Characterization of G-rich and T-rich oligonucleotides using ion-pair reversed-phase high-performance liquid chromatography/ tandem electrospray ionization mass spectrometry

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Characteristics of G-rich and T-rich oligonucleotides were investigated to compare their retention time, total ion current (TIC) intensity, charge-state distribution and product ions using ion-pair reversed-phase high-performance liquid chromatography/ tandem electrospray ionization mass spectrometry (IP-RP-HPLC/ESI-MS) at room temperature. Three commonly used mobile phases for the analysis of oligonucleotides, triethylammonium acetate (TEAA), triethylammonium bicarbonate (TEAB) and triethylammonium hexafluoroisopropanol (HFIP) have been utilized. Retention time of G-rich and T-rich oligonucleotide was significantly different in TEAA and TEAB buffer systems, while in the HFIP buffer system it was affected more by the length of oligonucleotides. On the other hand, the ESI-MS ion abundance in the HFIP buffer system was higher than that in both TEAA and TEAB buffers. The TIC intensity of T-rich oligonucleotides was much higher than that of G-rich oligonucleotides in all mobile phases. In addition, much higher charge-state fragments were observed in the HFIP buffer system than that in the case of TEAA and TEAB buffer systems. Product ions of both G-rich and T-rich oligonucleotides were affected by the charge state of the parent ions and collision energy.

Keywords: G-rich, T-rich, oligonucleotides, ion-pair reversed-phase high-performance liquid chromatography, electrospray ionization mass spectrometry

Introduction

G-rich and T-rich oligonucleotides have extensive biological significance. This is because G-rich segments are important as therapeutic compounds; CpG motifs are known to be immunomodulators; G-quartets are thought to be responsible for the therapeutic efficacy of some oligonucleotides. They are observed as tandem repeats in microsatellite DNA in sequences associated with human disease at the replication origin of single-stranded bacteriophages and viruses, in several transcriptional regulatory regions of important oncogenes and in other hot spots for genetic recombination. Furthermore, among DNA nucleic acid bases, G is the most easily damaged by toxic material and oxidants.[1–3] T-rich segments have an important function *in vivo* such as the promoter region of RPL36, the site of DNA drug binding, regulation rDNA replication and increasing expression at intron. Also, T-rich regions of DNA are important targets for UV damage, so that understanding the characteristics is a starting point for the study of the mutation profiles of UV damage to DNA.[4–6] Several methods have been used to study the characteristics of G-rich and T-rich oligonucleotides in recent decades, such as the structure information method by using atomic force microscopy (AFM), nuclear magnetic resonance (NMR) and bio-techniques.[3,7,8] However, characterization of G-rich and T-rich

DOI: 10.1255/ejms.666 ISSN 1356-1049 © IM Publications 2004

olionucleotides obtained from other tools such as liquid chromatography/mass spectrometry (LC/MS) have rarely been reported.[9]

The direct combination of liquid chromatography online mass spectrometry with electrospray ionization (ESI) has been an important technique for the characterization of proteins and nucleic acids.[10–13] Compared with traditional methods of oligonucleotide analysis, oligonucleotides can be desalted and separated by high-resolution high-performance liquid chromatography (HPLC) prior to mass spectrometric investigation; therefore HPLC/MS offers the greatest level of selectivity and specificity for oligonucleotide mixtures. However, it is very important to optimize the HPLC mobile phase system to obtain efficient mass spectrometric detection of the separated analytes. because the optimal mobile phases for HPLC are often incompatible with the optimum solvents for electrospray ionization (ESI) and a compromise condition must be found between them. Triethylammonium acetate (TEAA), usually used as an ion-pairing buffer, has better separation of oligonucleotides, but it is not compatible with ESI-MS detection. Some researchers introduced the use of the ion-pairing buffer triethylammonium bicarbonate (TEAB), which had better separation and improved ESI-MS signal with post-column addition of acetonitrile.[13–16] A significant improvement in the incompatibility between HPLC separation and ESI-MS in sensitivity and mass spectral peak quality has been made by Apffel *et al.*, in which an ion-pairing agent system composed of triethylamine (TEA) as the ion-pairing agent and hexafluoroisopropanol (HFIP) as the buffering acid was used and high ESI-MS sensitivity, suppressed cation adduction levels and excellent chromatographic peak dispersion were obtained in the analysis of all types of DNA.[17] However, little research has been done to date to characterize the behavior of G-rich and T-rich oligonucleotides on ion-pair reversed-phase high-performance liquid chromatography/tandem electrospray ionization mass spectrometry (IP-RP-HPLC/ESI-MS) with these three mobile phases.

In the present study, the oligonucleotides of G-rich and T-rich were analyzed by RP-IP-HPLC/ESI-MS with TEAA, TEAB and HFIP mobile phases to characterize the retention time, total ion current (TIC) intensity, charge-state distribution and the spectra of product ions.

Experimental

Chemicals and materials

Oligonucleotides were obtained from Asia Biochemistry (Shanghai, China) and used as received. The sequences investigated are listed in Table 1 and the average molecular

Table 1. Characteristics of oligonucleotides used in the experiments.

No.	Sequence	Calculated MW	Measured MW	Length	T%	G%
A1	5′-CGTAGCTGATCGTTCAAGCT-3′	6108.0	6107.9	20		
A2	5′-CGTAGCTGATCGTTCAAGCA-3′	6117.0	6117.8	20		
A3	5′-CGTAGCTGATCGTTCAAGCC-3′	6093.0	6092.6	20		
A4	5′-CGTAGCTGATCGTTCAAGCG-3′	6133.0	6133.1	20		
B1	5′-TTTTATTTGTTTT-3′	3926.7	3925.8	13	84.6	8.3
B2	5′-TCCCTAGCGTTGAATTGTCCCTTAG-3′	7598.9	7599.2	25	36	20
B3	5′-GACAGGAAAGACATTCTGGC-3′	6175.1	6175.3	20	15	30
B4	5′CGGCGGTGACGGCTGTTG-3′	5587.7	5586.9	18	22.2	50
B5	5′-AGGGTGGCGTGG-3′	3791.5	3791.6	12	16.7	66.7
C1	5′-TGGGGT-3′	1863.3	1863.2	6		66.7
C2	5′-ATGGGGAT-3′	2489.7	2490.3	8		50
C3	5′-GGGGATGGGGAT-3′	3806.5	3806.0	12		66.7
C4	5′-GGGGTTTGGGGGTT-3′	4421.9	4421.1	14		64.3
D1	5′-TATCTGTC-3′	2375.2	2375.8	8	50	
D2	5′-ATCTGT-3′	1782.2	1781.9	6	50	
D3	5′-TTTCCTTT-3′	2341.6	2341.2	8	62.5	
D4	5′-TTCTTTTTCT-3′	2949.9	2950.1	10	80	

mass of oligonucleotides was calculated using the formula: $M = 312.2 \times A + 303.1 \times T + 288.1 \times C + 328.2 \times G - 61.9$. Triethylamine (TEA, 99.5%), glacial acetic acid (99.99%) and HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Water was purified with an Elix-Milli-Q system (Millipore Corp., Bedford, MA, USA). CO_2 was used with a purity higher than 99.99%. 1,1,1,3,3,3-hexafluoro-2-propanol was obtained from Dupont (Delaware, USA).

HPLC mobile-phase preparation

The 50 mM triethylammonium acetate buffer, pH 7, was prepared by mixing 3.6 mL of TEA and 1.49 mL of acetic acid in 400 mL water and then carefully adjusting the pH to 7 using TEA or acetic acid. After adjusting the volume to 500 mL, the final concentration of triethylammonium acetate was 50 mM.

The triethylammonium bicarbonate buffer was prepared by passing CO_2 into the aqueous solution of 50 mM triethylamine at 5°C until the pH reached 8.4–8.8.

The 400 mM hexafluoroisopropanol-16 mM triethylammonium buffer, pH 7.7, was prepared by dissolving 22 mL HFIP in 400 mL water and then slowly titrating with 1.2 mL TEA.

All mobile phases were filtered through 0.45 µm film before being used in the HPLC analysis.

Instrumental analysis

All samples were analyzed using an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled with an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The liquid chromatograph was equipped with an online vacuum degassing system, a quaternary pumping system, an autosampler and a variable wavelength detector. The chromatographic separation was performed on a C18 column (150 × 3.0 mm i.d., 3.0 µm particle size, ZORBAX 300SB, Agilent Technologies, Palo Alto, CA, USA) at room temperature. Three mobile phases at a flow-rate of 300 $\mu L\ min^{-1}$ were used in the analysis and the conditions can be seen in the figure captions. The concentration of single oligonucleotide used in the experiments was 0.2 $nmol\ \mu L^{-1}$. Other concentrations of oligonucleotides in the solution mixture can be seen in the figure captions. The injection volume was 1 µL and UV detection was carried out at the 260 nm wavelength. The LC effluent was introduced into a Turbon IonSpray interface without splitting. Electrospray mass data were acquired in the negative-ion mode with a spray voltage of –4 kV and declustering potential of –100 V. The source temperature was 400°C. Nitrogen was used as the curtain gas (setting 16), nebulizer gas (setting 20) and turbo gas (setting 20). MS/MS was performed using nitrogen as collision gas (CAD gas setting 8). The mass spectrometer was operated at unit mass resolution for both Q1 and Q3. The data were acquired using Sciex Analyst software, version 1.3.1 (Applied Biosystems, Foster City, CA, USA).

Table 2. Retention time of oligonucleotides on HPLC with TEAA, TEAB and HFIP.

No.*	Retention time (min)		
	TEAA	TEAB	HFIP
A1	28.36	25.67	24.87
A2	27.28	24.62	23.83
A3	26.20	23.55	22.97
A4	24.70	22.00	21.20

*see Table 1

Results and discussion

Retention behavior characteristics of the oligonucleotides on HPLC with different mobile phases

To simplify, we first investigated a type of oligonucleotides: type A (A1–A4) with the same length but different at their 3′ end bases (Table 1). It is evident that the retention time of the one with the G base at the 3′ end was the shortest in all three mobile phases (Table 2) and the retention times were in the order of G < C < A < T (at the 3′ end). This elution order was different from some reported data.[18] This difference may be caused because the temperature used in the HPLC analyses was different. We can clearly see from Table 2 that the difference in the retention time was higher between A3 and A4 than between A1 and A2. The largest difference was between A1 and A4. This was due to the hydrophobic difference between the individual bases at the 3′ end of A1–A4. The addition of C or G does not increase oligonucleotide retention times for as long as the addition of A or T. This result was in good agreement with that obtained by Gilar *et al*. by using the hetero-oligonucleotide ladder on HPLC.[19]

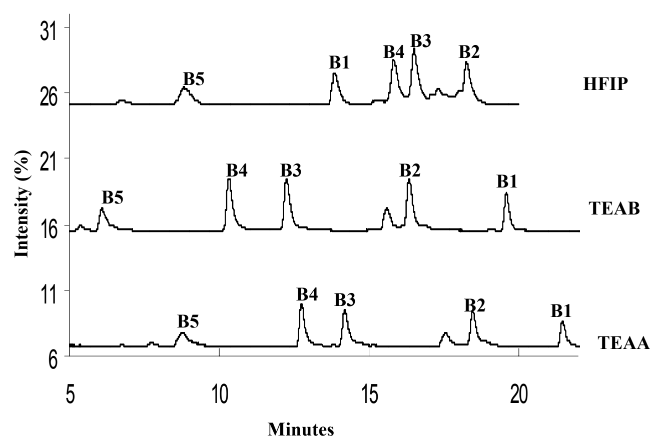

Figure 1. Retention time of oligonucleotides B1–B5 eluted by three mobile phases on IP-RP-HPLC. B1: 5′-TTTTATTTGTTTT-3′ B2: 5′-TCCCTAGCGTTGAATTGTCCCTTAG-3′ B3: 5′-GACAG-GAAAGACATTCTGGC-3′ B4: 5′CGGCGGTGACGGCTGTTG-3′ B5: 5′-AGGGTGGCGTGG-3′.

To learn more about the characterization of oligonucleotides eluted by different mobile phases, five oligonucleotides with different G and T content and with a length from 12 to 25 mer (type B) were analyzed by HPLC/MS. Figure 1 shows the retention time of oligonucleotides on HPLC with a TEAA, TEAB and TEA-HFIP buffer system, respectively. It is interesting to find that, among type B, the first elution was the G-rich oligonucleotide but the last elution was T-rich oligonucleotides in the TEAA and TEAB buffer systems. The analyses of other G-rich and T-rich oligonucleotides (C1–4, D1–4) gave similar results, i.e. the retention time of the G-rich in the TEAB and TEAA buffers is shorter than that of the T-rich when they had similar length (data not shown). However, it should be pointed out that the retention time in the TEAB and TEAA buffers increased with the decrease of G content, while in the HFIP buffer, the retention time increased with the increase in length (Figure 1). This may be explained by the following reasons. There are many polar groups including O and N atoms in the G-base molecule. So, if these bases were resolved into water, intermolecular hydrogen bonds would be formed easily, leading to a higher hydrophilicity. Since, during the separation of oligonucleotides on the HPLC column, ion-pair formation with TEA ions occurred, probably both in the mobile phase and on the stationary phase, which would determine the retention behavior,[20] high hydrophilic sample (G-rich) would easily be exchanged by the protonated or non-protonated TEA from the C18 surface, resulting in a shorter retention time. Unlike TEAA and TEAB buffer systems, in which G-rich oligonucleotides would easily form inter- and intramolecular complexes such as G-quadruplex second structures,[21] the buffer of TEA/HFIP was an efficient denaturant so the retention behavior in the HFIP buffer system was different from that in the TEAA and TEAB buffer systems.[15] In addition, the TEAA, TEAB and HFIP concentrations in the mobile phases had different effects on the retention time of oligonucleotides. In the TEAA and TEAB buffer systems, the gradient elution had less effect on the retention time of G-rich oligonucleotides than that on the T-rich oligonucleotides (data not given). This further indicated that higher hydrophobicity of T contributed more to the retention behavior than that of G when more organic mobile phase was used. Compared with TEAA and TEAB buffers, in the HFIP buffer system the change of the gradient had a significant effect on the retention time. This indicated that other reasons, such as the chain length, contributed more to the retention behavior of oligonucleotides. Gilar *et al*. also found that the retention time in the HFIP buffer was not accurately fitted for his model obtained by using TEAA; they thought that it was due to the substantial changes in ion-pairing interaction strength.[19]

ESI-MS characteristics of oligonucleotides on IP-RP-HPLC/ESI-MS with different mobile phases

It is well known that ions are generated in ESI by the application of a high voltage to the sprayer through which a solution containing analyte is infused. Several factors would influence the ESI response of an analyte in a mass spectrum. A significant factor is the composition of the electrospray solvent. During ionization, the used solvents would result in different surface-charge densities on the droplet and the charged offspring droplets, which have a crucial effect on the subsequent formation of the analyte ions. Second, the quantity and the type of the sample, mainly referred to as the surface activity and the free energy of the analyte. The surface activity affects the nature of ion evaporation from a charged droplet and the free energy is required to remove the ion from the droplet. The ESI performance of the analyte will also depend upon other factors such as ESI voltage, nebulizer gas, heater gas flow and temperature, which can be controlled by the mass spectrometrist. A lot of research has illustrated that different solvents and buffers would result in dramatically different mass signal response and the choice of a compatible mobile phase is very important to obtain both a good resolution of the analytes in the UV chromatogram and a higher mass signal intensity in ESI. However, very little research has focused on the ESI-MS signal response of G-rich and T-rich oligonucleotides with these three mobile phases.

In the present study, the MS signal response of oligonucleotides on IP-RP-HPLC/MS was investigated by using several mobile phases including TEAA, TEAB and HFIP systems. For the oligonucleotides with the same length but different bases at the 3′ end, it was found that their TIC intensities are highly related with the 3′ end base. For example, in the TEAB buffer system, the TIC intensities of A1–A4 were 4.99×10^7, 4.22×10^7, 3.18×10^7 and 2.38×10^7, respectively. Furthermore, the TIC intensity of the oligonucleotides decreased with the increase of G content. Additionally, compared with the TEAA and TEAB systems, the TIC intensities were increased by two orders of magnitude when the HFIP system was used (data not shown). Null *et al*. were the first to predict the relationship between ion intensity and G content using ESI-Fourier transform-ion cyclotron resonance (FT-ICR).[22] They found that the ESI signal decreased with the increase of G content by direct injection of the electrospray solution containing acetonitrile and 2-propanol, ammonium acetate, piperidine and imidazole, respectively. This result is due to the difference in the hydrophobicity and free energy of nucleotide bases.[23–27] The hydrophobic molecules will easily be absorbed on the droplet's surface because of their greater surface activity, so their chance to form analyte ions would be larger than others. So, the weakest hydrophobic analyte will have the smallest response on MS. A similar relationship was also observed with peptides on IP-RP-HPLC/MS-MS.[25]

Similar to the retention characteristics of the oligonucleotides on HPLC, it was also found that the TIC intensities of G-rich and T-rich oligonucleotides were different in the TEAA and TEAB buffer systems and the HFIP buffer system. That is, the TIC intensity of G-rich and T-rich oligonucleotides in the HFIP buffer system was much higher than those in the TEAA and TEAB buffer systems. HFIP

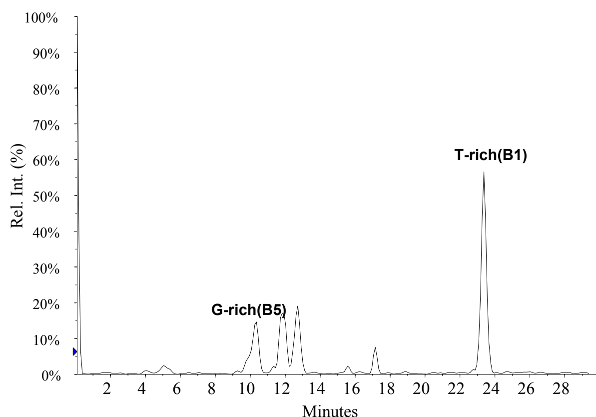

Figure 2. Comparison of IP-RP-HPLC-ESI-MS chromatogram for T-rich and G-rich oligonucleotides with TEAA buffer system. B5: 5′-AGGGTGGCGTGG-3′; B1: 5′-TTTTATTTGTTTT-3′.

is an acid weaker than HAC and H_2CO_3, so the linkage of the oligonucleotides with HAC and H_2CO_3 would be much stronger than that of the HFIP. Since weaker bonding would be beneficial to being broken to form more analyte ions in the ESI process, the ESI-MS response of oligonucleotides in the TEAA and TEAB buffer systems depends more on the binding action than that of the HFIP buffer system. Another reason for higher ESI response obtained in the HFIP buffer system may be that the boiling point of the HFIP was much lower than that of acetate and bicarbonate (HFIP = 59°C, TEA = 89°C, HAC = 118°C and Bicarbonate = 195°C). During the spraying, HFIP easily escaped from the solution so the TIC intensity was stronger than in the buffers of TEAA and TEAB. Therefore, ESI-MS response of oligonucleotides in the TEAA and TEAB buffer system would be much lower than in the case of the HFIP buffer system. The results also

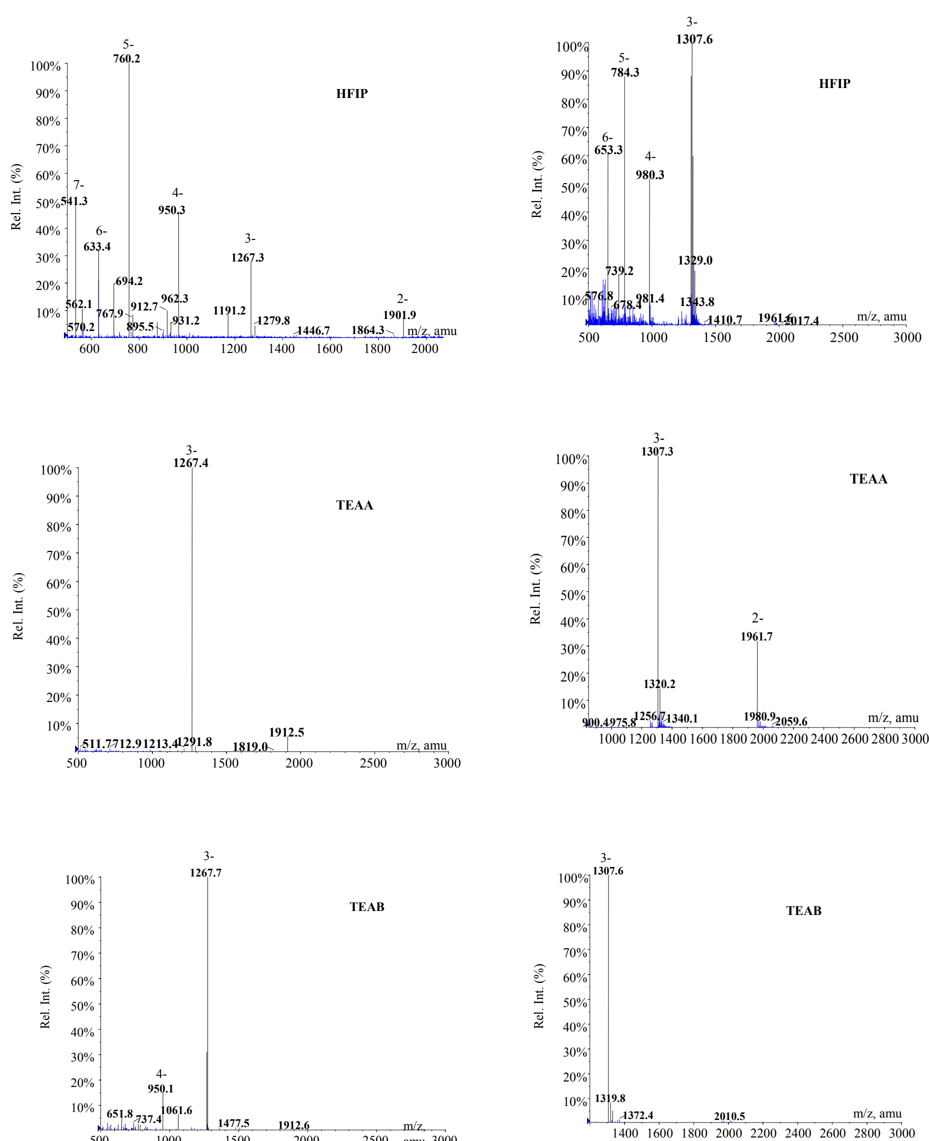

Figure 3. Charge-state distribution of T-rich and G-rich oligonucleotides in three mobile phases on IP-RP-HPLC-ESI-MS.The left is G-rich sample : 5′-GGGGATGGGGAT-3′ and the right is T-rich sample: 5′-TTTTATTTGTTTT-3′.

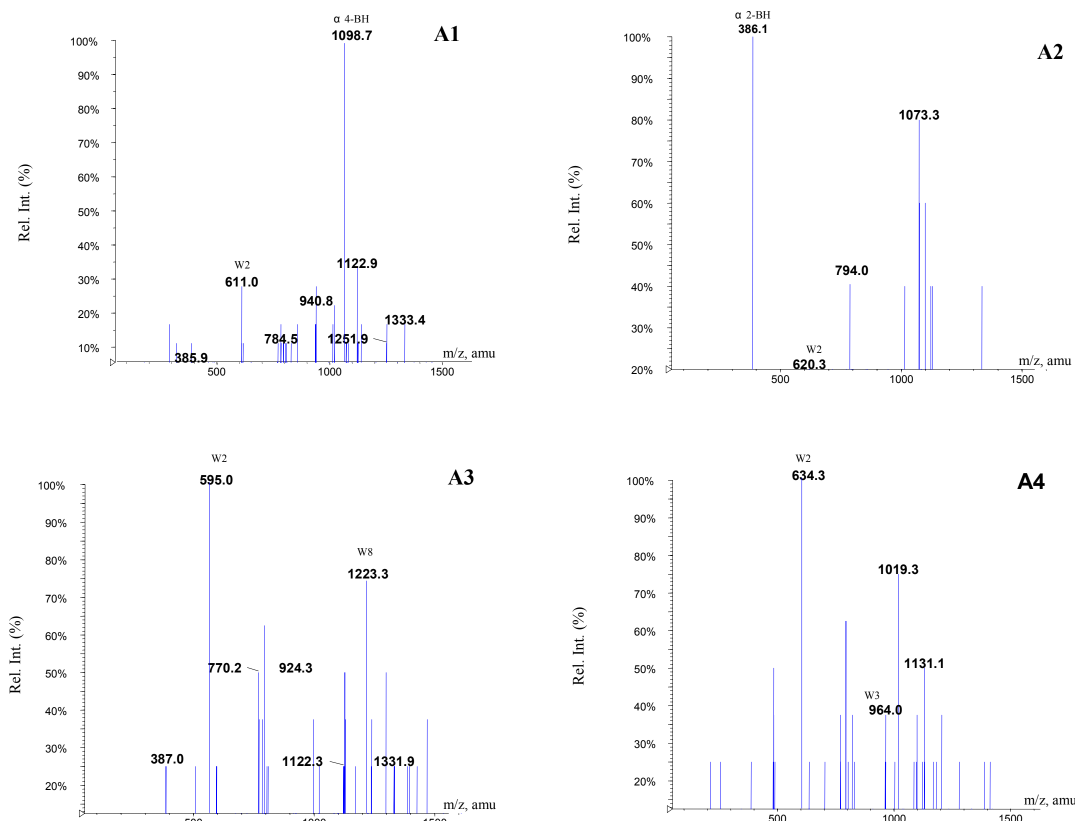

Figure 4. MS/MS spectra of oligonucleotides A1–A4 in HFIP buffer system. A1: 5′-CGTAGCTGATCGTTCAAGCT-3′; A2: 5′-CGTAGCT-GATCGTTCAAGCA-3′; A3: 5′-CGTAGCTGATCGTTCAAGCC-3′; A4: 5′-CGTAGCTGATCGTTCAAGCG-3′.

showed that the TIC intensity of T-rich oligonucleotides was much higher than that of G-rich oligonucleotides in all mobile phases and Figure 2 shows the TIC intensities of G-rich and T-rich oligonucleotides in the TEAA mobile phase system. Other G-rich and T-rich oligonucleotides (C1–4 D1–4) were further investigated and similar results were obtained (data not shown). Since, among the four bases, G has weakest hydrophobicity and T has the strongest hydrophobicity, the TIC intensity of G-rich oligonucleotide was lower than that of T-rich oligonucleotide.

The mobile-phase properties not only influenced chromatographic retention time and the ESI-MS response of oligonucleotides, but also affected the charge-state distribution. Figure 3 shows the charge-state of G-rich and T-rich

Table 3. The fragments of G-rich oligonucleotide (C4) at low collision energy.

Ion type	*m/z*	3-	4-	5-	6-
		Relative abundance			
T	125.3	nd	nd	1.5	4.7
G	149.6	nd	nd	4.8	16
α2-G	425.4	nd	39.3	7.4	1.4
W2	624.4	45.4	21.4	4.8	8.1
W12 6-	640.8	nd	53.6	35.4	10.9
α3-G	755.1	53.9	42.9	21.4	14.4
α4-G	1082	38.5	39.3	23.2	8.6
α9-G	1326.6	30.8	25.0	1.1	0.3
W5	1610.1	38.5	17.9	nd	nd

nd: not detected

Table 4. The fragments of T-rich oligonucleotide (D1) at low collision energy

Ion type	*m/z*	2-	3-	4-
		Relative abundance		
T	125.3	nd	92.8	99
G	149.6	nd	7.1	4.8
α2-A	400.4	nd	2.0	7.4
W2	609.2	27.9	21.4	10.1
W7 3-	712.3	nd	7.1	23.2
W6 2-	917.6	42.7	28.6	4.2
α4-C	1017.7	24.2	14.3	1.6
α6-G	1611.5	8.4	nd	nd

nd: not detected

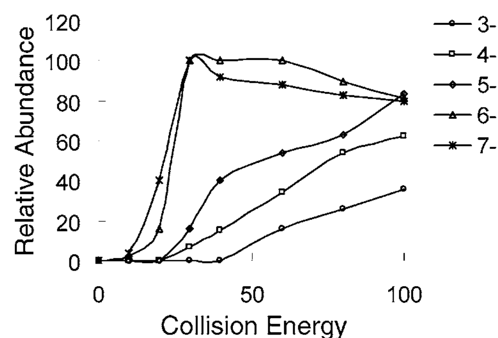

Figure 5. The relative abundance of G from different charge-state oligonucleotide C4: 5′-GGGGTTTGGGGGTT-3′ at different collision energy.

oligonucleotides eluted by three buffer systems, respectively. It can be observed that the lower charge state of 2- was predominant in the TEAA and TEAB buffer systems whereas, in the HFIP buffer system, the charge-state shifted to much higher, mainly to 5-, 6- and 7-, i.e. the HFIP buffer system can produce more abundant multiple charge-state fragments than the other two buffer systems, which agreed with Cheng *et al.*[28]

Although only different at the 3′ end, it is surprising to find that the fragmentation model of oligonucleotide A was much different in these three mobile phases. The product ion spectra of oligonucleotides A1–A4 are given in Figure 4. It is easy to find out the initial w series fragments due to the 3′ end base so that the oligonucleotides, different at the 3′ end base, could be distinguished from the spectra of product ions. Oligonucleotides with A and T bases at the 3′ end will form the α-BH series fragments while w_2 will be formed easily with C and G bases at the 3′ end. In addition, fragments of these oligonucleotides are also easily recognized by changing the collision-induced dissociation (CID) conditions (data not shown). So the product ions scan and the mass spectrum of the CID could be used to identify this type of oligonucleotide. For the three mobile phases, the product ion spectrum had approximate fragment types, indicating that the mechanism for the product ion formation was not significantly affected by the mobile phase.

The multiply-charged nature of the parent ion introduces complexities in the mass spectrum of the product ions,[29] so we compared the product ion spectra of G-rich and T-rich oligonucleotides with different charge states in order to understand which charge state is the compatible charge state for forming the sequence fragment. Tables 3 and 4 give the details of the fragments produced by different charge states. From the tables, we can see that the base ions will be produced easily at higher charge states of both G-rich and T-rich oligonucleotides, whereas the sequence fragment will be formed easily at the lower charge state. The reason for this may be that the energy surface and energy for break-up of the proton-bound intermediate is higher with higher charge state oligonucleotides.[29] As in the case of the charge state, the collision energy also affected the fragmentations of both G-rich and T-rich oligonucleotides. The relative abundance of the base increased with the increase in collision energy (Figure 5) and the single base fragment from the higher charge state by the lower collision energy in G-rich oligonucleotide was much higher than that of the lower charge state. We also observed that the relative abundance of G in G-rich oligonucleotides decreased slightly with the increase of collision energy at higher charge states (6- and 7-), which may be due to the fact that the T base was formed by increasing the collision energy in the product ion in this sample. This phenomenon indicated that lower collision energy was needed to produce fragment ions from the higher charge-state oligonucleotides than those from the lower charge state. Similar results were obtained in the case of T-rich oligonucleotides in our experiments (data not shown). In addition, the 5′ end base fragment in G-rich and T-rich oligonucleotides was found to form easily in the product ion spectra at higher collision energy (spectra not shown), which agreed with the results of Phillips and McCloskey[30] and will be helpful for the analysis of unknown oligonucleotides.

Conclusions

The behavior of G-rich and T-rich oligonucleotides on IP-RP-HPLC/ESI-MS was investigated with three mobile phases, including the TEAA, TEAB and HFIP buffer systems. The first elution was the G-rich oligonucleotide while the last elution was T-rich oligonucleotides in the TEAA and TEAB buffer systems; however, in the HFIP buffer system, the retention time was affected more significantly by the chain length. On the other hand, TIC intensities of G-rich and T-rich oligonucleotides in the HFIP buffer system were much higher than those in the TEAA and TEAB buffer systems and the TIC intensities of T-rich oligonucleotides were much

higher than that of G-rich oligonucleotides in all mobile phases. Much higher charge-state fragments were observed in the HFIP buffer system than those in the case of the TEAA and TEAB buffer systems. The abundance of product ions was significantly affected by both the charge-state of the oligonucleotide and collision energy.

Acknowledgements

This research was supported by a grant from NSFC (No. 40332024) and GIGCX-03-030311106.

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Received: 21 May 2004
Revised: 18 June 2004
Accepted: 22 June 2004
Web Publication: 27 September 2004