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Large-scale purification of haptenated oligonucleotides using high-performance liquid chromatography

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ABSTRACT

We report methodology for the succesful separation of unreacted oligonucleotide from end labeled material (fluorescein or biotin) on an milligram scale using high-performance liquid chromatography (HPLC). The oligonucleotides (19–24-mers) were synthesized on an automated instrument using cyanoethylphosphoramidite chemistry. These oligonucleotides possessed a primary amino group at either the 5'-end or the 3'-end. After trityl-on HPLC purification and detritylation, the amine-terminated oligonucleotides were treated with either fluorescein isothiocyanate or biotin-(aminocaproyl)$_2$-N-hydroxysuccinimide active ester to give the haptenated materials. After removal of excess labeling reagent, the labeled oligonucleotides were purified by reversed-phase HPLC using a polystyrene-based column, with C_{18} groups on the phenyl part of the polystyrene backbone. The terminally labeled oligonucleotides hybridized to their complementary sequences, as observed by size-exclusion chromatography.

INTRODUCTION

In connection with a program designed to commercialize non-radioactive DNA probe technology [1], we investigated the covalent labeling of oligonucleotides with non-radioactive reporter groups. Several technologies exist to accomplish this objective. Keller and coworkers [2,3] have introduced haptens onto bulk DNA using both nucleophilic attack and photochemistry. Another technique for introduction of haptens onto DNA is to synthesize terminally [4] and internally [5] aminated oligonucleotides which can then be specifically labeled with an appropriate hapten. Tous *et al.* [6] and Smith *et al.* [7] have obtained fluoresceinated, terminally labeled oligonucleotides in this fashion. Telser *et al.* [8] have prepared oligonucleotides which were internally labeled with biotin, fluorescein and pyrene, and studied their thermodynamic characteristics. Others have chemically labeled oligonucleotides with biotin [9–11]. While the synthetic chemistry of labeling DNA with small molecules has been dealt with in detail, the chromatographic techniques for adequate separation of starting oligonucleotide from product, especially on a milligram scale, are less well investigated. The aforementioned references use polyacrylamide gel electrophoresis (PAGE) or reversed-phase high-performance liquid chromatography (HPLC) columns for their oligonucleotide separations. However, PAGE suffers from a low loading capacity, while reversed-phase HPLC will give coelution of fluorescein isothiocyanate (FITC) labeled oligonucleotide with non-labeled oligonucleotide. Biotinylated

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oligonucleotides show no separation at all on reversed-phase HPLC. In this paper we describe the results of a comparative study of several separation conditions for haptenated oligonucleotides.

EXPERIMENTAL

Materials

Fluorescein isothiocyanate was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Biotin-(aminocaproyl)$_2$-*N*-hydroxysuccinimide ester and Aminomodifier II were obtained from Clontech (Palo Alto, CA, U.S.A.). Triethylamine was supplied by Aldrich (Milwaukee, WI, U.S.A.); glacial acetic acid by J. T. Baker (Phillipsburg, NJ, U.S.A.); ethanol by Aaper Alcohol and Chemical (Shelbyville, KY, U.S.A.); concentrated ammonium hydroxide by Mallinckrodt (Paris, KY, U.S.A.); HPLC-grade water and acetonitrile by Fisher (Fair Lawn, NJ, U.S.A.), acrylamide-N,N'-methylenebisacrylamide (Bis), tetramethylethylenediamine (TEMED), ammonium persulfate, xylene cyanol and Bromophenol Blue by Bio-Rad (Richmond, CA, U.S.A.); urea by Boehringer Mannheim (Indianapolis, IN, U.S.A.). NAP-5 Sephadex columns were obtained from Pharmacia (Piscataway, NJ, U.S.A.). Amino-controlled pore glass was purchased from Glen Research (Herndon, VA, U.S.A.).

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used for all HPLC separations. This consisted of a 600E system controller, U6K injector, 745B data module or NEC Powermate 386/20 and a 484 detector or a 991 photodiode array detector. The columns used were Waters μBondapak C_{18}, 30 × 0.39 cm, Hamilton (Reno, NV, U.S.A.) PRP-1, 25 × 0.41 cm, and EM Science (Cherry Hill, NJ, U.S.A.) Polyspher RP-18, 15 × 0.46 cm. Oligonucleotides were synthesized on Applied Biosystems (Foster City, CA, U.S.A.) 380A or 380B DNA Synthesizers using β-cyanoethylphosphoramidite chemistry with the SYN 11 program, for both the 5'-aminated (Clontech) and 3'-aminated (Glen Research) oligonucleotides. Deprotection of the oligonucleotides was performed at 55°C for 6 h in concentrated ammonium hydroxide. Gel electrophoresis was performed on a Bio-Rad Sequi-Gen apparatus, with an E-C apparatus (St. Petersburg, FL, U.S.A.) EC 650 power supply. All HPLC separations were performed at ambient temperature. A Beckman (San Ramon, CA, U.S.A.) DU-70 spectrophotometer was used for measuring ultraviolet (UV) absorption spectra. A Savant SpeedVac (Farmingdale, NY, U.S.A.) was used for concentration of samples and a Jouan (Saint Herblain, France) centrifuge was used for centrifugation.

HPLC purification of trityl-on oligonucleotides

The aqueous mobile phase (A) for the trityl-on HPLC purification was 0.1 *M* triethylammonium acetate (pH 7.0). The organic mobile phase (B) was acetonitrile. The following linear gradient was used: 10 to 40% B in 15 min, hold at 40% B for 10 min, then linear to 10% B in 5 min. A Waters μBondapak C_{18} at 1.5 ml/min was used for the trityl-on chromatography. The purified product is collected at approximately 14 min and concentrated to a residue on a SpeedVac.

Manual detritylation of oligonucleotides

The trityl-on purified oligonucleotides were taken up into 1.0 ml of acetic acid–water (80:20, v/v) and were left to stand at room temperature for 1 h. The solvent was removed on a SpeedVac and the residue was taken up into 100 μl of 0.3 *M* sodium acetate. Precipitation of the oligonucleotide was initiated by addition of 1.0 ml of −20°C ethanol and was effected by immersion of the suspension in a −78°C (dry ice–isopropanol) bath. Centrifugation of the suspension (−10°C, 12 560 *g*, 15 min) gave a pellet which was used for the haptenation reactions.

Synthesis of haptenated oligonucleotides

Fluoresceinated oligonucleotides. To a solution of 1 μmol of detritylated, terminally aminated 24-mer oligonucleotide (determined by UV absorption) in 250 μl sodium borate buffer (pH 9.0) 8 mg (20 μmol) fluorescein isothiocyanate dissolved in 250 μl of dimethylformamide (DMF) was added. The reaction was left to proceed in the dark at room temperature for 16 h. The reaction mixture was loaded onto a NAP-5 column which had been equilibrated with 10 ml water. The column was eluted with 1.0 ml water and the eluate was collected and concentrated on the SpeedVac.

Biotinylated oligonucleotides. To a solution of 1 μmol detritylated, terminally aminated 24-mer oligonucleotide in 250 μl sodium phosphate buffer (pH 7.2) 11 mg (20 μmol) of biotin-(aminocaproyl)$_2$-N-hydroxysuccinimide ester in 250 μl of DMF was added. The reaction was left to stand in the dark at room temperature for 17 h. The reaction was worked up as in the fluorescein case to give the concentrate, ready for HPLC or gel separation.

Polyacrylamide gel electrophoresis of haptenated oligonucleotides

A 150 ml solution of 12% acrylamide–Bis/8 *M* urea/89 m*M* Tris/89 m*M* sodium borate/2 m*M* EDTA, pH 8.0 was left to polymerize for 4 h in a 21 × 40 × 0.2 cm Bio-Rad Sequi-Gen apparatus, after addition of ammonium persulfate and TEMED. After pre-electrophoresis of the cell (1 h, 20 W), the oligonecleotides were loaded onto the gel. Generally, no more than 1 μmol of oligonucleotide in 150 μl of formamide could be applied at once. After 4–5 h at 40 W, the bromophenol blue dye had eluted off the gel, signaling the end of the run. At this point, the gel was overlaid on a silica gel plate and the DNA bands were visualized by UV shadowing. The product (slowest moving major) bands were excised and extracted with 1.0 *M* triethylammonium bicarbonate overnight. The extracts were concentrated, taken up into 0.5 ml of water and desalted on a NAP-5 column. The eluate was checked for DNA concentration by UV absorbance.

Reversed-phase HPLC purification of haptenated oligonucleotides (Hamilton PRP-1 procedure)

The aqueous mobile phase (A) for the purification of haptenated oligonucleotides was 0.1 *M* sodium phosphate (pH 8.9). The organic mobile phase (B) was 50% aqueous acetonitrile. The following linear gradient was used: 10 to 30% B in 23 min, then 30 to 10% B in 10 min. The flow-rate was 1 ml/min and the injection volume was 150 μl. The detector was adjusted to 290 nm and 2.0 a.u.f.s.

Reversed-phase HPLC purification of haptenated oligonucleotides (Polyspher RP-18, EM Science procedure)

The aqueous mobile phase (A) for the purification of haptenated oligonucleotides was 0.1 *M* sodium phosphate (pH 8.8). The organic mobile phase (B) was 50% aqueous acetonitrile. The following linear gradient was used: 20 to 50% B in 36 min, then 50 to 20% B in 14 min. The flow-rate was 0.6 ml/min and the injection volume was 150 μl. The detector was adjusted to 290 nm and 2.0 a.u.f.s.

Hybridization determination by size-exclusion HPLC

The ability of haptenated complementary single stranded oligonucleotides to hybridize was demonstrated by size-exclusion HPLC using a Bio-Rad Bio-Sil SEC-125 as follows: the mobile phase was 0.1 *M* sodium phosphate (pH 7.0), the flow-rate was 1 ml/min at 280 p.s.i. and the detector was adjusted to 260 nm and 0.5 a.u.f.s. An aqueous solution of each oligonucleotide, concentration approximately 1 μg per 40 μl, was injected individually and each retention time determined for the single stranded oligonucleotides. Equivalent amounts of complementary single stranded oligonucleotides were mixed at room temperature and injected under the previous conditions. Typical retention times for single stranded 24-mers were 7.7–7.9 min and changed to 7 min when hybridized.

RESULTS AND DISCUSSION

We have found that a 2-mm thick, 21 × 40 cm polyacrylamide gel will separate labeled from non-labeled material quite well on a 0.5–1.0 μmol scale upon electrophoresis. In all cases observed, labeled material migrates more slowly than non-labeled. However, efficiency of separation falls off drastically at higher oligonucleotide loadings. Our investigations of separation technology for large-scale work then shifted from gel electrophoresis to suitable HPLC columns and conditions. We first examined the separation afforded by a Waters μBondapak C_{18} HPLC column (30 × 0.39 cm). On an analytical scale (0.1 μmol), a C_{18} ODS column did not separate biotinylated oligonucleotide from the starting oligonucleotide. We then attempted a separation of FITC-labeled oligonucleotide from starting, amine-terminated oligonucleotide. Due to fluorescein's lipophilicity, one would expect good separation of labeled from non-labeled material for fluoresceinated oligonucleotide. In fact, separation was seen between fluoresceinated and non-fluoresceinated oligonucleotide (Fig. 1). However, upon further investigation of the fractions by denaturing gel electrophoresis, it was determined that peak 1, from Fig. 1, was starting oligonucleotide, while peak 2 was a mixture of starting material and product. Under the non-denaturing chromatographic conditions, it is possible that intercalation of the fluorescein label into a neighboring oligonucleotide is occurring. This supposition prompted us to investigate the HPLC of oligonucleotides under denaturing conditions.

One chromatographic support which is well suited for the large-scale separation of oligonucleotides under denaturing conditions is polystyrene reversed-phase (PRP) material, with divinylbenzene crosslinks. Several studies [12–14] have shown that PRP columns possess outstanding loading capacities and will function under extremes of pH (2–13), as well as at high salt concentration. As shown in Fig. 2, the Hamilton PRP-1 column (25 × 0.41 cm) will fractionate oligonucleotides. Unfortu-

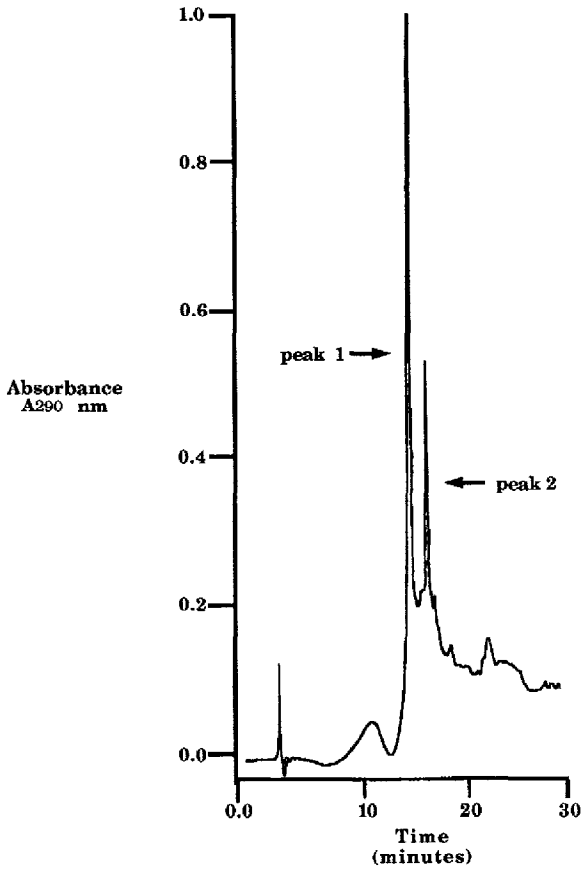

Fig. 1. Injection of 2.4 μg of a 23-mer, 5'-end labeled with FITC. Peak 1, eluting at 16.3 min, is starting 23-mer. Peak 2, eluting at 17.98 min, is a mixture of starting and fluoresceinated oligonucleotide. Column is Waters μBondapak C_{18}, flow-rate 1 ml/min. Gradient table: initial, 5% B, 95% A; 5 min, 20% B, 80% A; 35 min, 50% B 50% A. Solvent A is 0.1 *M* triethylammonium acetate, solvent B is acetonitrile.

nately, "ghosting" frequently occurs. Ghosting is a phenomenon in which some injected oligonucleotide is retained on the column during a sample run. The retained oligonucleotide is eluted in a subsequent run, when the gradient conditions that eluted the major part of the initial charge are attained. Ghosting was detected when, after thoroughly washing the HPLC injector port and syringe, the ghost peak still eluted in a blank injection. This even occurs after repeated column washes. Ghosting is seen in trityl-on as well as trityl-off HPLC and so is not limited to the case of haptenated oligonucleotides. The phenomenon is largely restricted to injections of 1 μmol and larger. This phenomenon is not a problem if the column is used for only one oligonucleotide. If the intended use for the column is for multiple oligonucleotide separations, the probability of contamination is unacceptably high. From the above experiences, the criteria for a suitable HPLC support were determined to be high loading capacity, good resolution, stability to extremes of pH and salt concentration and no ghosting. As the PRP-1 column fulfilled all of the criteria save the last one, it seemed a

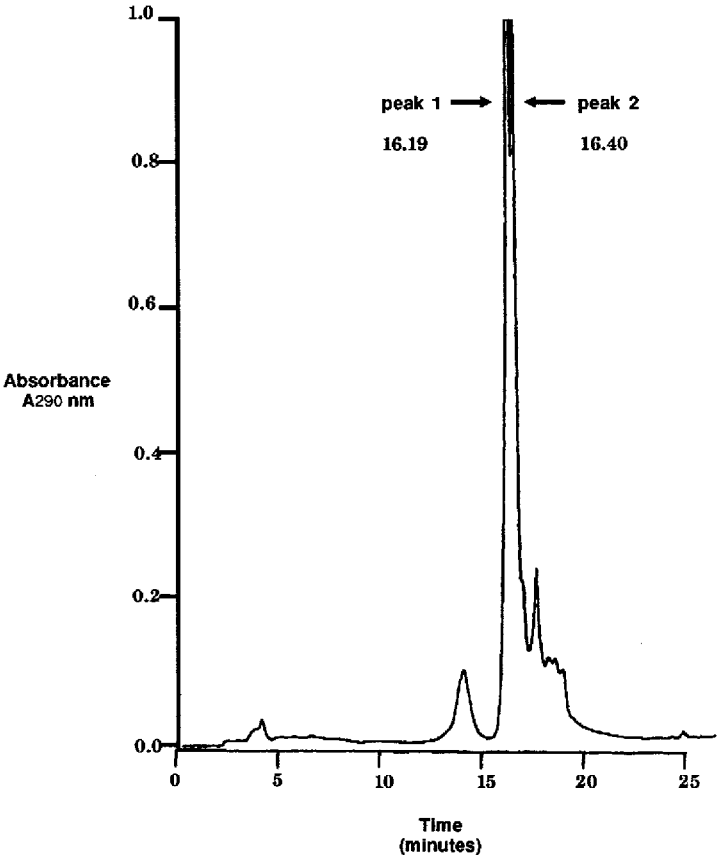

Fig. 2. Injection of 200 μg of a 25-mer, 5′-end labeled with FITC. Column is Hamilton PRP-1. Peak 1, eluting at 16.19 min, is unreacted starting material, while peak 2, eluting at 16.4 min, is haptenated oligonucleotide.

worthwhile exercise to examine other polystyrene-based supports which were covalently modified. The EM Science Polyspher RP-18 column (15 × 0.46 cm) is a 3-C_{18} styrene/3-C_{18}-1,4-divinylbenzene cross-linked polymeric support which is generally used in situations requiring ODS column resolution under unusual salt or pH conditions. We have found that this support is admirably suited to the task of resolving haptenated from non-haptenated oligonucleotide, as well as removal of excess haptenation reagent. The separation time between starting material and product ranges between 9 and 11 min, with a flow-rate of 0.6 ml/min on the analytical column. Fig. 3 shows the resolution seen for a typical preparation of a fluoresceinated, end-labeled oligonucleotide, while Fig. 4 depicts the separation between terminally biotinylated oligonucleotide from terminally aminated starting oligonucleotide. We have obtained 1.5 mg of purified 25-mer from the analytical EM column in a single crude injection of 2.2 mg, with recovery of unreacted starting material. Importantly, no ghosting is observed when blanks are injected onto an RP-18 column after a sample run. Best separation results are obtained on the RP-18 when the pH of the load matches of the pH of the eluent.

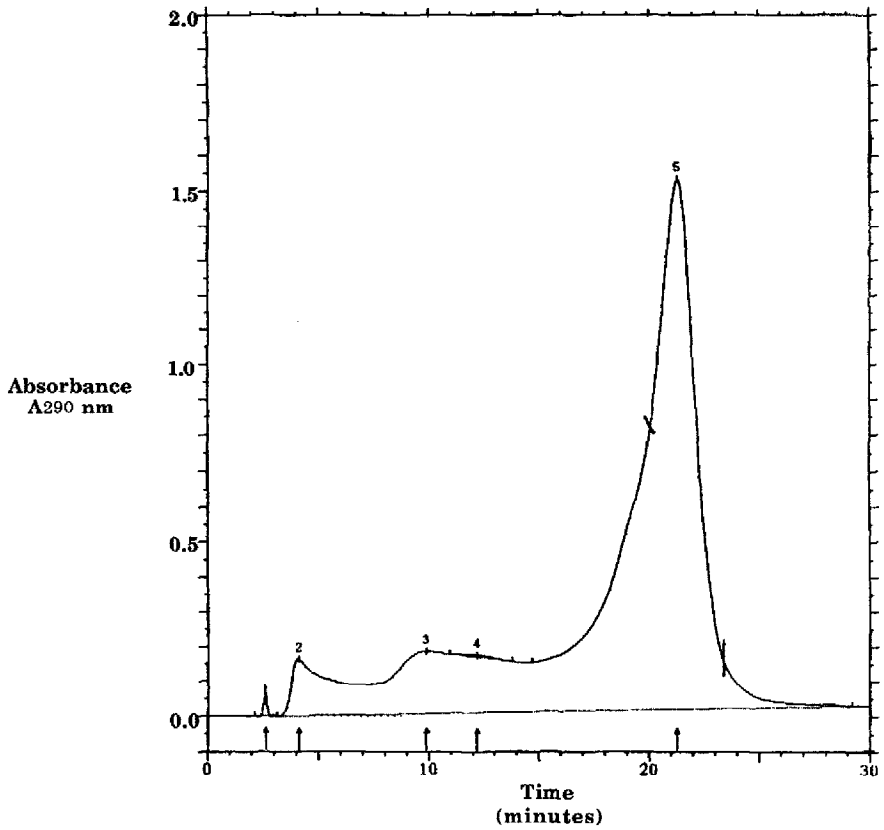

Fig. 3. HPLC trace of 249 μg loading of a 24-mer, 5′-end labeled with FITC. Column is EM Science Polyspher RP-18. Peaks 3 and 4, eluting at 9.9 and 12.3 min, are starting oligonucleotide. Peak 5, eluting at 21.3 min, is FITC-labeled material.

Finally, hybridization of the haptenated oligonucleotides to their complementary sequences has been examined qualitatively by hyperchromicity measurements and quantitatively by size-exclusion chromatography on a high-performance liquid chromatograph (Fig. 5). In all cases, the terminally haptenated oligonucleotides were able to hybridize to their complementary strands.

CONCLUSION

We have found that C_{18} column HPLC will not adequately separate fluoresceinated from non-fluoresceinated oligonucleotide, as checked by polyacrylamide gel electrophoresis. PAGE itself has the problem of low loading capacity. Unmodified polystyrene reversed-phase columns have high loading capacity and afford some separation of haptenated from non-haptenated oligonucleotide, but have "ghosting" as a setback. Taking all of these limitations into account, we now employ the RP-18 HPLC column from EM Science to separate haptenated from non-haptenated oligonucleotide. It has the characteristics of high loading capacity, good resolution, and no "ghosting" in its favor.

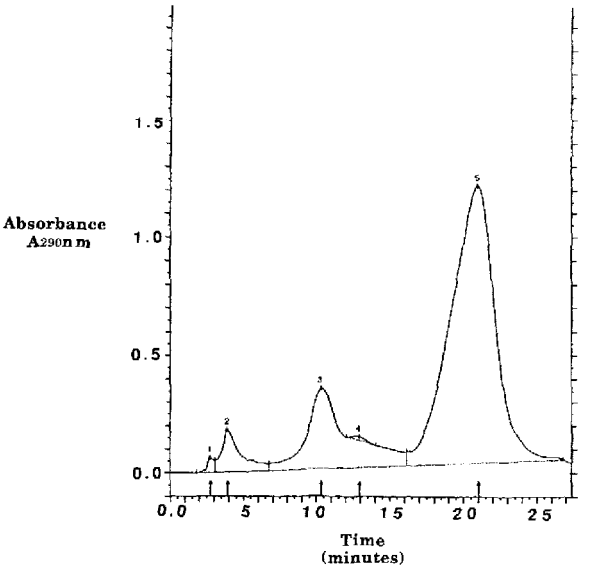

Fig. 4. Injection of 345 μg of a 25-mer, 3′-end labeled with biotin-(aminocaproyl)2-N-hydroxysuccinimide ester. Column is EM Science Polyspher RP-18. Peaks 3 and 4, eluting at 10.4 and 13 min, are starting oligonucleotide, while peak 5, eluting at 21 min, is biotinylated material.

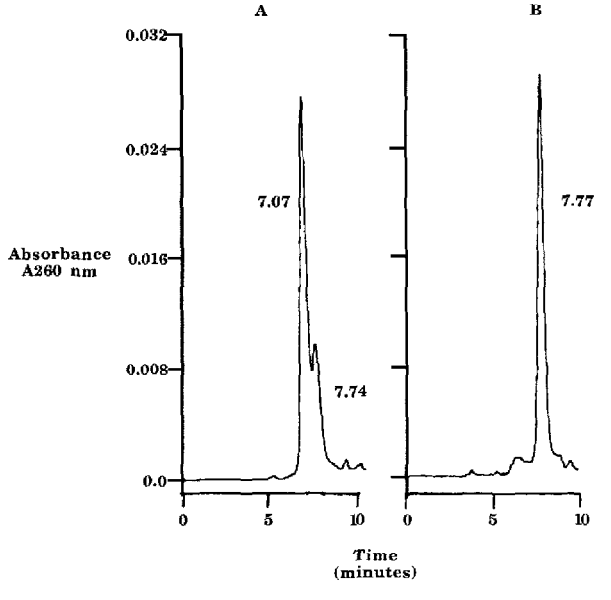

Fig. 5. (A) Injection of 24-mer, 5′-end labeled with FITC and hybridized to complementary strand, 7.07 min peak; (B) 24-mer by itself, 7.77 min retention time. Note the residual peak at 7.74 min from unhybridized material in (A). This disappears upon further admixture with complementary DNA. Column is Bio-Sil SEC-125.

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