NEW METHOD FOR THE SEPARATION OF OLIGONUCLEOTIDES BY CHROMATOGRAPHY ON ACRIFLAVIN–AGAROSE

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Received 29 January 1982

1. Introduction

In the fields of molecular genetics and nucleic acid research, the synthesis and preparation of homo- and hetero-oligonucleotides are of growing interest. Generally, oligonucleotide separation is performed by ion-exchange chromatography [1–5] or by high-performance liquid chromatography (HPLC) (review [6]). Separation based on ion exchange allows for limited resolution since the charge difference between successive oligomers is small. However, HPLC requires expansive apparatus. Such drawbacks led to the synthesis of new adsorbents for oligonucleotides and related compounds separation. Separation of aromatic and heterocyclic compounds may be performed by charge-transfer chromatography employing solid supports to which acriflavin has been covalently bound [7,8]. Such charge-transfer gels were used in [9] to separate 5′-AMP from cyclic 3′,5′-AMP. The separation of single from double-stranded DNA can also be achieved through the use of acriflavin–agarose gels [10]. Adsorbents consisting of similar dyes covalently bound to polyacrylamide gels have been used for base-pair-specific fractionation of sheared DNA [11] or for structure-specific separation of supercoiled DNA from released or linear forms [11,12]. Such separations are based on the effects of electron-charge transfer, hydrogen bonding and electrostatic interaction in promoting the formation of intercalating dye complexes.

We report here a new separation method for various oligonucleotides using an acriflavin-substituted agarose gel and compare the results to those obtained by the more classical DEAE ion-exchange method and to HPLC.

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2. Materials and methods

5′-TMP was purchased from Boehringer (France). Oligo(dT) was synthesized from the pyridinium salt of 5′-TMP under anhydrous conditions and in the presence of dicyclohexylcarbodiimide (Aldrich-Europe, Belgium) according to [13]. Oligo(rI) was a gift from Professor M. Michelson (Institut Curie, Paris). Hetero-oligonucleotides were synthesized using *Escherichia coli* polynucleotide phosphorylase as in [14,15]. Essentially, the reaction mixture contained 100 mM Tris–HCl buffer (pH 8.5), 0.3 M NaCl, 10 mM 2-mercaptoethanol, 10 mM $MnCl_2$ oligo(dT)$_4$ (Collab. Res. Mass.) as primer 5′-dNDP (dNDP/primer = 1:5) and 5 units polynucleotide phosphorylase/ml. The mixture was incubated at 37°C for several hours and stopped by the addition of 50 mM EDTA.

Acriflavin–ultrogel A4R type I was obtained from Réactifs IBF (France) or prepared by a modification of the original method in [7] using 4% agarose gel beads instead of dextran gel.

DEAE–Trisacryl M was obtained from Réactifs IBF. HPLC chromatography was performed with a RPC5 column [6] consisting of a polychlorotrifluoroethylene support coated with methyl-trialkyl-ammonium chloride. All other chemical agents were of analytical grade.

Chromatographic separations on acriflavin–agarose were done at room temperature using glass columns (1.6 × 60 cm or 0.38 × 40 cm). All experiments were done after equilibration of gel columns with sodium acetate–acetic acid or ethylmorpholine–HCl buffer as indicated in the figure legends. Chromatographic elutions were done with linear NaCl gradients using an Ultrograd apparatus (LKB, Sweden).

DEAE ion-exchange chromatography was performed

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

in a 1.6 X 20 cm column filled with DEAE-Trisacryl M. The column was equilibrated with 0.025 M sodium acetate buffer (pH 5.3) containing 0.2 M NaCl, and elution was performed using a linear NaCl gradient from 0.2–0.8 M in the same buffer.

Some oligodeoxyribonucleotides were analyzed on a RPC_5 column filled with DEAE-Trisacryl M. The column was equilibrated with 0.025 M sodium acetate buffer (pH 5.3) containing 0.2 M NaCl, and elution was performed using a linear NaCl gradient from 0.2–0.8 M in the same buffer.

Some oligodeoxyribonucleotides were analyzed on a RPC_5 column as in [16]. Nucleoside compositions of oligonucleotides were analyzed on a jacketed Aminex A_5 column after phosphodiesterase and phosphatase digestion [16]. The unidentified products which appear on some figures are the results of secondary products due to the chemical synthesis itself [13].

3. Results and discussion

We had determined that the adsorption of short oligonucleotides to acriflavin gel is favored at low ionic strength (not shown). Increasing the ionic strength decreases the extent of the interaction between the oligonucleotides and the immobilized acriflavin drug. We have applied the samples at low ionic strength in order to facilitate adsorption of the oligonucleotide onto the acriflavin gel; weak electrostatic interactions may help to promote the initial rate of association.

Fig.1a shows the elution profile of a series of oligo(dT) nucleotides at pH 4 from an acriflavin gel. The elution position varies as a function of the size of the oligomer (number of mononucleotides). Moreover, increasing the pH of the buffer decreases the resolving capacity of the acriflavin gel for oligo(dT) (fig.1b); the distance between the oligo$(dT)_n$ peak and oligo$(dT)_{n+1}$ peak decreases. An increase in pH may modify the ionization state of the chromatographic support as well as that of the solute (modification of the electron density of the aromatic rings) and thus alter the elution molarity value for NaCl. In any case, increasing the salt concentration progressively decreases electrostatic interactions. The elution positions of the oligomers vary as a function of the net charge of each oligonucleotide, which is proportional to the size of the oligomer. The chromatographic separation afforded by the acriflavin gel results from a combination of charge transfer and electrostatic interactions and perhaps from minor contributions by hydrophobic and hydrogen bonding interactions. This combination of interactions provides a better partition coefficient than that obtained with classical ion exchangers. We find a linear relationship between the elution position, in terms of [NaCl] and molecular size of the oligomer tested. This empirical relationship might facilitate oligomer identification when the method is used to resolve unknown mixtures. Using a DEAE ion-exchanger, the elution molarities of the oligonucleotides are also a function of their size, but follow a non-linear rule. Chromatography with acriflavin–agarose can be extended to the efficient separation of other oligonucleotide mixtures (fig.2). Fig.2a represents the separation of a mixture of oligoribopurines (oligo-rI) using

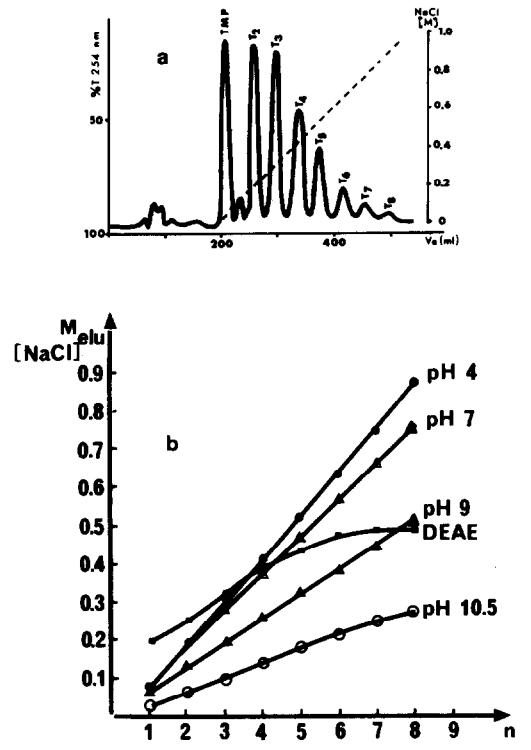

Fig.1. (a) Chromatographic separation of 5′-TMP oligomers (1 mg) on acriflavin–Ultrogel A4R type I (4.6 X 60 cm column) in 10 mM sodium acetate buffer (pH 4) at 34 ml/h flow rate with an NaCl gradient. (b) Relationship between the size of the oligo(dT) and their NaCl elution molarity (M_{elu}). Chromatographic separations are performed either on acriflavin–Ultrogel A4R: (●——●) pH 4, 10 mM sodium acetate buffer, (△——△) pH 7, (▲——▲) pH 9, (○——○) pH 10.5, 50 mM ethylmorpholine–acetic acid buffer; or (■——■) on DEAE-Trisacryl M; n = number of T residues in the oligomers.

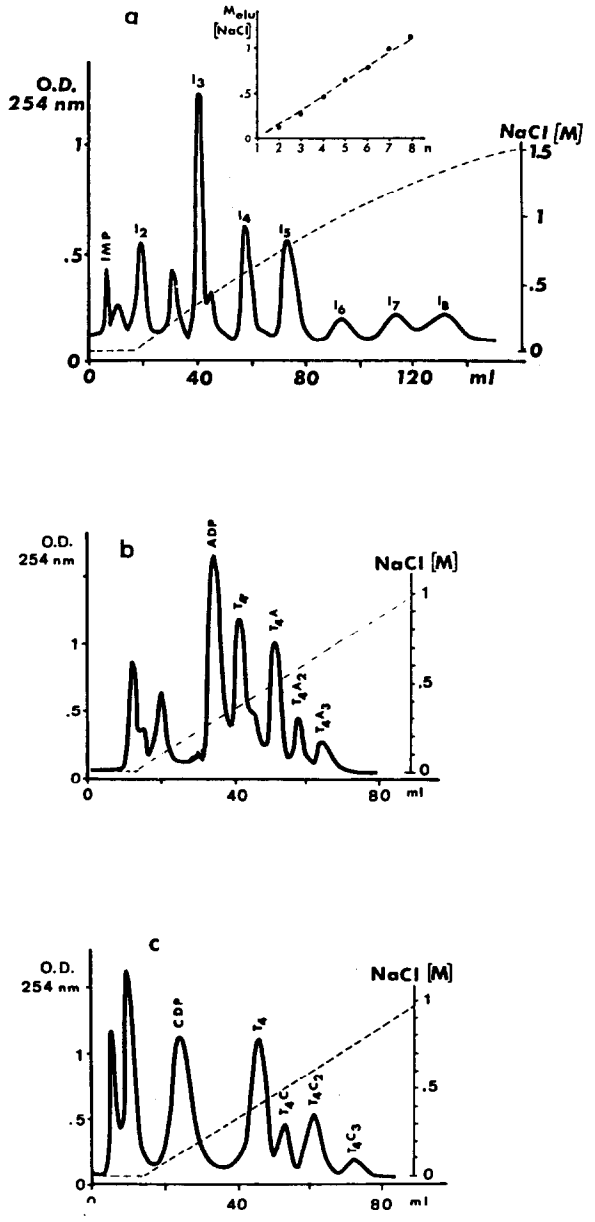

Fig.2. Chromatographic separation of various oligonucleotides (0.5 A_{260}) on acriflavin–Ultrogel A4R type I. (0.38 × 40 cm column) in 10 mM sodium acetate buffer (pH 4) and at 20 ml/h flow rate; NaCl gradients were as indicated in the figures. (a) 5′-IMP oligonucleotides: The inset represents the relationship between the NaCl elution molarity (M_{elu}) and the size of each 5′-IMP oligomer. n = number of purines in the oligomers. (b) Products of the enzymatic reaction between oligo(dT)$_4$ and 5′-ADP using polynucleotide phosphorylase. The two non-adsorbed peaks represent some nucleotide impurities and/or nucleoside monophosphates. (c) Products of the enzymatic reaction between oligo(dT)$_4$ and 5′-CDP using polynucleotide phosphorylase.

similar experimental conditions to fig.1a. A linear relationship is found between the elution volumes and the molecular size. A comparison of the elution molarity values for oligo(dT) and oligo(rI) molecules shows that oligopurines are preferentially retained (e.g., for n = 5).

Chromatography on acriflavin–agarose also appears to be an excellent method for the separation of hetero-oligonucleotides. Fig.2b,c show the resolution of 2 series of enzymatically synthesized hetero-oligonucleotides. From these and data not shown it appears that the retention order of purine and pyrimidine oligomers is:

$$C < T \sim U < G < I < A$$

We have performed a comparative experiment on the separation of 5′-TMP oligomers by using acriflavin–

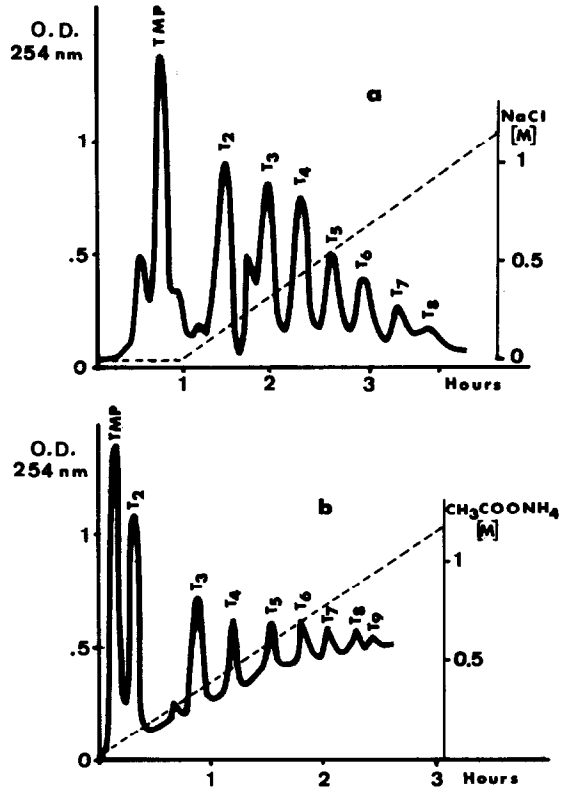

Fig.3. Chromatography of 5′-TMP oligomers on: (a) acriflavin–Ultrogel A4R type I in the same conditions as fig.2; (b) RPC$_5$ as in section 2, at room temperature, using a 0.3 × 25 cm column at 60 ml/h flow rate, and with an ammonium acetate gradient (pH 4.5).

agarose and RPC-5 high performance liquid chromatography. The separation efficiency is better on acriflavin gel than on RPC-5 HPLC whereas the time required to effect separation is shorter on the RPC-5 column (fig.3); 2.5 h instead of 4 h. Nevertheless the use of immobilized acriflavin chromatography for oligonucleotide separation offers several advantages: it is easy to use and does not require expensive auxiliary equipment; it permits excellent resolutions on an analytical scale and may be scaled up without difficulty.

Acknowledgements

We express our deepest gratitude to Professor P. Chambon for his useful recommendations during the development of this work, and to Professor J. Porath and Dr B. Davison, for valuable suggestions during the preparation of this manuscript. We also thank J. L. Plassat for his technical help.

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