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LIQUID CHROMATOGRAPHY MASS SPECTROMETRY OF ANTISENSE OLIGONUCLEOTIDES

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Abstract: The development of rapid, simple and accurate methods for characterizing and insuring the integrity of oligonucleotides is important for the development of therapeutic and diagnostic products. This study was performed to determine the utility of high performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry detection (LCMS) as an alternative or addition to the current techniques of HPLC, capillary electrophoresis and gel electrophoresis.

Introduction

The therapeutic use of oligonucleotides will require a high level of quality control to insure sample integrity and the identification of minor impurities. High-performance liquid chromatography (HPLC) provides some information on the purity of oligonucleotide preparations, but the length, homogeneity, base composition, and sequence must be verified by other methods. In this study, we have combined the separation capability of liquid chromatography (LC) and the characterization advantages of mass spectrometry (MS) to perform LCMS on oligos. Three types of oligodeoxyribonucleotides were studied: those with a phosphodiester backbone, a phosphorothioate backbone, and a methylphosphonate backbone. Increasing interest in modified synthetic oligonucleotides that perform poorly on reverse-phase HPLC and whose structural integrity must be verified has heightened the need for an analytical method that can provide information on purity and structure. Here we report the use of LCMS to distinguish oligonucleotides differing by as little as one base. Also demonstrated is the ability to detect contaminating species, at a 1% molar ratio, that coelute on reverse-phase HPLC.

Oligonucleotides containing 100 and more nucleosides are routinely assembled using automated solid phase DNA synthesis[1,2]. This technique is normally highly efficient, having stepwise coupling yields of nucleoside phosphoramidite monomers of 99%, however, even with such efficient coupling reactions the theoretical yield for a 20mer is only 81.8%. Errors in synthesis can also occur, resulting in a further decrease in the overall yield. For example, branching of the oligonucleotide chain, most frequently at the N-6 of adenosine and the O-6 of guanosine[3], results in molecules with higher molecular weight. Furthermore, low molecular weight impurities can arise through the initiation of chain synthesis at reactive sites on the solid support or from failure sequences that are capped, blocking further chain elongation. Synthetic oligonucleotides therefore need to

be separated from a large number of side products. Sometimes this can be difficult since the impurities frequently coelute on HPLC, making their detection and removal troublesome. Purification and characterization methods for oligonucleotides include HPLC, PAGE, capillary electrophoresis and dimethoxytrityl oligonucleotide purification cartridges. The usefulness of these methods is well studied[4,5,6], but they cannot unequivocally identify oligonucleotide composition or impurities. The use of mass spectrometry in the analysis of oligonucleotides[7,8,9] and the more recent application of HPLC and electrospray mass spectrometry to oligonucleotides[10] are promising approaches which can provide helpful information to verify base composition. Another reason LCMS is useful is that direct mass spectral analysis of unpurified oligonucleotides can give poor signals due to contaminating salts which form cation adducts with the highly polar oligonucleotides. LC serves to remove these salts and other contaminants prior to MS. For instance, a recent report by Reddy et al.[8] used a double-pass HPLC procedure and repeated dryings to purify the oligonucleotides for mass analysis. The LCMS method we report here allows for the simple and rapid analysis of oligonucleotides in one step.

Materials and Methods

The oligonucleotides were synthesized on ABI 380B DNA synthesizers (Applied Biosystems Inc., Foster City, CA.) using chemicals and protocol provided by the manufacturer. Samples were cleaved from the solid support with 30% ammonium hydroxide, deprotected by heating at 55 ^{o}C overnight followed by ethanol precipitation. HPLC was performed on an Ultra Micro Protein Analyzer (Michrom BioResouces, Inc., Auburn, CA.) with a flow rate of 50 μL min^{-1}. The buffer system consisted of acetonitrile/200 mM diisopropylethylamine/water, pH adjusted with acetic acid to 7.2. Buffer A and B were composed of 2/10/88 and 90/10/0 (v/v) respectively, used in conjunction with a PLRP-S 5μ 100 Å column, 0.5 X 150 mm (Michrom BioResources Inc.). The pneumatically-assisted electrospray experiments were performed on an API III triple-quadrupole mass spectrometer (Perkin Elmer SCIEX) with an upper limit of *m/z* 2400. Spectra were acquired in the negative mode with a step size of 1 amu and a dwell time of 2 ms step^{-1}. Molecular masses of the oligonucleotides were determined using the Sciex Hypermass program (Mass Spec 3.1). The LCMS connection was made with a splitless 1 m teflon tube (150 μm ID). Approximately 7 nanomoles of each oligonucleotide was used in each experiment.

Results and Discussion

The first LCMS experiment was performed on standard oligos that typically can be separated by reverse-phase liquid chromatography. This study was performed on a mixture of oligonucleotides, two 18mers and a 17mer, with standard phosphodiester bonds. Their sequences were: A 5'-ATCTTTCCAAGGCAAGG-3' (M_r = 5188), B 5'-TGGAGAAGGGAGAAGAAC-3 (M_r = 5670), and C 5'-GTGACCATCAAATTCCTT-3' (M_r = 5433). A gradient of 16% B to 24% B over 30 minutes was used. The separation obtained in the UV trace was approximately 60%, however, the LCMS data improved our ability to distinguish the individual oligonucleotides (Figure 1). The multiply charged ions (3-, 4-, and 5- species) were used to reconstruct the molecular weight of each oligonucleotide with an average error of 0.02%.

The value of LCMS is not limited to oligonucleotides that can be separated by reverse-phase HPLC. For instance, modified oligos can present a particular problem when performing LC separations, however the LCMS interface offers discriminating information.

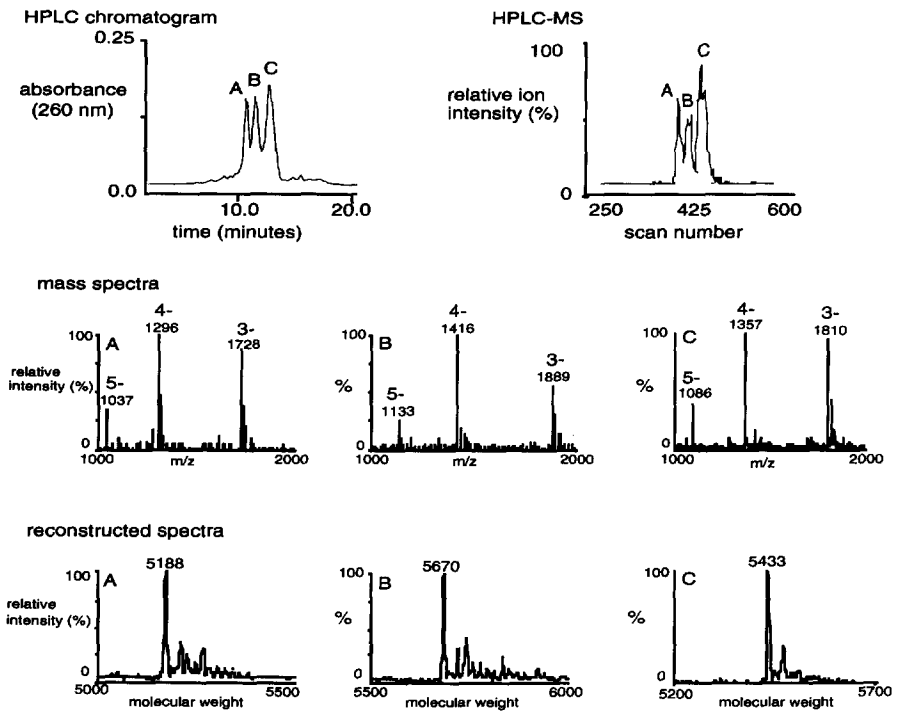

Figure 1. LCMS of three oligonucleotides containing the standard phosphodiester backbone. UV chromatogram from the HPLC detector and extracted ion chromatogram from the mass spectrometer, m/z range 1000-2000 (top). Electrospray mass spectra of peaks A, B and C (middle). Multiply charged negative ions representing negative charge states 3,4, and 5. Reconstructed molecular weight spectra of each oligonucleotide (bottom).

An important oligo modification for antisense drug design is one in which the phosphodiester bond has been modified by replacing a non-bridging oxygen with a methyl group. This chemical modification allows oligonucleotides to passively diffuse through cell membranes[11]. Because of the intended therapeutic use of antisense oligonucleotides, assurance of structure and purity of these modified oligos are very important. Reverse-phase HPLC data alone gives little structural or purity information because the broad peaks generated are typically not well resolved. The methylphosphonate oligonucleotides that were used for this study had a mixture of normal and modified backbones (approximately 50:50). The oligonucleotides had the following sequences

with x indicating bases linked by a methylphosphonate bond and p for phosphodiester links: A 5'-CxAxTpCxTpTxCpCxTpCxGpTx-3' (M_r = 3536) and B 5'-TxAxGpCxTpTxCpCxTpTxApGxCpTxCpCxTxGx-3' (M_r = 5399). The gradient used on the HPLC was 10% B to 20% B over 30 minutes. While the UV chromatogram of this mixture shows almost no separation (Figure 2), the combination of HPLC with mass spectrometry overcomes this problem because each time point (scan) contains information on the entire mass range. Oligonucleotides that are not separated may be distinguished when selected ions are monitored. For example, this LCMS experiment shows that by selecting ions corresponding to the different oligonucleotides, it is possible to distinguish and identify them (Figure 2). Mass spectral data of the multiply charged ions were used to obtain a molecular weight spectrum of each oligonucleotide with a mass accuracy of 0.03% and 0.04%, respectively. The relatively high error is due to the 1 amu step size used in these experiments which allowed for rapid scanning, but reduced the accuracy, accordingly.

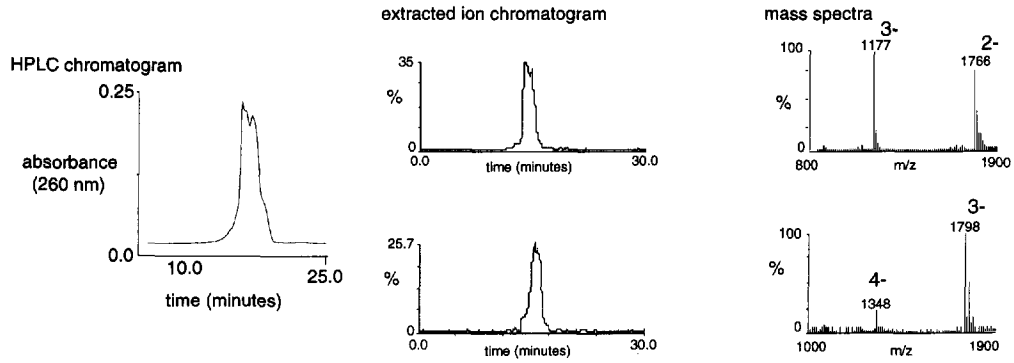

Figure 2. LCMS of two oligonucleotides (M_r = 3536 and 5339) containing a mixture of phosphodiester and methylphosphonate bonds. UV chromatogram from the HPLC detector (left). Extracted ion chromatograms using a single ion (-3 species) specific to each oligonucleotide (middle), and electrospray mass spectra (right).

The third class of oligonucleotides studied with LCMS were phosphorothioate deoxyribonucleic acids. These analogs contain a modified phosphodiester bond in which a sulfur has been substituted in place of an oxygen atom. Phosphorothioate DNA is less susceptible to cleavage by endonucleases and is therefore more stable inside cells, facilitating its use in antisense therapy[12]. As with the methylphosphonates, reverse-phase HPLC does not typically separate phosphorothioate oligonucleotides. Three phosphorothioate oligonucleotides representing n, n-1 and n-2 species were used in these experiments: A 5'- CCTGCTCCCCCCTGGCTCCC-3' (M_r = 6207), B 5'-CCGCTCCCCCCTGGCTCCC-3' (M_r = 5887) and C 5'-CCTGCTCCCCCTGGCTCC-3' (M_r = 5597). The UV and selected ion chromatograms were similar to the previous experiment (Figure 2) where these individual oligonucleotides were indistinguishable. The multiply charged ions were used to obtain the

molecular weight of each oligonucleotide (Figure 3). This study demonstrated the ability to determine the individual compounds from a complex unresolvable mixture of phosphorothioate oligonucleotides.

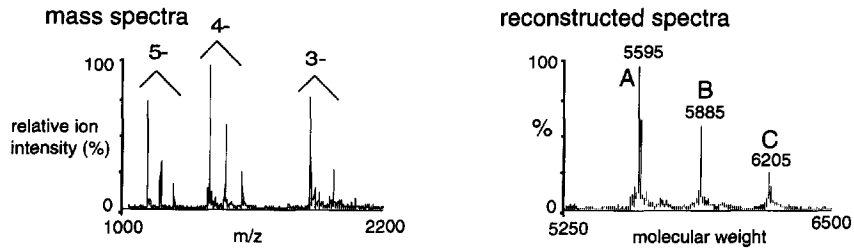

Figure 3. LC mass spectral data obtained from three phosphorothioate oligonucleotides which co-eluted off of the LC. Mass spectrum with multiply charged ions for each oligonucleotide (left) and reconstructed molecular weight spectrum (right) revealed the three oligonucleotides.

In addition to identifying major reaction products, the detection and characterization of minor contaminants is also important for oligonucleotides used as therapeutic agents. Because of the high yield of each synthetic step and efficient purification methods, contaminants such as incomplete and branched oligonucleotides are present only at low concentrations[3]. To test the ability of LCMS at detecting contaminants, we spiked phosphorothioate oligonucleotide C of the previous experiment (Figure 3) with a 1% molar ratio of oligonucleotide B, 2.3 nanomoles and 0.023 nanomoles, respectively. The presence of the minor component next to the larger signal of the major component is visible both in the multiply charged spectra and the reconstructed spectra (Figure 4), and further demonstrates the viability of this technique for detecting minor reaction products. Ion intensity should not be used to quantitate relative concentrations without prior knowledge of each components response factor.

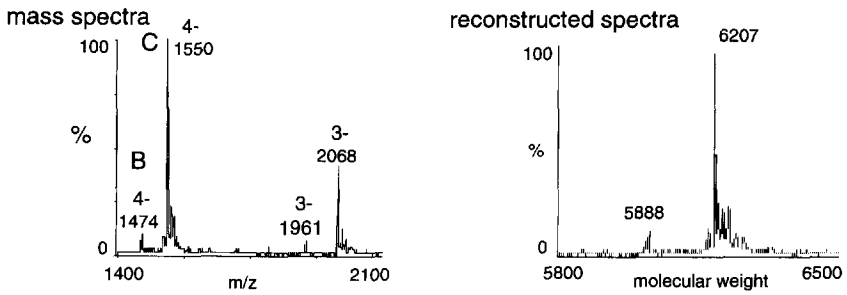

Figure 4. LCMS of two phosphorothioates at a molar ratio of 100:1. Electrospray mass spectrum (left) and reconstructed molecular weight spectrum (right) reveal the presence of the minor component M_r = 5888 (0.023 nanomoles) and the major component M_r = 6207(2.3 nanomoles).

Electrospray mass spectrometry has emerged as a powerful tool in the characterization of oligonucleotides. The combination of HPLC with ESI has previously demonstrated its utility in the analysis of small molecules, peptides, and proteins and with this study we have demonstrated the feasibility of directly coupling HPLC and ESI in the analysis of antisense oligonucleotides. In addition, the entire procedure for LCMS requires approximately 30 minutes, making it a rapid method of analysis that does not require sample preparation beyond deprotection and ethanol precipitation. Our results indicate that only 0.3% of a 0.2 micromole scale synthesis is sufficient for analysis, therefore eliminating the necessity to upscale the synthesis for analytical purposes.

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