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Note

Use of fast protein liquid chromatography for the purification of synthetic oligonucleotides

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Solid-phase methods made the synthesis of oligodeoxyribonucleotides rapid and allowed its widespread employment in molecular biology[1]. Oligonucleotides synthesized by means of these methods have to be purified from by-products after detachment from the solid matrix. The demand for a rapid and effective method of purification based on chain length and unaffected by base composition is based on the following: (i) the product of interest is generally the longest polymer among those present in the complex reaction mixture; and (ii) owing to the degeneration of the genetic code a set of oligonucleotides with the same length but different sequences often has to be synthesized.

It has been demonstrated that reversed-phase high-performance liquid chromatography (HPLC) and ion-pair HPLC are not adequate, as the separation of oligonucleotides depends on both the size and the base composition in a way that is difficult to predict[2]. Further, preparative polyacrylamide gel electrophoresis under denaturing conditions is not completely unaffected by the base composition and, moreover, is a time-consuming method that does not allow high recovery of the product[3].

Up to now HPLC on strong anion exchangers has been the most suitable method[2]. The columns commonly utilized (*e.g.*, Partisil 10 SAX) are based on a derivatized silica gel matrix. To reduce to the minimum the influence of the base composition on the chromatographic pattern, organic solvents can be included in the mobile phase[4]. However, the pH range (3.5–7.0) over which the columns are operative is very narrow, basic conditions being totally excluded. A different chromatographic absorbent having a wider operating pH range (2.0–12.0), stable to pressure and organic solvents, is now commercially available in the form of pre-packed columns (Mono Q, R 515; Pharmacia, Uppsala, Sweden), and is usually employed for protein purification[5].

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We have exploited the capability of this column to separate synthetic oligonucleotides of different lengths in order to establish conditions that allow separation only on the basis of chain length.

EXPERIMENTAL

Instrumentation

Two chromatographic systems were used: (a) A Pharmacia fast protein liquid chromatography (FPLC) system consisting of a gradient programmer with two P-500 pumps equipped with an LKB S UV monitor; and (b) A Varian 500 liquid chromatograph equipped with a Varichrom UV detector.

Chemicals and oligonucleotides

A mixture of polydeoxyribothymidilic acids with chain lengths of up to 15 nucleotides was prepared by the phosphotriester method[6]. By the same method were prepared two hexadecamers, $d(TGTACA_4C_2TACG_2)$ and $d(AT_3C_2ATC_2GTAG_2T)$, one octadecamer, $d(ATGT_3C_3TGATGT_2GC)$, and a mixture of four hexadecamers, $d(TA^A_GTCCATCCACCA^C_TT)$.

Columns and chromatographic conditions

Three columns were used: Whatman Partisil 10 SAX (250 × 4.6 mm I.D., 10 μm), Pharmacia Mono Q R 515 (50 × 5 mm I.D., 10 μm) and reversed-phase Hibar LiChrosorb RP-18 (Merck) (250 × 40 mm I.D., 5 μm).

For neutral elution, solvent A was 0.02 *M* KH_2PO_4 (pH 7.0)–20% acetonitrile and solvent B was 0.02 *M* KH_2PO_4 (pH 7.0)–1 *M* KCL–20% acetonitrile. For basic elution, solvent A was 0.02 *M* piperazine (pH 9.7)–20% acetonitrile and solvent B was 0.02 *M* piperazine–1 *M* KCl (pH 9.7)–20% acetonitrile. A linear gradient from 10% to 70% B in 70 min was used and the flow-rate was 0.5 ml/min.

For elution on the RP-18 column, solvent A was 0.1 *M* triethylammonium acetate (TEAA) (pH 7.0) and solvent B was acetonitrile. A linear gradient from 0% to 20% B in 80 min was used and the flow-rate was 1 ml/min.

The column effluents were monitored at 256 nm. All chromatography was carried out at room temperature.

RESULTS

To compare chromatographic analysis carried out under alkaline and neutral conditions, a saline gradient (KCl) buffered by phosphate (20 m*M*, pH 7.0) or piperazine (20 m*M*, pH 9.7) was used. Using the neutral eluent a mixture of oligo(dT)s was chromatographed on two columns, Partisil 10 SAX (Fig. 1A) and Mono Q (Fig. 1B). The chromatograms clearly indicate that the resolution capacity of the Mono Q column is comparable to that of Partisil 10 SAX.

Two hexadecamers with different nucleotide sequences were co-chromatographed on a Mono Q column at pH 7.0 (Fig. 2A) and pH 9.7 (Fig. 2B): two partially resolved peaks were observed under neutral conditions, whereas only one peak was detected under alkaline conditions. Further, when an octadecamer was added to the mixture it co-migrated with one of the hexadecamers at pH 7.0 (Fig. 3A) whereas it appeared as a separate peak at pH 9.7 (Fig. 3B).

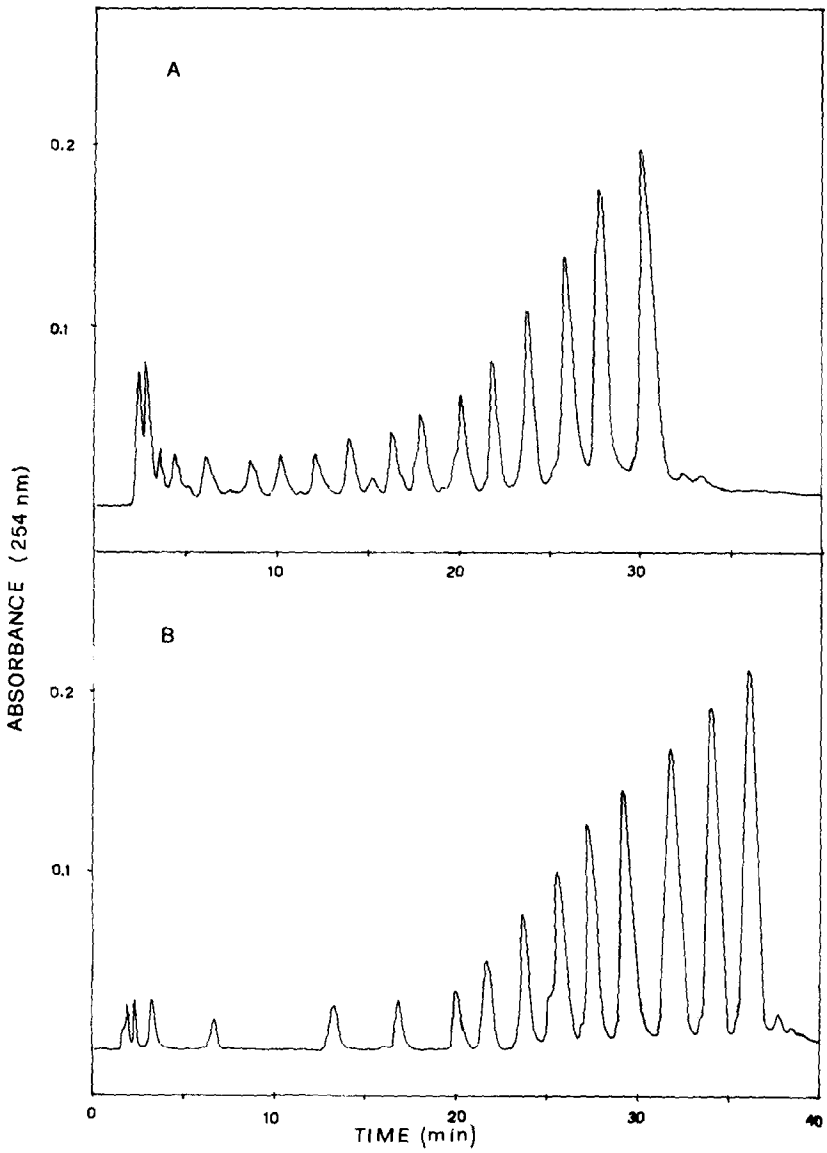

Fig. 1. Separation of a mixture of polythymidilic acids up to the pentadecaoligomer. (A) Partisil 10 SAX column (25 cm × 4.6 mm I.D., 10 μm); (B) Mono Q R 515 column (5 cm × 5 mm I.D., 5 μm). Eluent A, 0.02 *M* KH_2PO_4 (pH 7.0)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 7.0)–20% acetonitrile; linear gradient from 10% to 70% B in 70 min; flow-rate, 0.5 ml/min at room temperature.

To test the method in a real case, four hexadecamers were simultaneously synthesized and the crude reaction mixture was chromatographed on a Mono Q column at pH 9.7 (Fig. 4A). The last-eluted peak, which appeared homogeneous at pH 9.7 (Fig. 4B), was split into the expected four peaks when chromatographed in

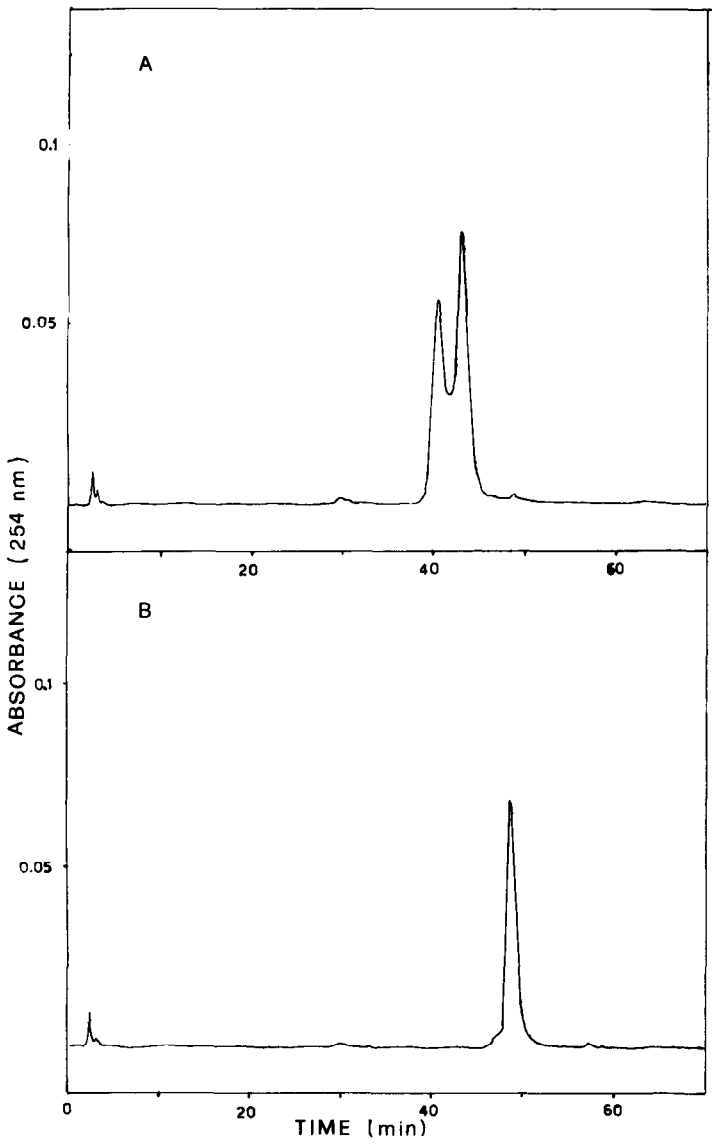

Fig. 2. Separation of two hexadecamers, d(TGTACA$_4$C$_2$TACG$_2$) and d(AT$_3$C$_2$ATC$_2$GTAG$_2$T), on a Mono Q R 515 column (5 cm × 5 mm I.D., 5 μm); linear gradient from 10% to 70% B in 70 min; flow-rate, 0.5 ml/min at room temperature. (A) Eluent A, 0.02 *M* KH_2PO_4 (pH 7.0)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 7.0)–20% CH_3CN. (B) Eluent A, 0.02 *M* KH_2PO_4 (pH 9.7)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 9.7)–20% acetonitrile.

the reversed-phase mode (Fig. 5B), in agreement with the chromatographic behaviour of the same hexadecamers purified by preparative electrophoresis under denaturing conditions[7] (Fig. 5A).

On the other hand, the same four hexadecamers, purified electrophoretically,

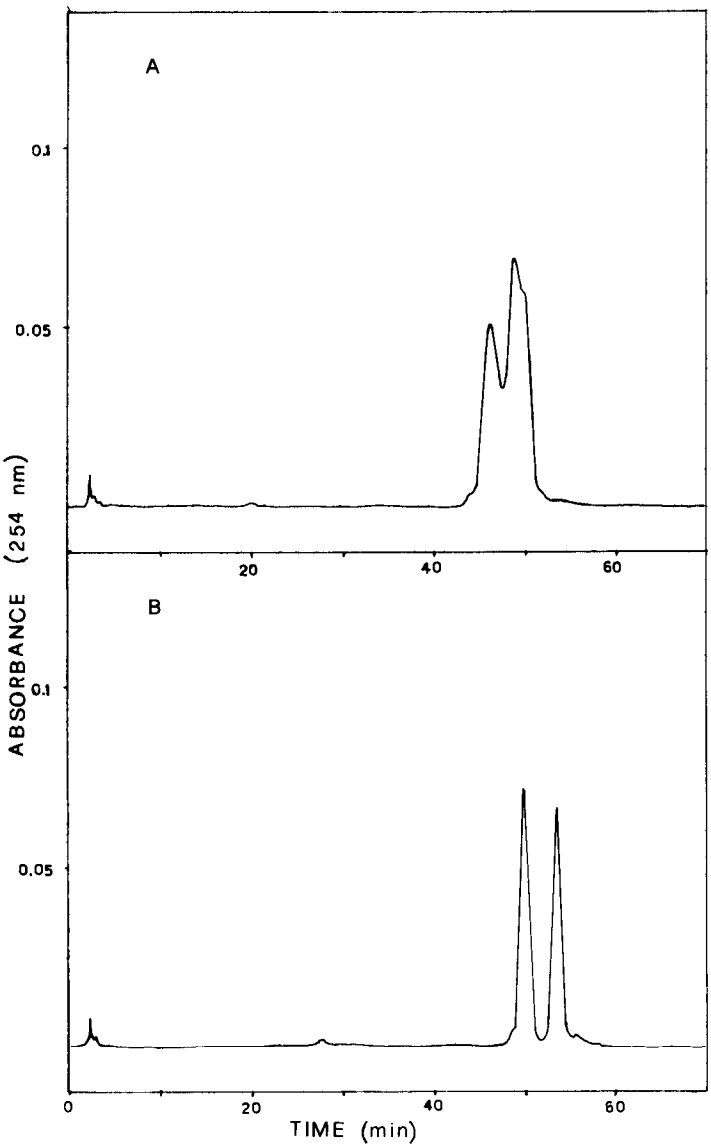

Fig. 3. Separation of two hexadecamers, d(TGTACA$_4$C$_2$TACG$_2$) and d(AT$_3$C$_2$ATC$_2$GTAG$_2$T), and one octadecamer, d(ATGT$_3$C$_3$TGATGT$_2$GC), on a Mono Q R 515 column (5 cm × 5 mm I.D., 5 μm); linear gradient from 10% to 70% B in 70 min; flow-rate, 0.5 ml/min at room temperature. (A) Eluent A, 0.02 *M* KH_2PO_4 (pH 7.0)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 7.0)–20% acetonitrile. (B) Eluent A, 0.02 *M* KH_2PO_4 (pH 9.7)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 9.7)–20% acetonitrile.

gave rise to partially resolved peaks when co-chromatographed on Partisil 10 SAX at pH 7.0 (Fig. 6), this pH being the upper limit for this column. Therefore, in this chromatographic system the collection of the last-eluting peak does not allow the complete recovery of oligonucleotides of interest when the crude mixture is purified.

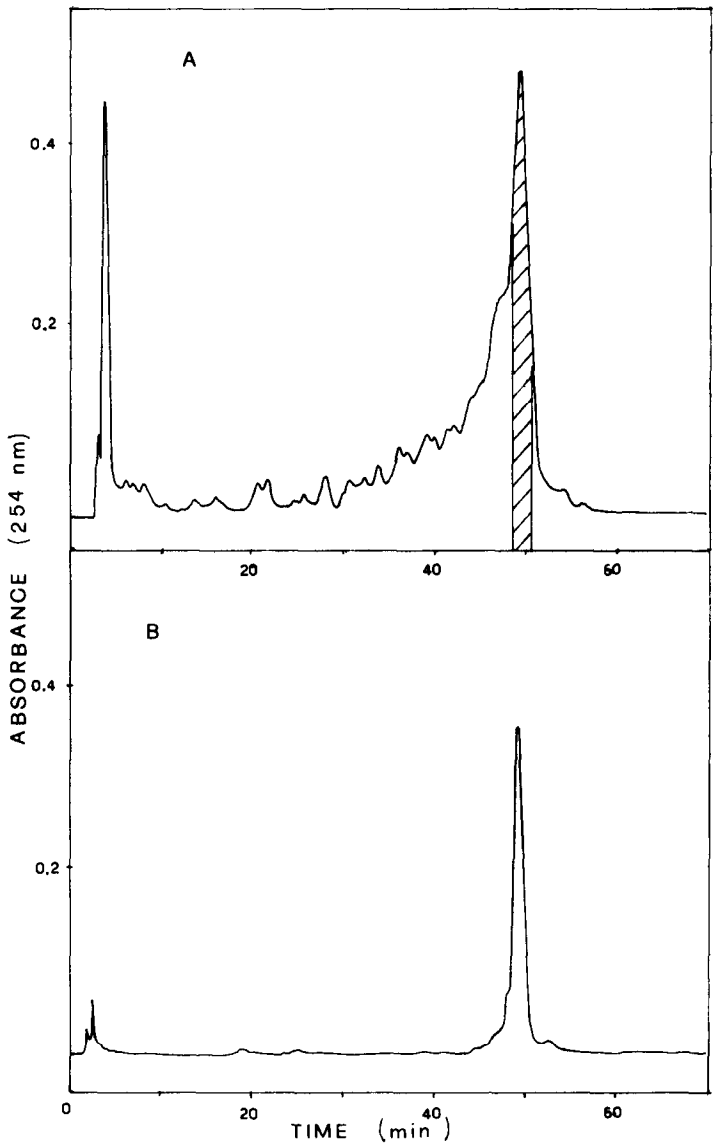

Fig. 4. Anion-exchange chromatography on a Mono Q R 515 column (5 cm × 5 mm I.D., 5 μm). (A) Chromatographic pattern of the crude reaction mixture of four hexadecamers, d(TA$^{A}_{G}$TCCATCCACCA$^{C}_{T}$T); (B) the peak indicated by shadowing in (A). Eluent A, 0.02 *M* KH_2PO_4 (pH 9.7)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 9.7)–20% acetonitrile; linear gradient from 10% to 70% B in 70 min; flow-rate, 0.5 ml/min at room temperature.

DISCUSSION

The phenomena governing the chromatographic behaviour of oligonucleotides are generally complex. This applies also to ion-exchange chromatography when the

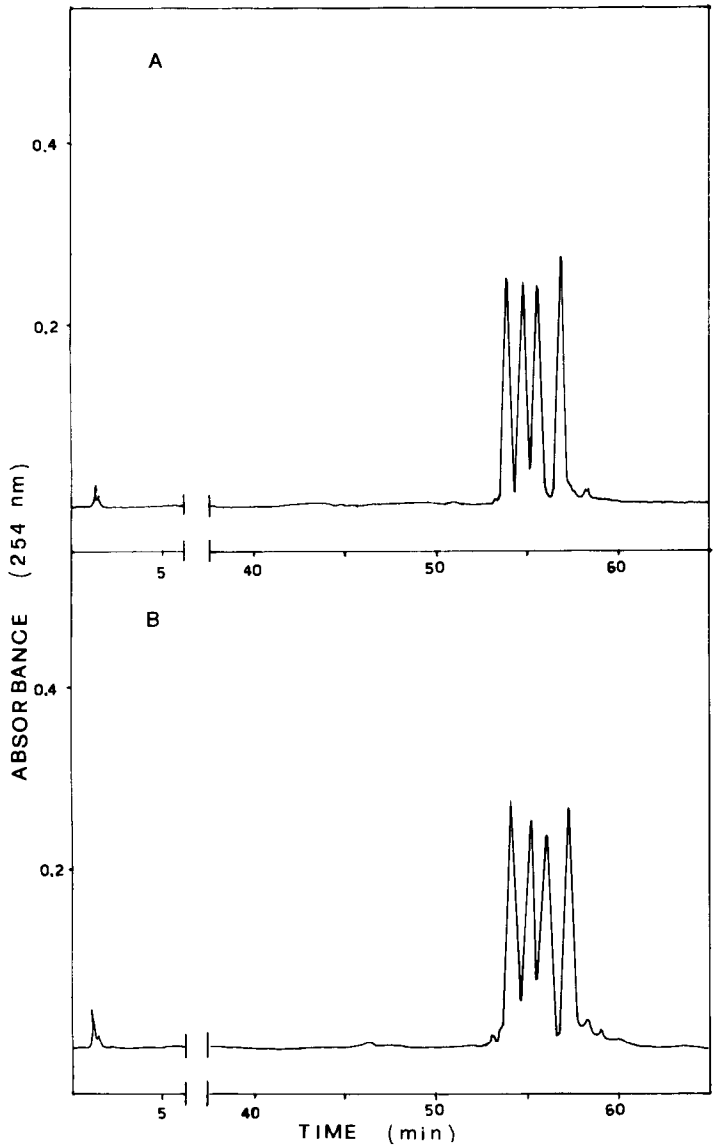

Fig. 5. Reversed-phase HPLC of the mixture of four hexadecamers, d(TA$^{A}_{G}$TCCATCCACCA$^{4C}_{T}$T) on a LiChrosorb RP-18 column (25 cm × 4 mm I.D., 5 μm). (A) Mixture of four hexadecamers purified by gel electrophoresis; (B) mixture of four hexadecamers purified by anion-exchange chromatography on a Mono Q R 515 column (the peak indicated by shadowing in Fig. 4A). Eluent A, 0.1 *M* TEAA (pH 7.0); eluent B, acetonitrile; linear gradient from 0% to 20% B in 80 min; flow-rate, 1 ml/min at room temperature.

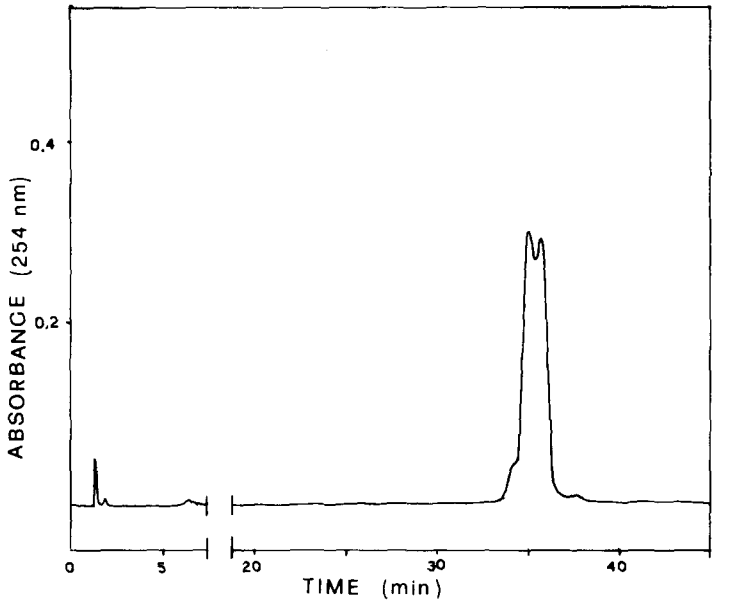

Fig. 6. Anion-exchange HPLC of the mixture of four hexadecamers, d(TA$^{A}_{G}$TCCATCCACCA$^{C}_{T}$T), purified by gel electrophoresis; Partisil 10 SAX column (25 cm × 4.6 mm I.D., 10 μm). Eluent A, 0.02 *M* KH_2PO_4 (pH 7.0)–20% acetonitrile; eluent B, 0.02 *M* KH_2PO_4–1.0 *M* KCl (pH 7.0)–20% acetonitrile; linear gradient from 10% to 70% B in 70 min; flow-rate, 0.5 ml/min at room temperature.

separating conditions do not allow operation according to the only easily predictable parameter, *i.e.*, chain length. The addition of organic solvents, suggested by several workers[4], reduces the influence of base composition. We preferred acetonitrile among others such as formamide, owing to its higher volatility, which simplifies the sample recovery.

Our results indicate that alkaline elution conditions can be used to obtain simpler separations based only on oligonucleotide chain length; however, this requires a stable chromatographic support such as Mono Q. With the proposed method four hexadecamers synthesized as a mixture on a solid matrix were purified as effectively as by preparative polyacrylamide gel electrophoresis but with a higher recovery and more rapidly.

Considering that the purification of the final products is the rate-limiting step in the preparation of synthetic oligonucleotides, the results reported here suggest that Mono Q columns, usually used for protein and peptide purification, are useful in nucleotide chemistry also.

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