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Separation of modified 2′-deoxyoligonucleotides using ion-pairing reversed-phase HPLC

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Abstract

A group of 18-mers of the same base sequence, but with differing alkyl modifications is used to investigate effects of these modifications on retention of oligonucleotides using ion-pairing reversed-phase liquid chromatography (IP-RPLC). It is shown that IP-RPLC is able to distinguish between oligonucleotides differing only by a single alkyl group. The identity of the nucleobase and position and length of the alkyl adduct affect retention of the oligonucleotide. These separation phenomena result from changes in charge and hydrophobicity upon alkylation. As demonstrated in this paper; chromatographic selectivity, unique to IP-RPLC, greatly facilitates the purification process of modified oligonucleotides.

Keywords: IP-RPLC; Oligonucleotides; Adducts; Purification; HPLC; Methylation

1. Introduction

The manufacture of synthetic oligonucleotides has increased significantly in recent years due to their use in a variety of applications such as PCR, genotyping, microarrays, therapeutics and pharmaceuticals, and structural studies [1–4]. Oligonucleotides, traditionally used as primers in PCR and DNA sequencing, have been investigated as drug candidates for regulation of the viral reproduction cycle and modulation of gene expression [1]. The high demand for oligonucleotides has led to improved phosphoramidite chemistry and driven synthesis to become a relatively fast and inexpensive process. However, synthesis of an oligonucleotide inevitably leads to truncated sequences and it is with some degree of difficulty that the desired product length is separated from failure sequences.

Purity is extremely important to the above mentioned studies, with many applications requiring primer purity of ≥99% [1,2,5]. Unfortunately, purification is often the most problematic step. Purification can be time consuming and sometimes requires complex procedures often resulting in low sample yield. Synthetic oligonucleotides have been purified by a number of electrophoretic and chromatographic techniques including polyacrylamide slab gel electrophoresis (PAGE), ion exchange and reversed-phase liquid chromatography, and solid phase extraction coupled to a variety of detectors [5–9]. PAGE allows for excellent separation of oligonucleotides ranging from 2- to 100-mers with greater than 98% purity in most cases [5,7]. However, this technique suffers from numerous complications including the necessity of manual band excision [5,7,9]. Also, gel-purified DNA has to be desalted prior to further use and loading capacity is low, limiting the usefulness of PAGE as a preparative method [9].

The two most commonly used chromatographic modes are anion exchange and reversed-phase. Anion exchange chromatography (AEC) separates oligonucleotides based on the number of charges and is very efficient for lengths from 2- to 30-mers with purity ranging between 95 and 98% [4,10,11]. Selectivity of the separation decreases as the length of the oligonucleotide increases and sometimes this causes overall yield to be sacrificed in order to obtain a high purity product [4,6,10]. Similar to PAGE, the samples purified by AEC must be desalted prior to further use.

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Purification by reversed-phase liquid chromatography (RPLC) is commonly used with the "trityl on" method [1,7,11]. The protecting group, dimethoxytrityl (DMT), used to protect 5′-deoxyribose hydroxyl group in the coupling and oxidation steps of synthesis is left on after the last coupling step [12]. This group is very hydrophobic and the full length product with the trityl group is highly retained on the reversed-phase column [1,5]. This purification is relatively quick and offers purities of ∼95% [5]. The trityl group must be removed by acid hydrolysis after purification. Impurities unresolved by this technique stem from failure sequences that also contain the DMT group as well as inefficient post-column detritylation [7,13].

Another chromatographic approach is ion-pairing reversed-phase liquid chromatography (IP-RPLC). IP-RPLC has been shown to be an efficient method for oligonucleotide purification, as well as for purification of "trityl on" oligonucleotides [8,13,14]. Recently, it was also shown by Fountain et al. that this separation technique is amenable to the purification of dye-labeled oligonucleotides [15]. Although the exact mechanism of separation has not been elucidated, it is known that the separation technique has characteristics of both reversed-phase and anion exchange techniques and is considered a mixed-mode separation [16]. The dynamic mechanism of IP-RPLC allows for selectivity based on charge as well as sequence.

This work will show the capability of IP-RPLC to separate and purify modified and unmodified oligonucleotides. Several alkyl adduct chain lengths at various base positions in sequences ranging from 18 to 32 bases in length will be used to demonstrate this phenomenon. In contrast, purification of modified oligonucleotides by the predominating methods (e.g., RPLC and AEC) is hindered by the lack of selectivity of these techniques for minor structural modifications.

2. Experimental

2.1. Chemicals and oligonucleotide samples

2.1.1. Chemicals

Acetonitrile (Fisher Scientific; Springfield, NJ, USA) used in this work was HPLC grade. Sodium perchlorate (Fisher) and Tris–HCl (J.T. Baker; Phillipsburg, NJ, USA) were reagent grade. The ion-pairing agent, triethylammonium acetate (TEAA) was purchased from Transgenomic Inc. (Omaha, NE, USA). Water was purified using a reverse osmosis system coupled with multi-tank/ultraviolet/ultrafiltration stations (US Filter/IONPURE, Lowell, MA, USA).

2.1.2. Oligonucleotides

Oligonucleotides were obtained from several sources. Unmodified oligonucleotides, as well as the sequences containing O^6-methyl dG, were purchased from Midland Certified Reagent Company (Midland, TX, USA). N^2-ethyl dG, O^6-ethyl dG, and N^6-*iso*-propyl dA modified phosphoramidites were gifts from James Fishbein's Lab (UMBC, Baltimore, MD, USA). The modified phosphoramidites were then sent to Midland Certified Reagent Company (Midland) and incorporated into the following 18-mer, 23-mer, and 32-mer sequences. One modified base was added to each sequence. The sequences were purified by Midland using reversed-phase chromatography and checked by MALDI. Several modified oligonucleotides such as O^6-methyl dG, O^4-methyl dT, N^6-methyl dA, and 5-methyl dC were also purchased as 18-mers from Trilink Biotechnologies (San Diego, CA, USA). The oligonucleotides purchased from Trilink were not purified by the company. The 18-mer sequence used is, 5′-TCGAGACTTCXAAGGGTT-3′ (X = O^6-ethyl dG, O^6-methyl dG, N^2-ethyl dG). Other modifications include 5′-TCGAGACTYXGWAGGGTT-3′ where (W = N^6-methyl dA, Y = O^4-methyl dT and X = 5-methyl dC). The 23-mer sequence is 5′-GAGACTTCGAXGGGTTCCGGAGC-3′ where (X = N^6-*iso*-propyl adenine). The 32-mer sequence is 5′-TCGAGACTTCXAAGGGTTCCGGAGCGGCCAAA-3′ (X = O^6-ethyl dG or N^2-ethyl dG). The 22-mer sequence is 5′-ACTTCCTGAAAACAAXGTTCTG-3′ where (X = 5-methyl dC). All of these modified bases, except N^6-*iso*-propyl dA and O^6-ethyl dG, can be readily purchased from oligonucleotide synthesis companies and are frequently used to study the effects of mutation which may lead to diminished protein expression, gene silencing, and/or cancer [17–19].

All oligonucleotides upon receipt were analyzed for purity by IP-RPLC, and in most cases additional purification was required and performed by IP-RPLC. Final purity of the oligonucleotides was confirmed by capillary electrophoresis (CE).

2.2. HPLC instrumentation and IP-RPLC conditions

Analytical separations were done on the WAVE® Nucleic Acid Analysis system (Transgenomic, Omaha, NE, USA) using the Hitachi System Manager software (HSM). The HSM program is interfaced with the pump (Model L7100), autosampler (Model L7200), oven (Model L7300), and UV detector (Model L7400) through the D-700 interface module. A semi-preparative system consisted of the same elements except it was plumbed to accept larger flow rates. The analytical and semi-prep columns were OligoSep®, 4.6 mm × 75 mm (P/N DNA-99-3510, Transgenomic) or 6.9 mm × 75 mm (P/N NUC-99-3866, Transgenomic), respectively. These columns are comprised of non-porous, C-18 modified polystyrene-divinylbenzene (PS-DVB). The oven temperature was set to 30 °C.

A two solvent gradient was used with a mobile phase consisting of an ion-pairing agent and organic modifier. Solvent A consisted of 0.1 M TEAA and solvent B consisted of 0.1 M TEAA and 25% ACN. The flow rates used were 0.75 and 1.2 mL min^{-1} for the analytical and semi-prep systems, respectively. The gradient used for the oligonucleotides is dependent on the length and the number of species being separated and can be found in the figure captions. The samples

were first run on an analytical scale to determine their purity and optimize the gradient.

Purification was carried out on either the analytical or semi-prep chromatography systems. The maximum quantity of oligonucleotide purified for each sample averaged around 1 mg. The lyophilized sample was brought up in 100 μL of deionized water and two injections of 50 μL were made on the analytical system. When the semi-prep system was used for purification, the sample was diluted in 1 mL and two injections of 500 μL each were made. Separation components were collected using a Model FCX-100 or FCW-180 Fraction Collector (Transgenomic). Collection took place when peak area exceeded a threshold value (i.e., when the absorbance increases above the running baseline) within the specified time window of peak elution. During this period, the eluent was collected in 0.4 mL fractions. The UV detector was set at 260 nm for the analytical analysis and for purification on the semi-prep column the detector was set off-wavelength at 300 nm to prevent saturation of the UV cell. Data was collected and integrated using Hitachi System Manager Software version 3.0–2.1 on a Dell Optiplex platform.

2.3. Anion exchange conditions

The same chromatography systems described previously were also utilized to perform anion exchange chromatography. Separations were performed on a 4.6 mm × 250 mm DNA Pac PA-100 (P/N 43010; Dionex, Sunnyvale, CA, USA). A triternary gradient profile used deionized water (solvent A), 0.25 M Tris–HCl (solvent B), and 0.375 M sodium perchlorate (solvent C). From time 0 to 1.0 min, the percentages were held at 88% A, 10% B, and 2% C. At 1.1 min the gradient changed to 70% A, 10% B, and 20% C and ended with 45% A, 10% B, and 45% C at 36.1 min. A 0.5 min wash of 100% C was incorporated into the method followed by equilibration at initial conditions from 36.6 to 50 min.

2.4. Capillary electrophoresis

Capillary gel electrophoresis (CGE) was performed on a P/ACE 5500 Series system using P/ACE Gold software (Beckman Coulter, Fullerton, CA, USA). The BioCap fused silica capillary, coated with acryloylaminoethylethoxyethanol (AAEE) had dimensions of 30 cm × 75 μm i.d. × 375 μm o.d. (Bio-Rad Laboratories, Hercules, CA, USA). The run buffer was composed of 25 mM Tris, 25 mM borate and 2 mM EDTA (Bio-Rad). The buffer was degassed by sonication every two hours during analysis. The injection voltage used was 10 kV for 8 s and the separation was performed at 12 kV at 40 °C. UV detection was set at 260 nm.

3. Results

The alkyl adducts used in the study are mainly methyl and ethyl; in addition, purification of an N^6-*iso*-propyl dA 23-mer is shown. The primary sequence for each set of oligonucleotides (18-mers, 32-mers e.g.) is the same. The oligonucleotides within a set differ only with respect to length and base position of the alkyl adduct. Fig. 1A–E presents the various modified bases used in the following experiments. The addition of alkyl adducts represent derivatives of each nucleobase which allows for the examination of the chromatographic effects of modification at a variety of sites.

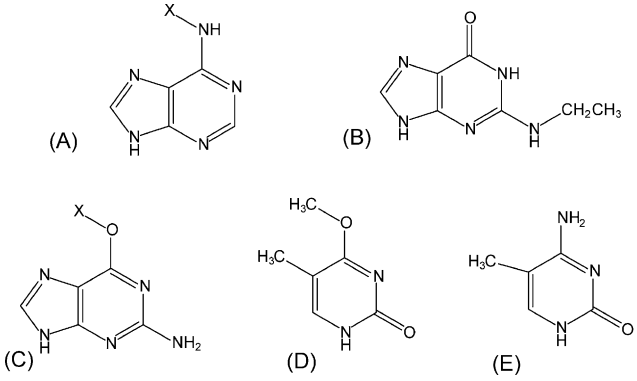

Fig. 1. This figure shows the location of all adducts present on the nucleic acid bases, which were incorporated into the oligonucleotide sequences: (A) N^2-ethyl dG; (B) O^6-X dG, where X = methyl or ethyl; (C) N^6-X dA, where X = methyl or *iso*-propyl; (D) O^4-methyl dT; and (E) 5-methyl dC.

3.1. Purification of a modified oligonucleotides by IP-RPLC

Fig. 2 shows the purification of a modified oligonucleotide, N^6-*iso*-propyl dA 23-mer. The first chromatogram (A) shows the crude injection of the N^6-*iso*-propyl 23-mer run on the IP-RPLC semi-prep system. Despite previous reversed-phase purification procedures, there are still significant amounts of impurities present in the sample. The second chromatogram (B) shows an aliquot of the purified product, which was injected onto the semi-prep system before concentration of the collected fractions. Gradient profiles used for purification purposes were less steep than those typically used for quantitative analysis, where rapid analysis is the focus and resolution is often compromised [15]. Still, the gradient profile used for N^6-*iso*-propyl was significantly steeper from those typically used for other sequences of similar length, because the *iso*-propyl group significantly increases the retention of the oligonucleotide. Post separation equilibration times were optimized to maximize peak resolution during the separation portion of the gradient run. This observation is attributable to more complete equilibration to initial conditions.

For unique conformer forming sequences, temperatures as high as 80 °C have been used for purification [15]. In general, it is our experience that lower temperatures improve resolution of separations where minor differences in charge density occur due to conformation, modification, and/or changes in length [20]. For our purposes, a temperature of 30 °C was used for purification. Overall, the resolution of modified

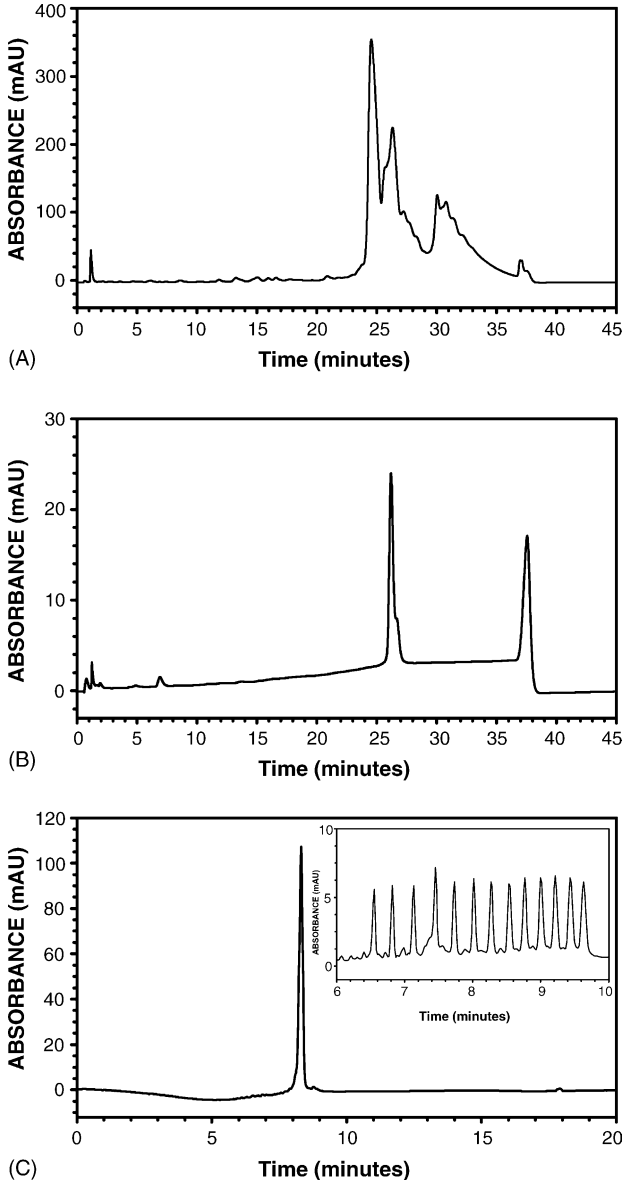

Fig. 2. This figure follows the purification of the N^6-*iso*-propyl 23-mer. The chromatograms show (A) modified 23-mer, which was previously purified by the company from which it was purchased, and (B) the purified 23-mer prior to final concentration. C is an electropherogram of the CGE purity confirmation, and the insert shows the standard ladder of a mixture of 8–32-mers. The gradient used for the N^6-*iso*-propyl 23-mer is 16% B at time 0 min, 35% B at 5 min, 62% B at 35 min, 60% B at 35.1 min, 100% B from 35.6 to 36 min, and 16% B from 36.1 to 48 min.

oligonucleotides and $n-1$ species decrease with an increase in temperature. This seems to be the case even with shallow gradients at high temperatures. The percent purity of modified 18-mers, purified by IP-RPLC, is approximately 98%. In addition, the IP-RPLC method does not require desalting after purification, which is a benefit over AEC. More importantly, purification of modified 18-mers was not possible by anion exchange chromatography (data not shown).

The purity of the N^6-*iso*-propyl oligonucleotide was confirmed by CGE coupled to UV detection and the results of this analysis are shown in Fig. 2C. The inset of Fig. 2C shows the standard ladder run on the CGE as a control. This ladder is a mixture of 13 oligonucleotides ranging from 8 to 32 bases in length. Percent purity will decrease as the length of the oligonucleotide increases. This is common to all chromatographic purification techniques [5]. Percent recovery of the major synthetic product, like purity, decreases with increasing oligonucleotide length.

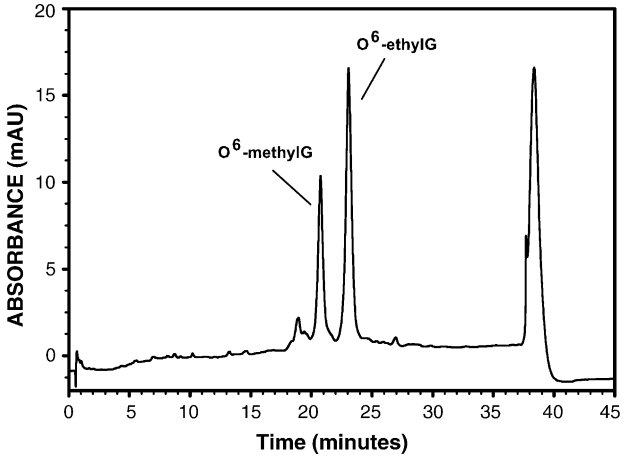

Fig. 3. The separation of two modified 18-mers, O^6-methyl dG and O^6-ethyl dG is shown. The gradient used to separate the modified 18-mers is 16% B at time 0 min, 24% B at 5 min, 38% B at 35 min, 60% B at 35.1 min, 100% B from 35.6 to 36 min, and 16% B from 36.1 to 48 min.

3.2. Separation of modified 2′-deoxyoligonucleotides

Through the process of purification, it was seen that an alkyl modified oligonucleotide can easily be purified from the synthesis mix. It is also possible to separate the same sequences with a different alkyl adduct at the same location or the same alkyl adduct on a different base, or a different location on the same base. For example, Fig. 3 shows the separation of O^6-methyl dG and O^6-ethyl dG 18-mers. The oligonucleotide containing the methylated guanine elutes first followed by the 18-mer containing the ethylated guanine. This result is expected; increasing the length of the alkyl substituent will increase the hydrophobicity of the nucleobase and/or increase the effect of an electron-donating group to the base. Separation of all modified bases was also attempted using an optimized anion exchange chromatography (AEC) method for comparison purposes (data not shown). Very little resolution (shouldering only) was obtained by AEC for the same 18-mers used in IP-RPLC, for which baseline resolution was achieved.

Fig. 4 shows the separation of four 18-mers of the same base sequence. One sequence is unmodified and the others contain O^6-methyl dG, O^4-methyl dT, and N^6-methyl dA (see Fig. 1B–D). Despite the fact that the same alkyl group is added to each sequence, the position on the base and the properties of the base itself must also make contributions to the retention of the 18-mer. The 18-mer without any modifica-

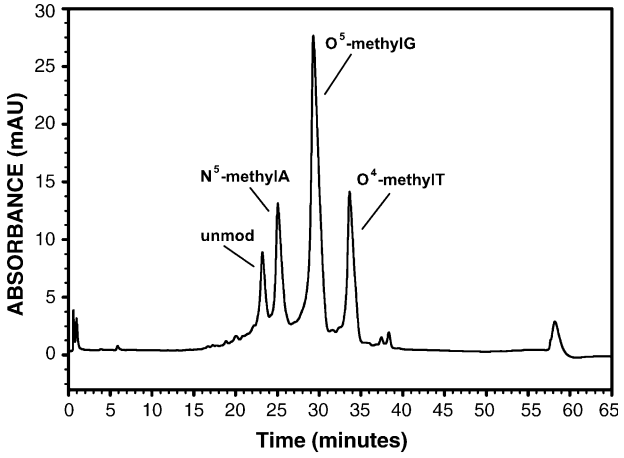

Fig. 4. In this chromatogram, a series of 18-mers with methyl modifications are separated—in order of unmodified 18-mer, N^6-methyl dA, O^6-methyl dG, and O^4-methyl dT. The gradient used for this separation is 16% B at time 0 min, 20% B at 5 min, 35% B at 55 min, 60% B at 55.5 min, 100% B from 55.6 to 56 min, and 16% B from 56.1 to 68 min.

tions elutes first, as would be expected. Next the 18-mer with the N^6-methyl dA adduct elutes, followed by the O^6-methyl dG, and O^4-methyl dT. The methyl group on adenine at N^6 may contribute to hydrophobicity, but it also does not significantly change the charge density in the ring; in contrast, the addition of the methyl group at O^6-dG and O^4-dT increase charge density in the ring of the nucleobase by forcing formation of the enol at the O^6 and O^4 positions, respectively. Thymine already has one methyl group at the C5 position and is considered the most hydrophobic nucleobase [21]. The addition at O^4 may further increase hydrophobicity of this base, causing this modification to elute later than the 18-mer modified at O^6-dG.

The chromatograms in Fig. 5 show the separation between (A) 18-mers and (B) 32-mers ethylated at two different positions on the same guanine base and the unmodified sequences. In Fig. 5A, baseline resolution is achieved between the unmodified sequence and N^2-ethyl dG and O^6-ethyl dG. Not only is the ion-pairing mechanism selective for the addition of an alkyl group, but it is also selective for the location of the alkyl group. Both of the alkyl groups are on the same guanine in the sequence, but on a different heteroatom. N^2 and O^6 are both exocyclic on the guanine base; however, charge distribution differs among the three. Guanine and N^2-ethyl dG keep the same localization around the ring with two double bonds in the ring and the keto group being the major resonance structure where as the addition of the ethyl group to the O^6 moiety leads to a fully conjugated ring. Fig. 5B shows a series of identical 32-mers, with the same alkyl groups as the 18-mer series. There is still separation between the 32-mer unmodified and the modified sequences; however, it is not as pronounced. Overall, the peak width is wider for the 32-mers than the 18-mers, with an average width of 4.1 and 2.3 min, respectively. Based upon the retention time and the average peak widths, the relative resolution factors are 0.6 for the 32-

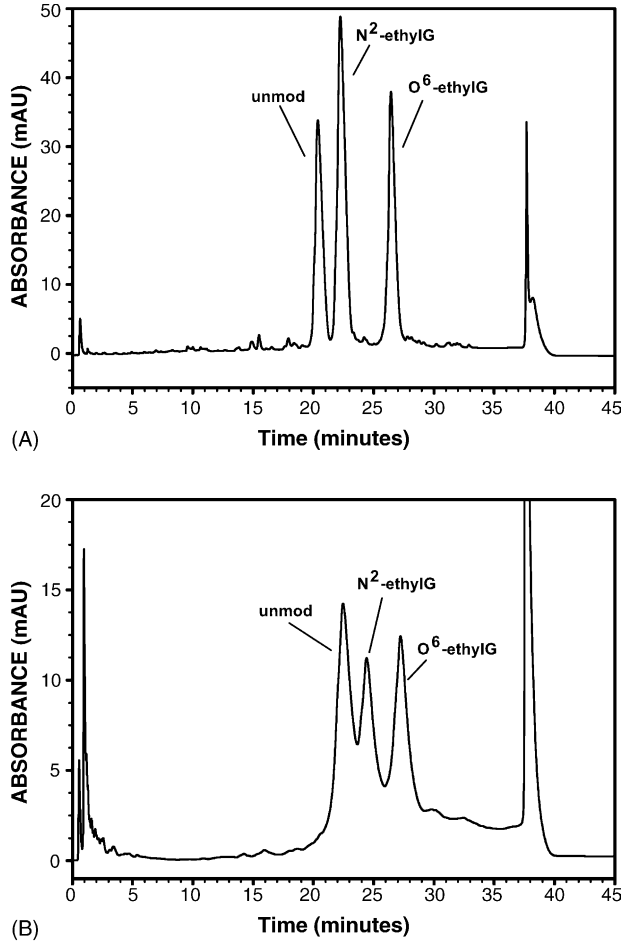

Fig. 5. These chromatogram show the separation of mixtures of unmodified, N^2-ethyl dG modified, and O^6-ethyl dG modified (A) 18-mers and (B) 32-mers. The separations show that not only can modifications on different bases be resolved, but that the same alkyl group on the same base on a different exocyclic heteroatom also changes the retention of the oligonucleotide. The gradient profile for the 18-mers is the same as the one used previously in Fig. 3. The gradient used for the 32-mers is 30% B at time 0–5 min, 50% B at 35 min, 60% B at 35.1 min, 100% B from 35.6 to 36 min, and 30% B from 36.1 to 48 min.

mers and 1.3 for the 18-mers. This is a resolution increase of over 200% for the 18-mer sequences. The addition of a hydrophobic moiety on a single base does not significantly increase the chemical potential of the compound in a chromatographic phase for a 32-mer as much as it does on the 18-mer [22].

Interestingly, a baseline separation could not be obtained for two 22-mers, one unmodified and one containing 5-methyl cytosine (Fig. 6A). The two oligonucleotides were run at varying gradients and percentages of acetonitrile, but the resolution was not significant enough to be amenable to purification on a larger scale than analytical. The methylated cytosine, which is common on many genes and is thought to act as a transcriptional activator/repressor, is located on an endocyclic carbon [20]. All of the other alkyl adducts are located on exocyclic heteroatoms. Methylation of C5 does not significantly affect the charge distribution or electron lo-

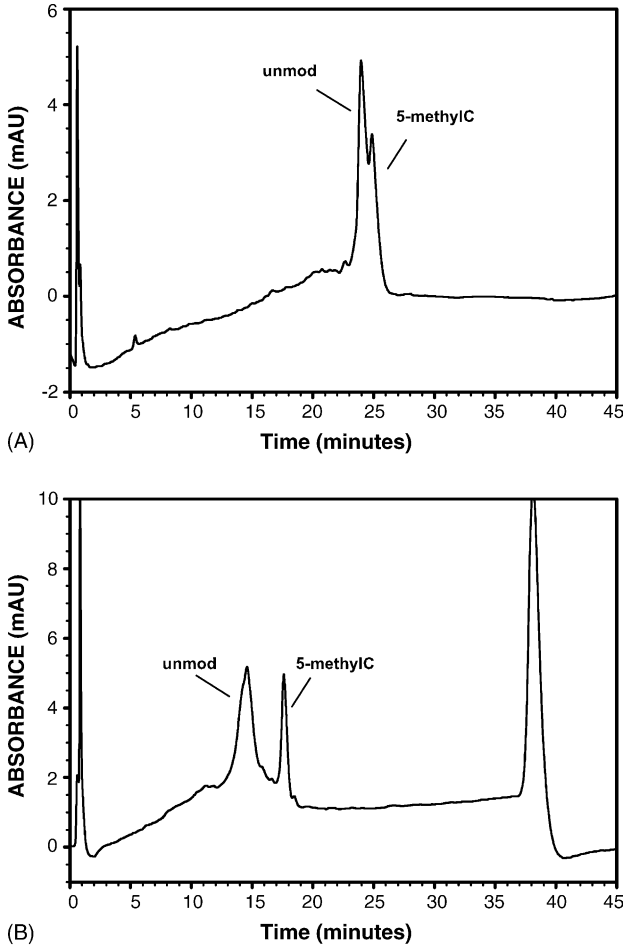

Fig. 6. A is a chromatogram of two 22-mers of the same sequence, one unmodified and one sequence with a 5-methyl cytosine modification present (see Fig. 1E). The two sequences can be resolved from one another; however, baseline resolution was not obtained. The gradient used for the 22-mer separation is 16% B at time 0 min, 24% B at 5 min, 50% B at 35 min, 60% B at 35.1 min, 100% B from 35.6 to 36 min, and 16% B from 36.1 to 48 min. Fig. 6B shows the attempt to separate a 5-methyl dC 18-mer from the unmodified 18-mer sequence. The gradient used initially was the same as the gradient used for the 18-mers in Fig. 3. Further optimization did not enhance the resolution.

calization within the ring. An 18-mer oligonucleotide of the same sequence used above in the previous experiments, but modified at C5 of cytosine, was run with the unmodified 18-mer sequence and can be seen in Fig. 6B. The C5 methylated 18-mer was baseline resolved from the unmodified sequence. Of the total variation in the chemical potential between the phases, the modification contributes to a greater percent of the difference between the 18-mer and the 22-mer [22]. The identity of neighboring bases, which surround the methylated cytosine, may play a role as well.

4. Discussion

The overall purity and recovery of IP-RPLC is equal to that of other chromatographic methods of purification for unmodified oligonucleotides and appears to be superior to all purification procedures for modified oligonucleotides. Separation is primarily based upon the difference in charge between oligonucleotides of varying length. The ion-pairing agent used, TEAA, interacts hydrophobically with the reversed-phase surface creating a dynamic “ion exchange” like atmosphere [9,17]. The negatively charged phosphates then interact with the positively charged amine groups creating the ion pair. Most models focus only on charge interaction and do not take into account any hydrophobic contribution made to the separation [23–25]. It seems that a more accurate model for retention would fall somewhere in between these two extremes. It has been shown that sequence as well as conformation plays a role in determining the final retention of the DNA or RNA [11,15]. Even the simplest experiment, using polydT and polydA oligonucleotides show that retention is altered because of the increased hydrophobicity due to thymine and the bending caused by long poly d(AT) tracts [11,26]. If IP-RPLC was simply a charge based mechanism, then retention would not be a consequence of base identity. In a previous paper, we described the separation of RNA conformational isomers [20]. In this case, a stem-loop was enzymatically synthesized and then purified from the transcription mix. However, several conformations of the stem-loop exist in equilibrium. IP-RPLC is sensitive enough to the shape or conformation of the secondary structures to cause a change in retention based upon structure [20]. Another area in which IP-RPLC exhibits high selectivity is the separation of modified oligonucleotides. Fountain et al. have shown the purification of oligonucleotides with covalently linked fluorescent dye labels [15]. Their work attributes the change in retention to the hydrophobicity of the labels.

It is shown that the addition of an alkyl group will increase the retention of the oligonucleotide when compared to its unmodified sequence, but the cause of this shift in retention is not obvious. It seems likely that the mechanism is a mix of electrostatic and hydrophobic contributions of each individual base; however, steric effects may also play a role, as it is thought that the identity of neighboring bases may be important to the separation. The addition of an alkyl group adds a factor of hydrophobicity, but separation of alkylated oligonucleotides at the exocyclic heteroatoms is better than at the endocyclic carbon. A hydrophobic effect is observed with the lengthening of the alkyl chain as seen in Fig. 3. When methylation at O^6-dG is extended to ethyl, the retention increases. However, it is also apparent from the results in Figs. 4 and 5 that base identity and location on the base play a role in determining overall retention. Fig. 4 shows the effects of a methyl group on A, G, and T. While thymine is the most hydrophobic base and adding another methyl group only increases this, it seems that charge density has a stronger effect on the retention [22]. Methylation at N^6-dA does not force the enolization of adenine as methylation does at O^6-dG and O^4-dT. Here it seems that charge density has a greater effect on retention than hydrophobicity. Further proof of this is seen in Fig. 5 where the ethyl modification is found at either N^2-

dG or O^6-dG. Again, ethylation at N^2 does not change the charge density in the ring to the same degree as ethylation at O^6 where enolization must occur.

Another trend appears from the comparisons made above. When the alkyl group is placed on a heteroatom, the retention shifts depending on the length of the alkyl chain, but when the alkyl group is placed on the ring carbon, as on the cytosine, the change in retention is not as significant. Separation was seen between the 18-mers, but only a slight separation was obtained for the 22-mers. This observation may be attributed to steric effects caused by neighboring base identity. The methylated cytosine in the 18-mer sequence has a thymine on the 5′ side and guanine on the 3′ side where the methylated cytosine in the 22-mer has an adenine on the 5′ side and a guanine on the 3′ side. Thymine is a smaller, more hydrophobic base [22]. Perhaps the increased hydrophobicity of cytosine, making it similar to thymine contributed to the increased resolution of the 18-mers, where the cytosine in the 22-mer was surrounded by two purines, which are less hydrophobic than thymine. In general, exocyclic modifications allow greater access to the surface of the column than endocyclic modifications and should thus be expected to have a greater effect on the retention time (partition coefficient) which is precisely the trend which is observed.

5. Conclusions

The separation of modified DNA oligonucleotides by IP-RPLC provides several advantages over existing purification procedures. In addition to the alkylated oligonucleotides seen here, other types of modifications such as oxidations, halogenations, and cross-linked species can also be separated using this technique. Each type of adduct affects the charge density, aromaticity, hydrophobicity, and steric bulk of the nucleobase on which it is located. Whereas traditional separative techniques are relatively insensitive to many of these parameters, IP-RPLC capitalizes upon all of these various physical changes to enhance the purification process.

The technique also offers advantages for quantitative analysis of alkylated nucleobases in medical diagnostics. Preparation of samples from biological sources will be minimized, because base hydrolysis is not necessary for adduct identification. Additionally, as the technique is both non-denaturing and non-destructive, structural isoforms of DNA can be discriminated by IP-RPLC and scarce analytes can be recovered for later use. Importantly, the recovered oligonucleotides are relatively salt-free, and the mobile phase is compatible with LC–MS.

Although the separation of unmodified oligonucleotides can be modeled, the detailed separation mechanism of modified sequences in IP-RPLC remains to be elucidated. Analysis of the separation between a greater variety of modified and unmodified sequences may provide further insight into the mechanism of retention. Any further clarification of the retention mechanism will be useful for optimizing purification parameters and may also enhance the diagnostic utility of the technique.

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