks in the ion-pair systems. A typical chromatogram is shown in Fig. 3: the reduced plate height ($h = H/dp$) is about 8 and the asymmetry factor is very close to 1.0. In systems with a low counter ion concentration, highly retained sample might give slightly tailing peaks (Fig. 4). Application of a lower amount of sample

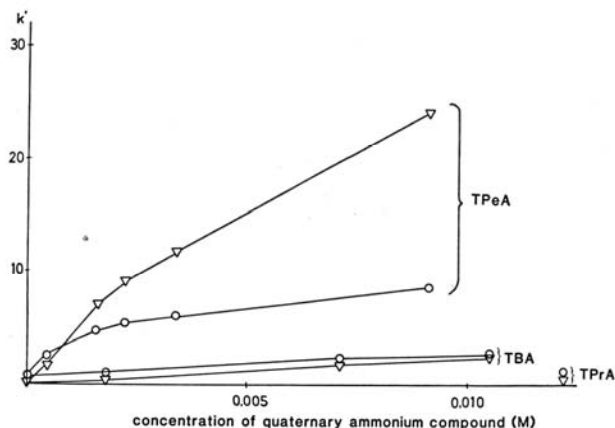


Fig. 1. Effect of different tetraalkylammonium ions on retention.
Eluent: Tetraalkylammonium in methanol + phosphate buffer pH 7 (1 + 1)
Solid phase: LiChrosorb RP-18, 5 μm (column 100 × 4.5 mm)
Samples: ▽ = ApAp
○ = Ap

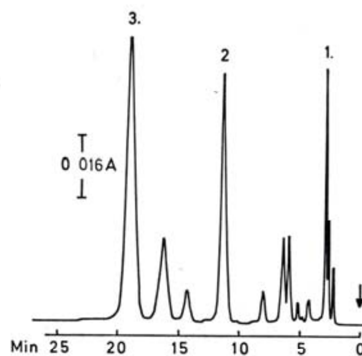


Fig. 3. Separation of oligodeoxyribonucleotides.
Eluent: TPeA 0.0016 M in methanol + phosphate buffer pH 7.03 (1 + 1); 0.84 mm/sec, 140 – 150 bars
Solid phase: LiChrosorb RP-18, 5 μm (column 100 × 4.5 mm)
Peaks: 1 = ApT
2 = Ap
3 = TpTp

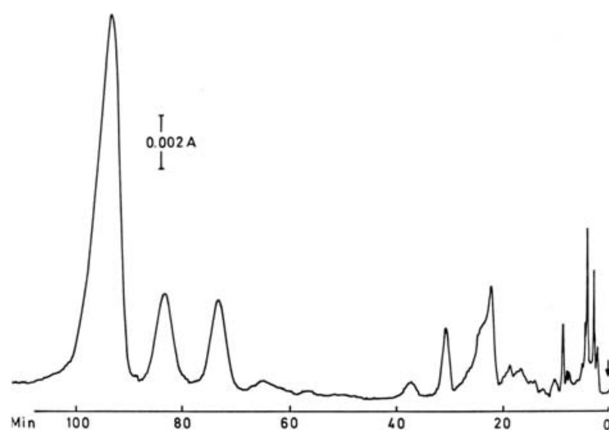
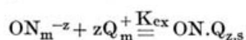


Fig. 4. Chromatogram of TpTpTpTp.
Eluent: TPeA 0.0016 M in methanol+phosphate buffer pH 7.03 (1+1); 0.84 mm/sec, 140−150 bars
Solid phase: LiChrosorb RP-18, 5 μm (column 100×4.5 mm)

or use of a less hydrophobic counter ion in higher concentration should improve the peak symmetry.

Retention mechanism

The retention is assumed to be an ordinary ion-pair adsorption process [14] based on the following equilibrium:



$$ON_m^{-z} + zQ_m^+ \overset{K_{ex}}{\rightleftharpoons} ON.Q_{z,s}$$

ON_m^{-z} is the anionic oligonucleotide in the mobile phase and Q_m^+ is the counter ion present in the mobile phase. $ON.Q_{z,s}$ is the ion-pair adsorbed to the stationary phase.

By changing the kind and concentration of the counter ion it is possible to regulate the distribution of the oligonucleotide between the phases. A quantitative expression for the distribution is based on the equilibrium constant for the process

$$K_{ex} = \frac{[ON.Q_z]_s}{[ON^{-z}]_m[Q^+]_m^z} \tag{1}$$

The magnitude of the constant depends on the nature of the ion-pair components and the properties of the phases. From eq. (1) it is easy to derive an expression for the distribution ratio of the anion.

$$D = \frac{[ON.Q_z]_s}{[ON^{-z}]_m} = K_{ex} \cdot [Q^+]_m^z \tag{2}$$

It shows that the higher the numerical charge of the sample, the stronger is the effect of a change of kind and concentration of the counter ion.

However, in adsorption processes it is also necessary to take into consideration the capacity of the adsorbent. The ion pairs of the sample will compete with other mobile phase components for the limited number of adsorption sites giving the following expression for the capacity ratio

$$K' = \frac{q.K^o.K_{ex}.[Q^+]_m^z}{A + K_{ex}.[ON^{-z}]_m.[Q^+]_m^z} \tag{3}$$

q is the phase ratio in the column, K° the capacity of the adsorbent while A includes the influence of the competing agents.

The curves in Fig. 2 indicate that the retention of the oligonucleotides might follow a relationship of this kind. The retention might, however, also be influenced by other effects. When using an eluent without any tetraalkylammonium counter ion, some sample containing two or more nucleotide units show a retention that is lower than the hold-up volume of the column. This indicates an influence from a size exclusion mechanism which may counteract the effect of the ion-pair retention.

Column stability

When using an eluent containing methanol-phosphate buffer pH 7 (1:1) with the addition of tetrapentylammonium ions, voids occurred in the top of the analytical column after the column had been in continuous use for one week. By refilling the void with fresh support it was possible to use the column for one or two days more but then a new void occured. After each refilling the pressure drop increased and when the column had been refilled 5 to 6 times the pressure drop reached such a level that the column had to be discarded. It was also observed that the retention decreased when the column had been used for more than one week and the separating efficiency of the column decreased gradually in spite of the refilling of the void in the top of the column. Normally the columns had to be discarded after two weeks.

The degradation of the adsorbent could be delayed by inserting a guard column containing the same material before the analytical column and the injection valve. No significant change in retention or separating efficiency and no voids in the top of the column were then observed after the column had been in continuous use for 5 weeks. However, on prolonged use of the column the usual signs of degradation appeared.

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Department of Analytical Pharmaceutical Chemistry
University of Uppsala
Biomedical Center
P.O. Box 574
S-751 23 Uppsala, Sweden

Department of Microbiology
University of Uppsala
Biomedical Centre
P.O. Box 581
S-751 23 Uppsala, Sweden