TIDES 2004

Low, Medium and High-Throughput LC-MS Methods for the Analysis of Oligonucleotides

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Overview

- METHOD 1: LC-MS characterization of therapeutic oligonucleotides in 15-30 minutes.
- METHOD 2: LC-UV quality control of oligonucleotides (N-1 resolution) in 5 minutes.
- METHOD 3: LC-MS quality control of 10-110mer oligonucleotides in 1.5 minutes.

Introduction

Three methods using liquid chromatography with mass spectrometry (LC-MS) or ultra-violet (UV) detection have been developed for the analysis of oligonucleotides.

METHOD 1 is suitable for sensitive characterization of therapeutic oligonucleotides (*Gilar et al., Oligonucleotides, vol.13, p.229, 2003; Fountain et al., Rapid Commun. Mass Spectrom, vol.17, p.646, 2003*). Phosphorothioate, 2'-O-Me, and G-rich oligonucleotides can be easily analyzed (**Figure 1**).

METHOD 2 was designed for the fast quality control (QC) of synthetic oligonucleotides by LC-UV (*Fountain et al., LC-GC North America Supplement, February, p. 26, 2004*). Oligonucleotides are separated in less than 4 minutes (<30mer) with good resolution of the target product from N – 1 impurities (**Figures 2, 3, and 4**).

METHOD 3 utilizes LC for on-line oligonucleotide desalting prior to electrospray ionization (ESI) MS analysis (*Fountain et al., Rapid Commun. Spectrom., accepted for publication*). High throughput QC of synthetic oligonucleotides up to 110mer was performed with a duty cycle of 1.5 minutes (**Figures 5 and 6; Table 1**). Analysis of approximately 950 samples per 24-hour time period is possible. This method was also applicable for analysis of PCR products and single nucleotide polymorphism (SNP) genotyping fragments .

Experimental

HPLC Systems: METHODS 1 & 3: Waters Alliance® HT Separations Module (Milford, MA)
METHOD 2: Waters CapLC™ Separations Module

Columns: METHOD 1: XTerra® MS C_{18}, 50 × 1.0 mm, 2.5 µm
METHOD 2: XTerra® Intelligent Speed (IS™) MS C_{18}, 20 × 4.6 mm, 2.5 µm
METHOD 3: XTerra® MS C_{18}, 10 × 2.1 mm, 3.5 µm (guard column)

Flow Rate: METHOD 1: 23.6 µL/min.; METHOD 2: 1.0 mL/min.; METHOD 3: see Figure 5.

Mobile phases: METHOD 1: A: TEA/HFIP (16.3 mM/400 mM), pH 7.9; B: 30 % MeOH in TEA/HFIP (16.3 mM/400 mM), pH 7.9
METHOD 2: 5% MeOH in TEA/HFIP (16.3 mM/400 mM), pH 7.9; B: 30 % MeOH in TEA/HFIP (16.3 mM/400 mM), pH 7.9
METHOD 3: Load/wash buffer consisted of 5% acetonitrile and 95% 5 mM dimethylbutylammonium acetate (DMBAA), pH 7. Elution mobile phase consisted of 25 % acetonitrile and 75 % 5 mM DMBAA, pH 7.

MS: METHODS 1 & 3: An orthogonal ESI-TOF mass spectrometer (Micromass® LCT™, Waters) was used for analysis of all oligonucleotides eluting from the HPLC systems. The system was operated by the manufacturer's software. Raw spectra were deconvoluted using the MaxEnt1™ option.

UV detection: METHODS 1 & 3: Waters 996 PDA detector
METHOD 2: Waters® 2487 Dual λ Absorbance Detector; 254 nm

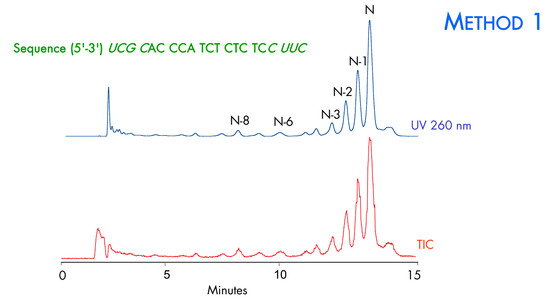
Buffer prep.: METHODS 1 & 2: Dissolve 41.5 mL of HFIP (hexafluoroisopropanol) in ~ 950 mL of water. While mixing vigorously, add 2.3 mL of TEA (triethylamine). Adjust final volume to 1 L with water. The pH of the solution should be about 7.9.

Samples: METHOD 1: The 21mer PS with 2'-O-methyl-modified terminal nucleotides (four from each end) was provided by Hybridon, Inc. (Cambridge, MA).
METHODS 2 & 3: Oligonucleotides were provided by Midland Certified Reagents (Midland, TX), One Trick Pony (Ramona, CA), IDT (Coralville, IA), Qiagen (Valencia, CA), and Sigma-Genosys (The Woodlands, TX).

Other: HPLC and MS conditions such as temperature, gradient slope, injection volume (mass), oligonucleotide sequences, and MS conditions can be found in the figure captions.

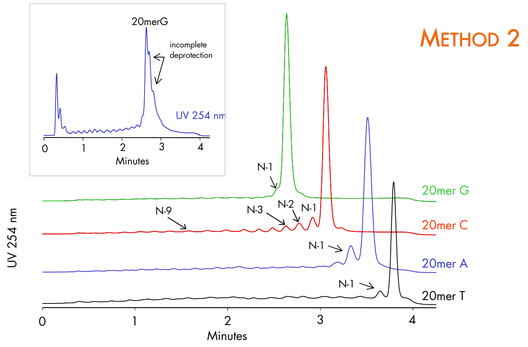
Results and Discussion

Figure 1: LC-MS analysis of a 21mer phosphorothioate (PS) oligonucleotide with 4 x 4 2'-O-methyl termini. 3' truncated metabolites were generated by *in vitro* enzymatic digestion. Baseline resolution of the target (N) from N-1, 2, 3, etc. metabolites was achieved. Italicized nucleotides are 2'-O-methylated.



Conditions: XTerra® MS C_{18}, 50 × 1.0 mm, 2.5 µm column. 60 °C, 23.6 µL/minute. Mobile phase A: 16.3 mM TEA, 400 mM HFIP, pH 7.9. Mobile phase B: 30% MeOH in buffer A. Gradient from 53.3% - 73.3% B in 6 minutes (1% MeOH per minute) followed by 73.3% - 93.3% in 24 minutes (0.25% MeOH per minute). Injection load was 67 pmole (0.45 µg).

Figure 2: Separation of the four different 20mer homooligonucleotides by ion-pair reversed-phase HPLC. The short column (20 mm) allows for this separation in under 5 minutes for each oligonucleotide. Separation of closely eluting impurities (i.e. N-1, 2, 3, etc.) from the target oligonucleotide was routinely achieved. The figure inset shows an example of a poor synthesis of 20merG, and the ability of the method to separate incomplete deprotection products from the main peak.



Conditions: XTerra® MS C_{18}, 20 × 4.6 mm, 2.5 µm column. 60 °C, 1.0 mL/minute. Mobile phase A: 5% MeOH in 16.3 mM TEA, 400 mM HFIP, pH 7.9. Mobile phase B: 30% MeOH 16.3 mM TEA, 400 mM HFIP, pH 7.9. Gradient from 0-60% B in 4.25 minutes; gradient profile #4 (concave gradient). Injection volume was 2 µL (200 pmole mass load).

Figure 3: Quantification of impurities in a 20mer oligodeoxycytidine ($(dC)_{20}$) synthesis. Since detection is performed by UV, the amount of impurities in each synthesis is calculated as an area % at 254 nm. Routine detection of 0.5 % (~ 5 pmoles) of failed products in the target synthesis was achieved. Conditions identical to Figure 2.

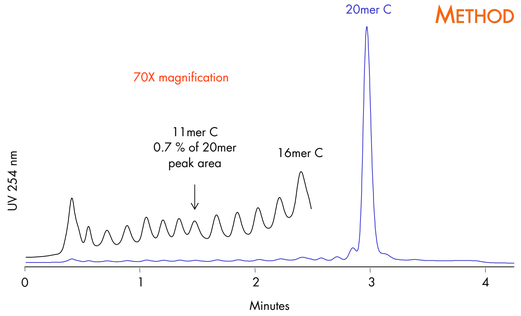
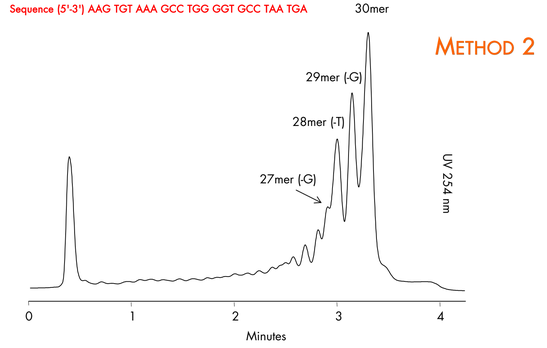


Figure 4: Separation of a 30mer mixed sequence oligonucleotide that was digested with 3' exonuclease to generate "failure sequences". Adequate resolution of N-x impurities is routinely achieved.



Conditions: XTerra® MS C_{18}, 20 × 4.6 mm, 2.5 µm column. 60 °C, 1.0 mL/minute. Mobile phase A: 5% MeOH in 16.3 mM TEA, 400 mM HFIP, pH 7.9. Mobile phase B: 30% MeOH 16.3 mM TEA, 400 mM HFIP, pH 7.9. Gradient from 0-65.8% B in 4.25 minutes; gradient profile #4 (concave gradient). Injection volume was 2 µL (200 pmole mass load).

Figure 5: Configuration of switching valve for on-line desalting of oligonucleotides

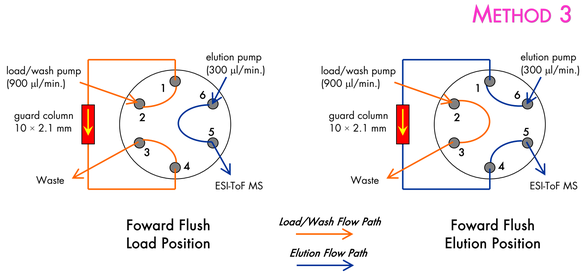
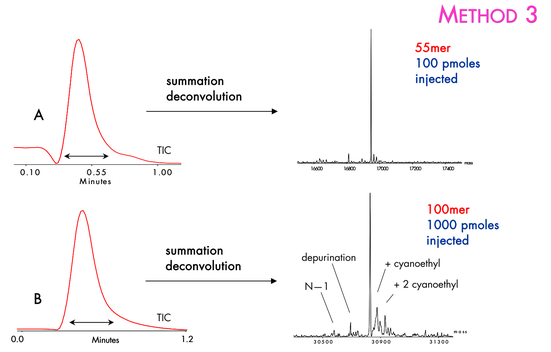


Figure 6: Rapid LC-MS analysis of a synthetic (A) 55mer and (B) 100mer oligonucleotide. Alkali cation adduction is minimized, giving a clear and easily interpreted mass spectrum. Notice the identification and characterization of synthesis impurities and incomplete deprotection products by ESI-MS.



Conclusions

- Three different methods were developed for the separation and characterization of native and chemically modified oligonucleotides.
- METHOD 1 is well suited for sensitive analysis of antisense oligonucleotides, G-rich oligonucleotides, and oligonucleotides greater than 50 bases in length.
- METHOD 2 was utilized for routine QC of synthetic oligonucleotides. The 5 minute duty cycle per sample allows for fast analysis of DNA synthesizer performance.
- METHOD 3 is a high throughput solution for the analysis of oligonucleotides up to 110mer in length, and DNA genotyping fragments.