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Immobilized thymine chromatography–mass spectrometry of oligonucleotides

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Abstract

New rapid, sensitive and specific methods are needed to determine the purity of synthetic oligonucleotides and their analogs, such as antisense and antigene oligonucleotide drugs. Synthetic oligonucleotides are typically separated using HPLC with UV absorbance detection at 260 nm. However, positive identification of oligonucleotides requires more specific detection, such as that offered by electrospray mass spectrometry. Size-exclusion, ion-exchange and reversed-phase HPLC columns are usually used for the analysis and purification of oligonucleotides. Among these, only reversed-phase ion-pair chromatography is suitable for LC–MS of oligonucleotides, but incomplete resolution of oligonucleotide mixtures sometimes limits this approach. Therefore, a mixed-mode stationary phase was developed for electrospray LC–MS of oligonucleotides incorporating reversed-phase, weak anion-exchange and possibly affinity properties in order to provide different selectivity than reversed-phase alone. The thymine derivative, 3-(1-thymidyl)propanoic acid, was synthesized, purified and then immobilized as a possible affinity ligand on an aminopropyl silica HPLC column using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, for activation. Unreacted amino groups on the column were end-capped by reaction with acetic anhydride. Next, mobile phase conditions were optimized for the separation of oligonucleotide mixtures up to 18-mers in length. The parameters investigated included gradients of temperature, pH, ionic strength, reversed-phase, normal-phase and combinations of these parameters for mixed-mode separations. Optimum column performance was achieved using gradients that utilized the potential affinity properties of the column. Finally, HPLC–electrospray mass spectrometric analyses of oligonucleotide mixtures were obtained using the immobilized thymine column and compared to reversed-phase LC–MS analyses.

Keywords: Stationary phases, LC; Immobilized thymine; Oligonucleotides; Nucleotide

1. Introduction

Oligonucleotides are used as primers for DNA amplification by the polymerase chain reaction [1], as linkers for DNA ligation and gene splicing [2], as antisense and antigene oligonucleotide therapeutic agents for mRNA or gene regulation [3,4], and numerous other biochemical and genetic engineering procedures of interest in biotechnology. In addition, a rapidly expanding new area in drug discovery and development concerns the use of analogs of oligonucleotides as drugs for gene regulation called antisense and antigene drugs [3]. Combinatorial libraries consisting of oligonucleotide analogs are also being screened as part of on-going drug discovery programs to identify potential new pharmacological targets for these compounds.

Purification of oligonucleotides is usually carried out by using high-performance liquid chromatography (HPLC) with UV absorbance detection at 260

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nm. However, verification of oligonucleotide structure, including molecular mass determination, presence or absence of protecting groups, identification of base modifications such as deamination, etc., requires more specific mass spectrometric detection and analysis. In addition, on-line liquid chromatography–mass spectrometry (LC–MS) also facilitates the characterization of impurities or unexpected compounds detected in a chromatogram.

The first LC–MS analysis of oligonucleotides was reported by van Breemen et al., in 1991 [5] using continuous-flow fast atom bombardment (FAB) MS. However, FAB-MS has been limited to oligonucleotides containing no more than 13 nucleotide [6], which excludes many important compounds such as antisense oligonucleotides in the range of 17–20 nucleotide. Recently, electrospray MS has been applied to the analysis of oligonucleotides containing up to 120 nucleotide [7]. Although electrospray LC–MS has not been demonstrated for oligonucleotides of such high molecular mass, electrospray LC–MS of oligonucleotides up to 20-mers in length is considered routine. Although capillary zone electrophoresis and related techniques are also compatible with electrospray MS, only HPLC separation methods are also useful for oligonucleotide purification.

HPLC methods for the analysis and purification of oligonucleotides, DNA and RNA usually use size-exclusion [8], ion-exchange [9], or reversed-phase columns [10]. Because of large elution volumes and dependence on large mass (volume) differences between analytes, size-exclusion chromatography is not generally suitable for LC–MS analysis of oligonucleotides. Although ion-exchange chromatography provides excellent resolution of oligonucleotides of different chain length (and therefore different charge), the elution buffers are often not volatile and are therefore incompatible with electrospray MS. Therefore, reversed-phase ion-pair HPLC with volatile ion-pair agents such as triethylammonium acetate has been the most suitable HPLC method for LC–MS analyses of oligonucleotides.

In order to provide different column selectivity and to complement reversed-phase LC–MS analysis of oligonucleotides, we investigated the use of an immobilized thymine HPLC column for affinity separation of oligonucleotides during LC–MS. Although there have been a few reports of affinity HPLC of oligonucleotides, no affinity LC–MS studies of oligonucleotides have been carried out. For example, Yashima et al. [11], prepared immobilized adenine or thymine stationary phases for the separation of oligomers of adenylic and uridylic acids. Other groups have used immobilized homopolymers of thymidylic acid for the affinity purification of poly(A)-containing mRNA [12,13]. The immobilized thymine column reported here differs from that of Yashima et al. [11] by the chemistry of immobilization, which includes the insertion of a propylamidopropyl spacer group between the thymine and silica solid support, and by the resulting mixed-mode stationary phase properties which include potential affinity, weak anion-exchange and reversed-phase characteristics.

2. Experimental

2.1. Chemicals

Reagents and solvents for organic synthesis were purchased from Aldrich (Milwaukee, WI, USA). Aminopropyl silica, 300 Å pore size and 5 μm particle size, and an aminopropyl silica HPLC column, 25 cm×2 mm, were obtained from YMC (Wilmington, NC, USA). Oligodeoxyribonucleotides and thymine were purchased from Sigma (St. Louis, MO, USA), except for polyadenylic or polythymidylic deoxyribonucleotides (containing mixtures of 12 to 18-mers), which were obtained from Pharmacia Biotech (Piscataway, NY, USA). HPLC solvents were purchased from Fisher Scientific (Springfield, NJ, USA).

2.2. Immobilization of thymine

3-(1-Thymidyl)propanoic acid [or 1-(3-propanoyl)thymine] was synthesized as shown in Fig. 1. Briefly, thymine (1 g, 8 mmol) was reacted with an equimolar amount of ethyl acrylate in sodium ethoxide. Following deesterification using 6 *M* hydrochloric acid and purification using flash chromatography, the desired product, 3-(1-thymidyl)propanoic acid, was obtained in quantitative yield. The structure was

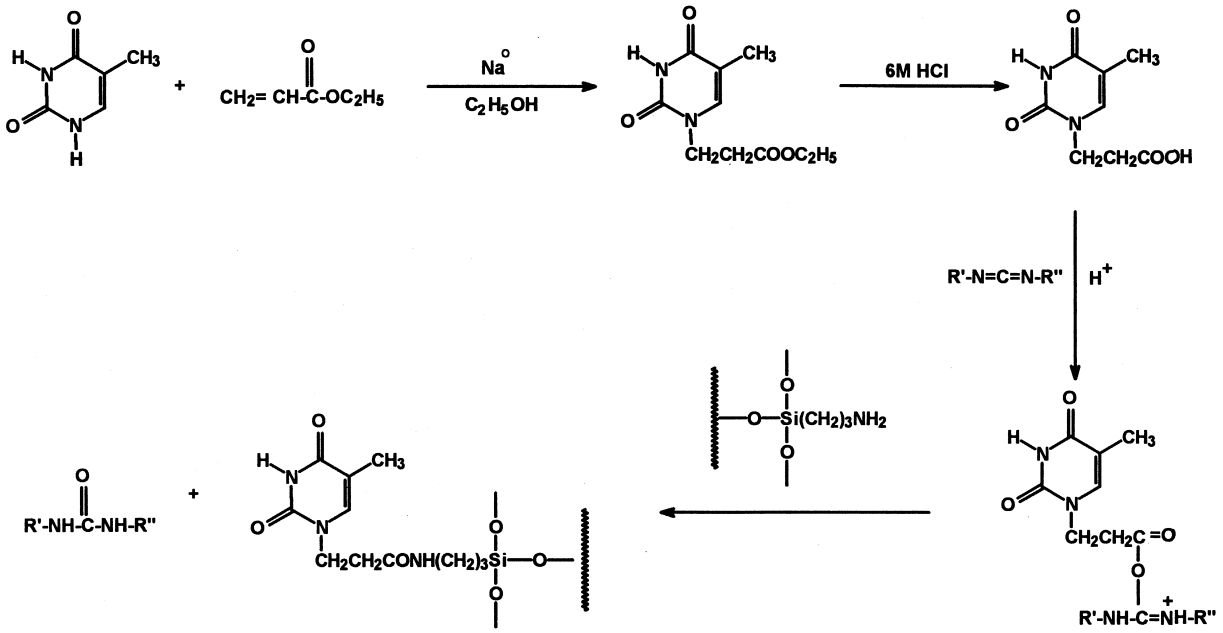

Fig. 1. Synthesis of 3-(1-thymidyl)propanoic acid followed by carbodiimide carboxyl group activation and immobilization on aminopropyl silica.

confirmed using electrospray MS and proton nuclear magnetic resonance (NMR).

A single deprotonated molecule was observed at m/z 197 in the negative-ion electrospray mass spectrum of 3-(1-thymidyl)propanoic acid, which was obtained using a Hewlett-Packard (Palo Alto, CA, USA) 5989B MS Engine quadrupole mass spectrometer as described below. NMR analyses were carried out using a Varian (San Fernando, CA, USA) XL-300 NMR system operating at a proton frequency of 300 MHz and a spectral width of 4000 Hz. Approximately 2 mg of 3-(1-thymidyl)propanoic acid was dissolved in dimethylsulfoxide in a 5-mm diameter NMR tube, and tetramethylsilane (TMS) was added as an internal standard. Proton bands in the NMR spectrum were observed as follows: δ= 1.74 ppm (s, 3H, methyl); δ=2.61 ppm (t, J=6.84 Hz, 2H on C-2); δ=3.82 ppm (t, J=6.84 Hz, 2H on C-3); δ=3.45 ppm (br s, 1H on N); δ=7.51 ppm (s, 1H on ring C); δ=11.24 ppm (s, 1H on carboxylic acid).

Next, the carboxylic acid group of 3-(1-thymidyl)propanoic acid (200 mg, 1.0 mmol) was activated using 1.9 molar equivalents of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide in 25 ml of potassium phosphate buffer (0.4 *M*, pH 4.3) (see Fig. 1). After reacting for 2 h, the pH was adjusted to 7.0 using potassium hydroxide. This solution was recirculated through the aminopropyl silica HPLC column (see description above) in a closed system at 300 μl/min in order to carry out on-column immobilization of the thymine. Based on information provided by the manufacturer, the aminopropyl silica column was estimated to contain 0.457 mmol aminopropyl groups. Therefore, the thymine derivative used for immobilization was present in a more than two-fold molar excess compared to the aminopropyl groups. The progress of the reaction was continuously monitored by measuring the absorbance at 260 nm of 3-(1-thymidyl)propanoic acid in the mobile phase using an HPLC absorbance detector. After 3 h, the absorbance of 3-(1-thymidyl)propanoic acid in the mobile phase stopped decreasing, but the reaction was allowed to continue overnight for a total of 11 h. The column was flushed extensively with water and then methanol.

In parallel, an identical reaction was carried out except that the aminopropyl silica was not packed in

a column. After derivatization, the free thymidyl aminopropyl silica was rinsed thoroughly with water and then assayed for the presence of any unreacted primary aminopropyl groups. To a suspension of 1 mg derivatized silica in 1 ml of a saturated aqueous solution of sodium tetraborate, three drops of 3% 2,4,6-trinitrobenzenesulfonate solution was added. After 2 h of reaction, the silica was rinsed with deionized water and examined for orange or yellow color indicative of primary amino groups. Since a slight yellow color was observed, the derivatization reactions described above were repeated a second time after which no orange or yellow color could be detected.

Because steric hindrance might have prevented some aminopropyl groups from reacting with the carbodiimide-activated 3-(1-thymidyl)propanoic acid, residual aminopropyl groups were end-capped by reaction with acetic anhydride. The acetic anhydride (100 m*M* in 100 m*M* ammonium acetate) was recirculated through the column at 300 μl/min overnight. The reaction was judged to be complete based on continuous monitoring of the disappearance of acetic anhydride at 245 nm in the mobile phase.

2.3. LC–MS

Negative-ion electrospray mass spectra were obtained using a Hewlett-Packard 5989B electrospray mass spectrometer equipped with a nebulizer-assisted electrospray interface, and a Hewlett-Packard 1090L gradient HPLC system containing an autoinjector and photodiode array UV–Vis absorbance detector. The quadrupole rods were maintained at 120°C, and the range m/z 500–1500 was scanned over approximately 8 s at unit resolution. The effluent from the HPLC column, which was flowing at 200 μl/min, was mixed with 5% aqueous ammonia in 50% methanol at 200 μl/min in order to enhance formation of deprotonated molecules during electrospray. The entire solution (flowing at 400 μl/min) was split 1:50 so that 8 μl/min flowed into the mass spectrometer. Electrospray was carried out using nitrogen nebulizing gas at 80 p.s.i. and bath gas at 350°C with a flow-rate of 10.0 l/min (1 p.s.i.=6894.76 Pa). The bath gas temperature was selected to facilitate evaporation of solvent from the sample droplets and prevent condensation from forming on the capillary entrance.

2.4. HPLC

The HPLC system consisted of either a Hewlett-Packard 1090L system integrated with the electrospray mass spectrometer, or an Applied Biosystems (Foster City, CA, USA) Model 140A dual syringe pump and 1000S diode array detector, Rheodyne (Cotati, CA, USA) Model 7725I injector, and Galactic Industries (Salem, NH, USA) Lab Calc data acquisition system with GRAMS/386 chromatography software. Various mobile phase compositions and gradients were investigated to facilitate possible affinity separations of oligonucleotides that are compatible with electrospray mass spectrometric detection. Optimum separations of adenyl homopolymers using the thymidylpropylamidopropylsilica column were obtained using a 40 min gradient from 300 m*M* (or 1.5 *M*) ammonium acetate in methanol to water (adjusted to pH 4.2 with acetic acid) at a flow-rate of 200 μl/min. Mixtures containing oligonucleotide heteropolymers and homopolymers were resolved using aqueous 0.1 *M* ammonium acetate and acetonitrile with the following gradient: 35–40% acetonitrile in 8 min, then to 56% acetonitrile at 18 min, then back to 35% acetonitrile at 20 min. For comparison, reversed-phase separations were carried out on a Vydac (Separations Group, Hesparia, CA, USA) C_{18} silica (5 μm diameter) reversed-phase column (25 cm×2.1 mm) with a 40 min gradient from water to acetonitrile (each solvent containing 20 m*M* ammonium acetate or triethylammonium acetate) at a flow-rate of 180 μl/min. Oligonucleotide standard solutions were prepared in water and the concentrations were standardized based on absorbance units at 260 nm. Typically, 1–10 mAU of each oligonucleotide were injected per analysis, which was approximately 8–80 pmol of each oligonucleotide (depending upon molecular mass).

3. Results and discussion

Using volatile mobile phase constituents such as ammonium acetate, water and methanol, the effects of ionic strength, pH, temperature and organic

modifiers were investigated on the separation of oligonucleotides using the immobilized thymine column. In general, gradients of decreasing ionic strength, decreasing pH and decreasing concentration of organic modifiers tended to provide optimum separations of adenyldeoxyoligonucleotides. For example, the separation of a mixture of adenyldeoxyoligonucleotides, $d(pA)_{12-18}$, (note that ''pA'' refers to the presence of a terminal 5′-phosphate group) using the thymine HPLC column is shown in Fig. 2. The mobile phase consisted of a 40 min gradient from 1.5 *M* ammonium acetate in methanol to water, which had been adjusted to pH 4.2 using acetic acid. The initial high ionic strength of the mobile phase and the lower solubility of oligonucleotides in methanol compared to water facilitated interactions between the adenine homopolymer and the immobilized thymine. As the ionic strength decreased and the concentration of water increased during the gradient, either the mobile phase more effectively solvated the oligonucleotides or else repulsion between phosphate groups on the oligonucleotides and negative charges on underivatized sites on the silica stationary phase disrupted binding (this effect is analogous to lowering the melting temperature of DNA duplexes by lowering the ionic strength of the solution). As a result of affinity and/or hydrophobic interactions with the stationary phase, the adenyldeoxyoligonucleotides eluted in order of increasing number of adenine residues.

In addition to ionic strength and solvent gradients, the pH was decreased during the separation, which progressively disrupted hydrogen bonding between complementary nucleic acid bases by protonating substituent groups. For example, the primary amino group of adenine, which is involved in forming one of the two hydrogen bonds in the adenine–thymine base pair, has a pK_a of 3.5 [14] and would be partly protonated at pH 4.2. Less efficient separations of the $d(pA)_{12-18}$ mixture were obtained if the pH was not lowered, if the mobile phase ionic strength was not initially high and then decreased over time, or if a methanol to water gradient was not used. Also, attempts to disrupt base pair formation and elute oligonucleotides by carrying out a column temperature gradient from 0–50°C during a separation had negligible effect. Therefore, all subsequent analyses were carried out at room temperature.

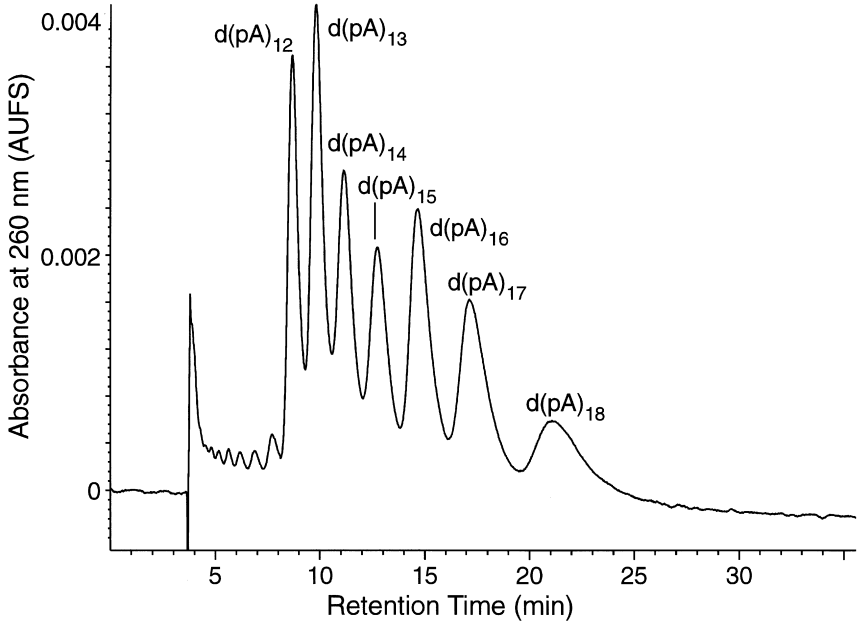

Fig. 2. Separation of a mixture of adenyldeoxyoligonucleotides, $d(pA)_{12-18}$, using an immobilized thymine HPLC column. The mobile phase consisted of a 40 min gradient from 1.5 *M* ammonium acetate in methanol to water, which had been adjusted to pH 4.2 using acetic acid.

An analogous mixture of thymidyloligonucleotides, $d(pT)_{12-18}$ and $d(T)_{12-18}$ (with and without a terminal 5′-phosphate group), was chromatographed using the same mobile phase conditions described in Fig. 2 for the adenyldeoxyoligonucleotides. As expected, no separation of this mixture was obtained under these conditions, consistent with the anticipated absence of an affinity interaction between immobilized thymine and the thymidyloligonucleotides. However, a different solvent system, which consisted of a 40 min gradient from 0.1 *M* ammonium acetate, pH 4.75, to 1.5 *M* ammonium acetate, pH 7, was found to provide complete separation of the thymidine homopolymer mixture on the immobilized thymine column (see chromatogram in Fig. 3). Apparently, this gradient exploited selectively the weak anion-exchange properties of the stationary phase. Note that the oligonucleotides containing an extra phosphate group on the 5′-terminus were retained longer on the column, which is consistent with an anion-exchange interaction.

After mobile phase conditions had been developed for chromatography of adenyldeoxyoligonucleotides using the immobilized thymine column (Fig. 2), electrospray LC–MS of heterogeneous oligonucleotide mixtures were carried out. For example, the immobilized thymine LC–MS analysis of a mixture of d(CAAGCTTG), d(GGAATTCC), $d(pT)_8$ and $d(pA)_8$ is shown in Fig. 4. Computer reconstructed mass chromatograms of each doubly charged deprotonated molecule and the corresponding UV absorbance chromatogram are shown in Fig. 4. The UV absorbance detector was placed in series between the immobilized thymine column and the mass spectrometer. During electrospray ionization under

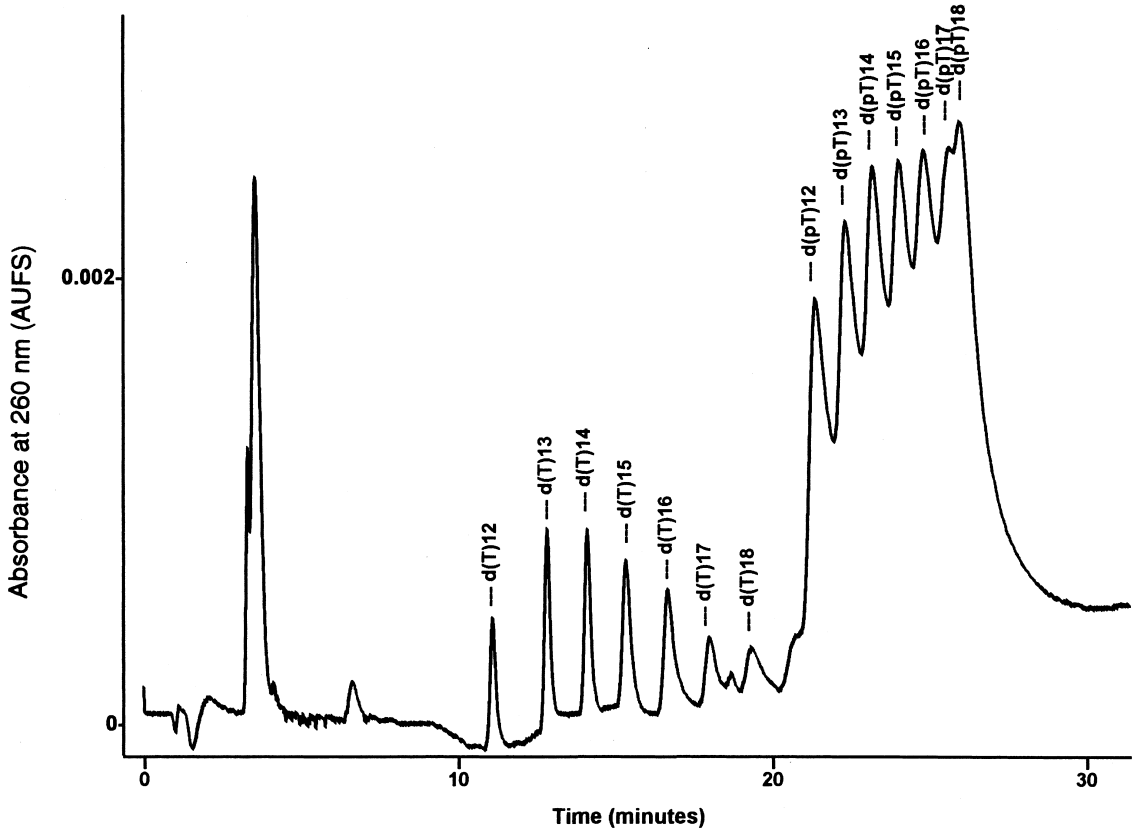

Fig. 3. Weak anion-exchange separation on the immobilized thymine column of a mixture of thymidyloligonucleotides using a 40 min gradient from 0.1 *M* ammonium acetate, pH 4.75, to 1.5 *M* ammonium acetate, pH 7.0.

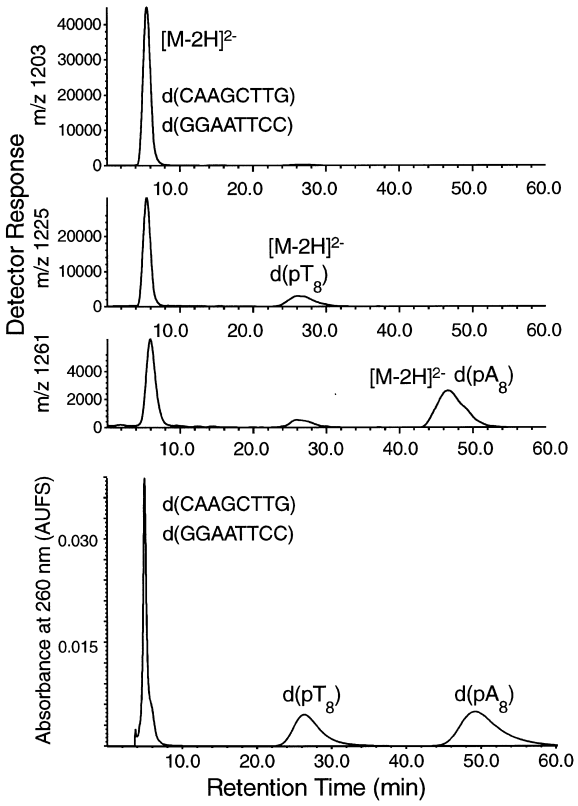

Fig. 4. LC–MS analysis using negative-ion electrospray of an equimolar mixture of d(CAAGCTTG), d(GGAATTCC), $d(pT)_8$ and $d(pA)_8$. Computer-reconstructed mass chromatograms of the doubly deprotonated molecules of each oligonucleotide are plotted as well as the UV absorbance chromatogram, which was recorded in-line between the immobilized thymine column and the mass spectrometer. A 30 min gradient was used from 90:10 to 40:60 (v/v) 300 m*M* ammonium acetate in methanol–water (pH 4.2).

these conditions, doubly deprotonated molecules predominated over singly or triply charged ions. Although necessary for optimum separations, the high ionic strength of the mobile phase (initially 300 m*M* ammonium acetate) suppressed ionization during electrospray and resulted in lower signal-to-noise levels than would be obtained if using water and methanol alone.

During the analysis shown in Fig. 4, d(CAAGCTTG) and d(GGAATTCC) coeluted near the beginning of the chromatogram. Because these compounds are isomeric, they produced identical deprotonated molecules, $[M-2H]^{2-}$ of m/z 1203. Under the initial high ionic strength mobile phase conditions of 300 m*M* ammonium acetate, each of these self complementary compounds self-associated in a head-to-tail arrangement to form duplexes. Probably because these duplexes were unable to form base pairs with thymine on the stationary phase, they were not retained by the column and eluted near the solvent front. Also detected at the solvent front (retention time of 6 min in Fig. 4) were ions of m/z 1225 and 1261, which were part of the chemical noise typically observed at the solvent front during LC–MS.

Next, oligonucleotide $d(pT)_8$ eluted at 26.2 min and was detected at m/z 1225, corresponding to the deprotonated doubly charged ion, $[M-2H]^{2-}$ (see the computer reconstructed mass chromatogram in Fig. 4). The corresponding mass spectrum of $d(pT)_8$ is shown in Fig. 5. Besides the doubly deprotonated molecule, $[M-2H]^{2-}$, at m/z 1225, related adduct ions were detected at m/z 1244 and 1263 corresponding to $[M-3H+K]^{2-}$ and $[M-4H+2K]^{2-}$,

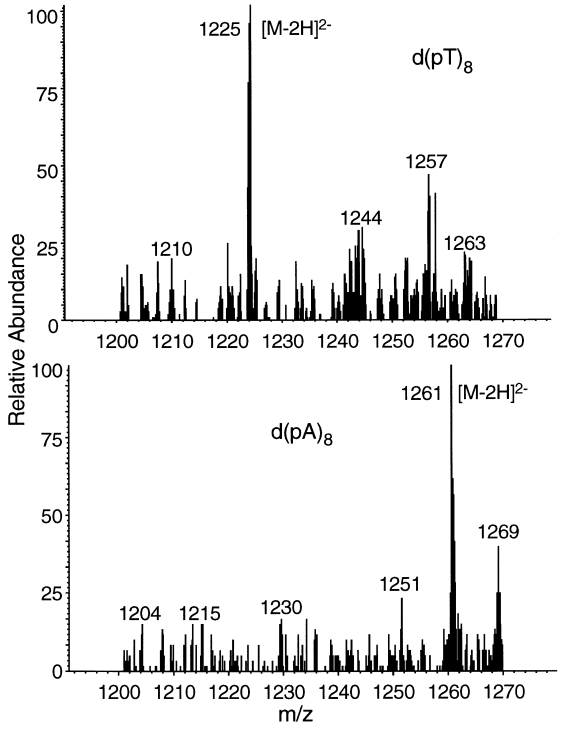

Fig. 5. Negative-ion electrospray mass spectra showing the doubly deprotonated molecules of $d(pT)_8$ and $d(pA)_8$, which eluted at retention times of 26.2 and 46.2 min, respectively, in the LC–MS analysis shown in Fig. 4.

respectively. The ion of m/z 1257 might be another adduct, but was not identified. Although $d(pT)_8$ showed some interaction with the column, presumably due to reversed-phase or weak anion-exchange characteristics of the column, this thymidyloligonucleotide was not retained as long as the oligonucleotide containing multiple adenine residues, since the thymine residues cannot base pair with immobilized thymine. Last to elute from the immobilized thymine column at a retention time of 46.2 min was $d(pA)_8$, which was detected at m/z 1261, corresponding to the doubly charged deprotonated molecule, $[M-2H]^{2-}$. (See computer reconstructed mass chromatogram in Fig. 4 and corresponding mass spectrum in Fig. 5). Possibly because of strong specific base pairing between immobilized thymine and adenine of the analyte, $d(pA)_8$ was retained much longer than the other oligonucleotides in this mixture. For comparison, the opposite elution order of $d(pA)_8$ and $d(pT)_8$ was observed during C_{18} reversed-phase ion-pair HPLC analysis, which provides additional support for affinity interaction as a mechanism of separation of the immobilized thymine stationary phase. The weak signal at 26.2 min in the chromatogram of m/z 1261 corresponds to ions produced in this range by $d(pT)_8$ such as the adduct ion, $[M-4H+2K]^{2-}$, at m/z 1263 (see Fig. 5). In addition, the ion of m/z 1269 in the mass spectrum of $d(pA)_8$ (Fig. 5) probably corresponds to a monooxidation product (possibly formed during electrospray) in which a single adenine residue was oxidized to 8-oxo-adenine or perhaps 2-oxo-adenine (also called 8-hydroxy-adenine and 2-hydroxy-adenine, respectively) weighing 16 u higher than the native molecule [15].

Additional separations of oligonucleotide mixtures with UV absorbance detection are shown in Figs. 6 and 7 to illustrate the selectivity of the immobilized thymine column. During these separations, the ionic strength of the mobile phase was maintained at a constant 0.1 *M* of ammonium acetate in order to reduce the ion suppression effects observed in the LC–MS example shown in Figs. 4 and 5. Because the signal-to-noise obtained during LC–MS under these conditions was no better than the results shown in Figs. 4 and 5, only UV absorbance data are shown to illustrate the selectivity of the column. It should be noted that ammonium acetate concentrations in excess of 30 m*M* were found to significantly suppress electrospray ionization, but higher concentrations were necessary to achieve optimum separations using the immobilized thymine column. In an effort to overcome this suppression effect, 100 m*M* 1,1,1,3,3,3-hexafluoro-2-propanol (adjusted to pH 7.0 with triethylamine) was used in place of ammonium acetate as reported by Apffel et al. [16] for reversed-phase LC–MS of oligonucleotides. Although Apffel

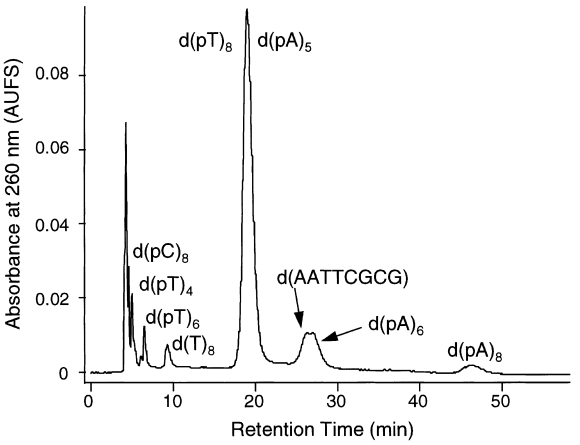

Fig. 6. HPLC separation using the immobilized thymine column of a heterogeneous mixture of eight oligonucleotides. The mobile phase consisted of a gradient from 35–70% acetonitrile in 0.1 *M* ammonium acetate (aq.) over 18 min to elute the oligonucleotides containing no adenine, and then back to initial conditions to elute adenine-containing oligonucleotides (see gradient details in Section 2.4).

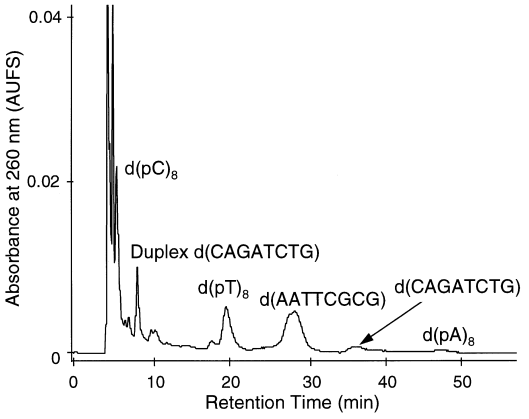

Fig. 7. HPLC separation with UV absorbance detection of a mixture of 8-mers containing either homogeneous or heterogeneous bases. The solvent system was the same as in Fig. 6.

et al. found that suppression of electrospray ionization may be overcome by using hexafluoropropanol in place of other ion-pair agents (such as ammonium acetate or triethylammonium acetate) during reversed-phase LC–MS, no separations could be achieved using hexafluoropropanol as a mobile phase additive.

In Figs. 6 and 7, a gradient of increasing acetonitrile concentration was used to separate (in reversed-phase mode) the oligonucleotides that contained no adenine residues. For example, in Fig. 6, $d(pC)_8$, $d(pT)_4$, $d(pT)_6$ and $d(pT)_8$ were resolved from each other and eluted before any oligonucleotides containing adenine. Next, $d(pT)_8$ coeluted with $d(pA)_5$ as the amount of acetonitrile in the mobile phase was reduced to the initial concentration. The remaining compounds eluted in order of increasing numbers of adenine residues (and presumably in order of their affinities for immobilized thymine), i.e., d(AATTCGCG) eluted first followed by $d(pA)_6$ and $d(pA)_8$.

In general, the length of the oligonucleotide chain determines the extent of hydrophobic interaction, weak anion-exchange and affinity chromatography that will be possible. Since adenyldeoxyoligonucleotides were retained longer on the immobilized thymine column than other oligonucleotides of identical length, these compounds demonstrated higher affinity for the stationary phase. Fig. 7 shows the separation of a series of 8-mers. Because chain length was constant, the retention time of each 8-mer was determined primarily by strength of the interactions between the nucleic acid bases and the stationary phase. In particular, $d(pA)_8$ was retained the longest because it contained the most adenine bases, which showed the strongest hydrophobic interaction and/or affinity interaction with the immobilized thymine residues. Two isomeric 8-mers, d(AATTCGCG) and d(CAGATCTG), were separated by more than 7 min. The 8-mer, d(CAGATCTG), was probably retained longer because its two adenine groups were spaced far enough apart to permit simultaneous binding to two different immobilized thymine residues whereas the two adjacent adenines on d(AATTCGCG) could only bind to one thymine group on the column at a time. Finally, a duplex of two d(CAGATCTG) molecules (formed by head-to-tail base pair complexation) was detected eluting much earlier (8 min) than the single stranded form (see Fig. 7). Like the duplexes shown in Fig. 4, the duplex of d(CAGATCTG) could not form hydrogen bonds with immobilize thymine, since the adenine groups were already bound to thymines on the complementary 8-mer.

4. Conclusions

An immobilized thymine HPLC column has been prepared and evaluated for compatibility with on-line electrospray LC–MS. Direct immobilization of a thymine derivative on a standard, factory-packed aminopropyl silica column allowed for the custom preparation of a high-performance column without the need for specialized tools or high pressure column packing equipment. Instead, only a standard HPLC system was required. Separation of oligonucleotide mixtures was demonstrated using the immobilized thymine column, which provided significantly different selectivity than conventional reversed-phase ion-pair chromatography. Like reversed-phase HPLC using volatile solvents and ion-pair agents, mobile phase conditions were developed for possible affinity chromatography of oligonucleotides which were compatible (although some suppression of ionization was produced by the high ionic strength mobile phase) with on-line electrospray mass spectrometric detection, thus providing another HPLC stationary phase suitable for electrospray LC–MS.

Acknowledgements

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References

[1] R.A. Gibbs, Anal. Chem. 62 (1990) 1202–1214.
[2] R. Wu, C.P. Bahl, S.A. Narang, Prog. Nucl. Acid Res. Mol. Biol. 21 (1978) 101–141.
[3] A.M. Thayer, Chem. Eng. News 68 (1990) 17–20.

[4] S.T. Crooke, Annu. Rev. Pharmacol. Toxicol. 32 (1992) 329–376.
[5] R.B. van Breemen, L.B. Martin, J.C. Le, J. Am. Soc. Mass Spectrom. 2 (1991) 157–163.
[6] L. Grotjahn, H. Blöcker, R. Frank, Biomed. Mass Spectrom. 12 (1985) 514–524.
[7] P.A. Limbach, P.F. Crain, J.A. McCloskey, J. Am. Soc. Mass Spectrom. 6 (1995) 27–39.
[8] M. Polverelli, L. Voituriez, F. Odin, J.-F. Mouret, J. Cadet, J. Chromatogr. 539 (1991) 373–381.
[9] J.M. Munholland, K.A. Bright, R.N. Nazar, Anal. Biochem. 178 (1989) 320–323.
[10] J.A. Thompson, R.D. Wells, Nature 334 (1988) 87–88.
[11] E. Yashima, T. Shiiba, T. Sawa, N. Miyauchi, M. Akashi, J. Chromatogr. 603 (1982) 111–119.
[12] C. DiRusso, R.P. Rogers, H.W. Jarrett, J. Chromatogr. A 677 (1994) 45–52.
[13] H.W. Jarrett, J. Chromatogr. 618 (1993) 315–339.
[14] R.M.C. Dawson, D.C. Elliott, W.H. Elliot and K.M. Jones, Data for Biochemical Research, Oxford University Press, New York, 3rd ed., 1986, pp. 76–77.
[15] N. Murata-Kamiya, H. Kamiya, M. Muraoka, H. Kaji, H. Kasai, J. Radiat. Res. 38 (1997) 121–131.
[16] A. Apffel, J.A. Chakel, S. Fischer, K. Lichtenwalter, W.S. Hancock, Anal. Chem. 69 (1997) 1320–1325.