NOVEL STRATEGIES FOR BIOANALYSIS OF THERAPEUTIC OLIGONUCLEOTIDES

by

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(Under the Direction of Michael G. Bartlett)

ABSTRACT

Oligonucleotide therapeutics have emerged as a promising class of drugs to treat a wide range of diseases caused by genetic abnormalities. They have also become indispensable tools for genomic studies allowing for specific knockdown of proteins to study signaling pathways and identify therapeutic targets. Two DNA therapeutics, Fomivirsen and Pegaptanib, have been approved by the US FDA. Replacement of the phosphodiester linkage with a phosphorothioate is one of the most successful modifications made to oligonucleotides to enhance their in vivo stability. The longer elimination phase of phosphorothioates and other modified oligonucleotides requires sensitive and selective methods to quantify the parent drug and their metabolites simultaneously. Traditional hybridization assays are highly sensitive but does not provide selectivity between the parent drug and the metabolites. LC-MS based assay is a promising approach for bioanalysis of oligonucleotide therapeutics due to its selectivity and structure identification capability. However, the lack of sensitivity and complicated sample preparation procedure remains the bottle neck for application of LC-MS based assays to preclinical and clinical studies. Chapter 1 is the introduction and describes the layout of the dissertation. Chapter 2 reviews the literature for analysis of oligonucleotides using LC-MS based methods. Numerous studies have shown that the mobile phase composition has a significant impact on the MS

sensitivity of oligonucleotides. In Chapter 3, various ion-pairing agents and counter-ions were evaluated for their effect on electrospray desorption efficiency of oligonucleotides and a novel model of electrospray behavior of oligonucleotides was proposed. The biological sample extraction remains a formidable challenge in developing quantitative LC-MS methods for oligonucleotides. In Chapter 4, a one-step solid phase extraction (SPE) method was developed to retrieve an oligonucleotide from rat plasma. Various issues such as non-specific binding and oxidation of the phosphorothioate linkages were addressed. UHPLC has the potential of greatly increasing the throughput of bioanalysis of oligonucleotide. However, the extensive sample-clean up required for operation under high system pressure and the low flow rate required for sensitive mass spectrometric detection of oligonucleotide poses major hurdles for transferring HPLC-MS methods to UHPLC platforms. In Chapter 5, an ethanol precipitation step was added to the commonly used phenol-chloroform extraction of oligonucleotide and resulted in minimal residual substances in the extracted samples. Other issues such as selecting the optimum organic modifiers, column dimensions, flow rate and scan rate for best chromatographic separation and LC-MS sensitivity of oligonucleotides were also addressed.

INDEX WORDS: Ultra high performance liquid chromatography, mass spectrometry, LC-MS/MS, solid phase extraction, liquid-liquid extraction, oligonucleotide, quantitation

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DEDICATION

I would like to dedicate this dissertation to my father Dr. Guanhua Chen. He taught me perseverance and composure when facing frustrations in scientific research. I must also thank my mother for giving me the strength and faith to succeed. Last but not least, I would like to thank my boyfriend Bo, for spending the last four Christmases with me in the lab and standing by me through the countless cycles of my mood swings between frustrations and successes.

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TABLE OF CONTENTS

Page

Chapter 1

Introduction

Since the approval of the first antisense drug Fomivirsen in 1998, tremendous efforts have been made to develop a sensitive and selective bioanalytical method for oligonucleotide therapeutics that would meet regulatory requirements. A variety of techniques have been used for preclinical and clinical studies of oligonucleotides. Radioactivity and hybridization methods have the advantage of ultra-high sensitivity but lack selectivity between the parent drug and the metabolites. qPCR assays are also extremely sensitive but developing a primer that specifically anneals with the parent drug can be problematic. Capillary electrophoresis methods provide superior separation between the parent drug and its metabolites. The electrokinetic injection allows small sample amounts to be enriched prior to separation. However, the method robustness and extensive sample clean-up required to prevent injection discrimination between oligonucleotide and anionic matrix substances pose formidable challenges for application in preclinical and clinical studies. LC-MS has been considered a promising technique due to its robustness, specificity and structural characterization capability. However, LC-MS has suffered from a lack of sensitivity and has not been able to capture the terminal elimination phase of oligonucleotide therapeutics for pharmacokinetic studies. In addition, biological sample extraction remains a major challenge in developing quantitative LC-MS methods for oligonucleotides. Chapter 2 reviews the recent LC-MS based assays for oligonucleotides and their application in bioanalysis.

Various strategies has been attempted to enhance the ionization efficiency and detection sensitivity of oligonucleotides by ESI-MS. Altering the chemical composition of the mobile phase was reported to have a significant impact on the ionization efficiency of oligonucleotide by numerous studies. In Chapter 3, the effect of different organic modifiers on the electrospray desorption efficiency of oligonucleotide and the corresponding mechanisms were investigated.

Another major hurdle for LC-MS methods is the extensive sample clean-up procedures required to achieve the necessary reproducibility and recovery needed for quantitation. Due to the extensive protein and non-specific binding, traditional solid phase extraction (SPE) and liquid-liquid extraction (LLE) were reported to have unsatisfactory recoveries. Assays with higher recoveries involve multiple steps of sample extraction and transferring, which could become challenging when applied in large-scale studies. Chapter 4 reported a one-step weak anion exchange (WAX) solid phase extraction (SPE) assay for the bioanalysis of a model oligonucleotide. Chapter 5 reported a phenol-chloroform liquid liquid extraction (LLE) method followed by ethanol precipitation. Chapter 5 also reported the systematic optimization of LC-MS conditions to enhance the sensitivity of bioanalytical assays for oligonucleotides.

Chapter 2

Chromatographic Methods for the Determination of Therapeutic Oligonucleotides

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Abstract:

Both DNA and RNA are being explored for their therapeutic potential against a wide range of diseases. As these new drugs emerge, new demands arise for the analysis and quantitation of these biomolecules. Pharmacokinetic and pharmacodynamic analysis requirements for drug approval place enormous challenges on the methods for analyzing these therapeutics. This review will focus on bioanalytical methods for DNA antisense and aptamers as well as small-interfering RNA (siRNA) therapeutics. Chromatography methods employing ultraviolet (UV), fluorescence and mass spectrometric (MS) detection along with Matrix-Assisted Laser Desorption/Ionization (MALDI) will be covered. Sample preparation from biological matrices will be reviewed as well as metabolite analysis and identification. All of these techniques are important contributions toward oligonucleotide therapeutic development. They will also be important in microRNA (miRNA biomarker discovery and RNomics in general, as more non-coding RNAs are inevitably discovered.

Introduction:

Therapeutic oligonucleotides (oligos) have emerged as promising candidates for drug therapies for a wide range of diseases, including cancer, AIDS, Alzheimer's disease and cardiovascular disorders[1]. They have also become indispensible tools for genomic studies allowing for specific knockdown of proteins to study signaling pathways and identify therapeutic targets. Currently, there are two DNA therapeutics approved by the US FDA. Fomivirsen is an antisense oligonucleotide for the treatment of cytomegalovirus retinitis[2]. Pegaptanib is an aptamer for the treatment of neovascular age-related macular degeneration[3]. Table 1 shows the many DNA

aptamer and antisense drugs, as well siRNA therapeutics that have been or are currently being used in human clinical trials[4].

There are numerous challenges for analyzing DNA and RNA as therapeutics and as laboratory tools. Therapeutic oligonucleotides are generally 19 – 27 nucleotides (nt) long. They are characterized by their size (5 – 14 kDa) and high degree of negative charge. The IC_{50} is generally in the low nanomolar range (0.2 – 5 nM)[5]. Once inside a cell, they are powerful regulators of gene expression, but measuring these low levels requires sensitive, quantitative methods, especially for the determination of pharmacokinetic parameters. Sensitive methods are required to determine metabolism, toxicological profiles and terminal phase elimination for therapeutic use. Laboratory use of oligonucleotides requires methods capable of measuring intracellular levels of these biomolecules. Since the mechanism of action of many oligonucleotides requires delivery of a sense-antisense duplex, the measurement of both the individual strands and the duplex are needed. Furthering the complexity and challenge of these biomolecules is sample preparation in plasma, tissues and cells. Exceptional techniques are required to extract oligos from biological matrices, separate them from interfering compounds, and then quantify, and characterize them.

Microbiological and imaging methods are available for analyzing RNA and DNA. Although some of these methods are quite sensitive, they often cannot distinguish between the parent molecule and their metabolites. Importantly, these methods also cannot differentiate unmodified from the modified oligos that are widely used in the lab and the clinic. Because they cannot distinguish between modified or unmodified small oligos, they cannot duplicate or directly

measure whether these modifications remain intact as the molecules are metabolized. A thorough review of these methods, including immunoassays and qRT-PCR, has been previously reported and therefore will not be covered here[6]. This review will focus on bioanalytical methods to analyze RNA and DNA with an emphasis on therapeutic oligonucleotides. Specifically, sample extraction from biological matrices will be explored. Chromatographic separation methods with UV and fluorescence detection will be reviewed, as well as mass spectrometric and hyphenated methods involving combinations of these techniques. The mechanisms for DNA and RNA therapeutics will be discussed due to their relevance in selecting the most appropriate approach for analyzing particular oligonucleotides.

Mechanisms of Oligonucleotide Therapeutics:

The antisense DNA mechanism was first reported in 1978 by Zamecnik and Stephenson, who found that short complimentary DNA sequences could be used to suppress the expression of many disease-causing genes[7]. Antisense DNAs bind with the complimentary mRNA sequence following entry in to the cell and translocation to the nucleus (Figure 1). The antisense DNA/mRNA duplex activates the nuclear enzyme RNase H, which then degrades the mRNA strand. The recognition site of RNase H is still not well understood, but DNA oligonucleotides as short as tetramers have been shown to activate RNase H[8]. Modifications to DNA, especially to the sugar/phosphate backbone can alter the mechanism of action, especially if they shift the structure of the DNA from B-form to a more RNA-like A-form configuration. These modified oligonucleotides typically no longer recognize RNase H but instead directly compete with mRNA substrates for interaction with many of the proteins involved in protein translation. A secondary mechanism for many modified DNA oligonucleotides is splicing inhibition, which involves interference with the production of mature mRNAs. One specialized application for

oligonucleotides involves sequences containing unmethylated CpG motifs. These oligos have been shown to activate an immune response via stimulation of B cells and acceleration of monocyte maturation. This unexpected, highly sequence specific enhancement of the immune system was discovered during the development of antisense oligonucleotides. This mechanism leads to activation of natural killer (NK) cells, dendritic cells, macrophage and B cells and holds great promise for the treatment of many diseases and as vaccine adjuvants[9].

RNA therapeutics include siRNA and miRNA. Both use the RNA interference (RNAi) pathway, which is a natural defense mechanism against double stranded RNA (dsRNA)[10, 11]. This silencing mechanism inhibits protein synthesis by destroying messenger RNA (mRNA) before it can be translated. This mechanism of inhibition is sequence specific and highly selective[11].

Figure 2 shows the RNAi pathway[12, 13]. RNA enters the pathway when the protein, Dicer[14], cleaves it in to 21 - 25 nucleotide (nt) strands[15]. These small RNAs (siRNA or miRNA) are then incorporated into the RNA induced silencing complex (RISC)[16]. This is where most synthetic RNAi molecules enter the pathway. Synthetic siRNAs require phosphorylation of the 5' end to be active. This occurs through the action of Clp1 kinase[17]. Following phosphorylation of the 5' end, RISC binds to the 3' end of the guide strand on the thermodynamically least stable end of the double strand[18, 19]. The passenger strand is then unwound, cleaved and released leaving the guide strand bound to RISC [20-22]

Recognition of the target mRNA begins with a "seed" region that is 2 – 8 nt from the 5' end of the guide strand[23]. This seed region pairs with the target mRNA. If the siRNA or miRNA is

perfectly complementary, then the target mRNA will be cleaved between the 10th and 11th nt from the 5' end[24]. If it is not perfectly complementary then translational repression will occur through competition with proteins involved in translational elongation or termination[19, 25, 26].

Knowledge of the RNAi mechanism allows us to make some general conclusions about the specificity for analytical methods that determine siRNA or miRNAs. These molecules are introduced as dsRNA, but once they are incorporated in the RISC complex, they exist as the single guide strand and possibly cleavage products of the passenger strand. Thus methods to determine siRNA levels should optimally measure both the single and double stranded forms. Measuring intracellular levels of siRNA will further challenge analytical methods because of the low levels of dsRNA and single stranded RNA (ssRNA).

Modifications

Therapeutic DNA and RNA are routinely modified to enhance their stability against the ubiquitous nucleases found in plasma, tissues and cells[27]. The structures of the most common modifications are shown in Figure 3. Besides decreasing degradation, modifications can also decrease toxicity and increase potency by increasing the volume of distribution and enhancing the binding specificity[23, 27, 28]. RNAs are routinely modified at the 2' ribose position because it does not interfere with the A-form duplex structure that is necessary for activity[29]. Substitutions of this 2' hydroxyl group protect against nucleases and increase affinity for the target mRNA. These include 2'-O-methyl (2'-O-Me), O-ethyl (2'-O-ethyl), O-methoxyethyl (2'-MOE), and fluorine (2'-F) substitutions as well as locked nucleic acids (LNA). LNAs have a bridge connecting the 2' oxygen and the 4' carbon. They enhance base stacking and are used on the passenger strand of duplex RNAs to enhance the binding specificity of the guide strand in the

RISC complex[30] The most frequently used 2' modifications are the 2'-O-Me, and 2'-F substitutions[5, 31, 32]. The positions of 2' modifications are often made after testing the oligonucleotide to locate the most labile linkages.

Besides enhancing binding selectivity of the guide strand, 2'-O-Me modifications also dramatically increase the elimination half-life of oligonucleotides up to 30 days in plasma and tissues from a wide-variety of species. 2'-F modifications reduce the hydrophobicity of the oligonucleotide which aids with entry into the cell[27]. Both modifications increase binding affinity and stability against nucleases. In general, modifications of the 2' position of ribose decrease an oligonucleotides affinity for the enzyme RNase H, thus favoring the RNAi pathway over the antisense pathway.

Methyl ether and allyl ethers have been used to end-cap the 3'-overhangs of siRNAs to protect them against exonucleases[33, 34]. Interestingly, although these bulkier allyl groups do not work well for internal modifications of siRNA, they retain excellent activity when used in anti-miRNAs that are used to down-regulate endogenous miRNA[35].

Backbone modifications including phosphorothioate modification are also commonly used[36]. Besides increasing the hydrophobicity, these modifications also increase resistance to exonucleases and increase plasma protein binding to improve tissue distribution and prevent renal excretion[37]. Phosphorothioate modifications maintain the oligos DNA-like properties and are excellent substrates for the enzyme RNase H[38]. Phosphorothioate containing oligonucleotides also possess low protein binding for serum albumin[39]. Phosphorothioates are

rapidly absorbed and distributed following intravenous administration and they have a long elimination half-life (greater than 60 hours in humans)[40]. Their clearance is primarily due to metabolic degradation via several nucleases. Modifications to DNA or RNA bases are not widely used in therapeutics, but there is an example where they were shown to increase potency[29].

Sample Preparation:

Bioanalytical techniques begin with sample extraction from a biological matrix. Sample preparation is critical to separate oligos from interfering compounds. The challenge lies with maximizing recovery of the analyte while removing unwanted components. Included in these challenges are minimizing sample transfers, minimizing degradation, and avoiding non-specific binding to containers. RNA extractions add additional complexity because these analytes must be protected against RNases. This requires that all glassware, plastic ware, instrument tubing and reagents be RNase free.

One of the simplest approach is dilute and shoot. Using this approach, Chen et al. directly injected plasma and urine samples after diluting with 1% Nonidet P-40 in saline[41]. While the separation and detection limit (0.21μg/ml) were significantly compromised in this assay, the recovery of the oligonucleotide was reported to be 100%. Bourque and Cohen used an on-line method for preparation of a phosphorothioate DNA[42]. Plasma and urine samples were injected directly on to a strong anion exchange column. A lithium bromide mobile phase was used to wash the serum proteins from the samples and then elute the analyte. The analytes were then fraction collected for subsequent analysis. This method provided excellent linearity with a rapid 5 minute sample preparation time.

Several studies use a proteinase K digestion or methanol precipitation to remove the protein from the biological samples[41, 43-46]. These approaches avoid issues with the nonspecific and irreversible binding of oligonucleotide to either SPE cartridges or many containers. The recovery of oligonucleotides when using the proteinase K digestion with liquid-liquid extraction (LLE) has been reported to be as high as 98%[43, 44].

Many of the extraction methods employed in the literature involve phenol/chloroform extractions or modifications thereof [47-49]. These extractions can be time consuming and laborious, sometimes involving six or more steps and three sample transfers[44, 46, 50]. Other methods use solid-phase extraction (SPE) cartridges[51-53]. These significantly reduce sample preparation time, but can still involve multiple sample transfers and time-consuming evaporation steps. Most of the SPE methods employ similar buffer systems as those used in chromatographic separations. Gaus et al. employed a sample extraction strategy where biological samples were purified by anion-exchange HPLC following phenol-chloroform LLE[54]. Ion-exchange peaks were fraction collected followed by C18 SPE for desalting. This study was conducted together with a capillary gel electrophoresis (CGE) assay, therefore the extraction procedure was almost the same as those used in most CGE applications. Dai et al. proposed a much more straightforward sample clean-up procedure for a phosphorothioate DNA[52]. The biological samples were mixed with a loading buffer containing the ion-pair agent, triethylammonium bicarbonate (TEAB) and extracted using a C18 SPE cartridge. The proteins and salts were easily eluted with TEAB and water whereas oligonucleotides were retained on the cartridge due to their increased hydrophobicity and pseudo-neutral properties following ion-pairing with TEAB. The ammonium ion also shields the

oligonucleotide from binding with sodium and potassium ions. The recovery of this procedure is reported to be 43-64% depending on the oligonucleotide concentration. The break-through from the SPE cartridge was measured by UV and found to be negligible, indicating that irreversible binding to the SPE support may be the cause for the reduced recovery. A similar method using triethylammonium acetate (TEAA) as the ion-pair agent was employed for the quantitation of a liposome entrapped antisense DNA[55] .

The study of quantitation of phosphorothioate DNA by LC-MS conducted by Zhang et al. is by far the most extensive research on biological sample preparation and handling[56]. Various issues have been addressed in this study including chromatographic reproducibility, non-specific binding to containers, enhancing the recovery from a biological sample, and stabilizing the analyte during evaporation. The authors suggest preconditioning the LC column with repeated injections of the analyte to improve the chromatographic reproducibility at lower concentrations. Silanizing glass autosampler vials and using 7mM triethlyamine (TEA) in 10% methanol was recommended to reduce the non-specific binding of oligonucleotides to sample containers. LLE followed by SPE was performed to increase the recovery. During LLE, 5% ammonium hydroxide was added to the phenol-chloroform to aid the distribution of oligonucleotides to the aqueous phase. The residual organic solvent was removed from the aqueous extract by C18 SPE using a TEA-hexafluoroisopropanol (HFIP) and TEAB buffer. Significant gains in recovery were observed when 100mM TEA was added to 60% acetonitrile in water as the elution solvent.

RNA sample extraction presents a unique challenge because of the ubiquitous nature of RNases in the environment. All laboratory materials that will be exposed to RNA need to be free of

RNases, including sample containers, mobile phase bottles, benches, pipette tips and even water. Traditionally two types of sample extraction are used for RNA, 1) chemical extraction using denaturing agents and organic solvent precipitation and 2) solid-phase extraction by immobilizing the RNAs on a glass support. Very few applications of the determination of RNAs from biological samples have been reported. Beverly et al. reported extraction procedures of a 23mer siRNA from vitreous fluid samples and retina/choroid samples as part of a stability study from ocular tissues[49]. For vitreous fluid, a one-step chemical extraction was employed by using a mixture of phenol/ chloroform/isoamyl alcohol (25:24:1) and 1mM Tris/EDTA at pH 8.0. The recovery was between 14%-40% depending on the concentration. A commercially available solid-phase extraction kit was used for retina/choroid sample clean up. Briefly the sample was lysed and homogenized and most of the cellular components and DNA were removed by a mixture of phenol:chloroform (5:1) isoamyl alcohol at pH 4.7. The aqueous layer was then mixed with ethanol to increase affinity toward the glass support and finally the mixture was twice passed through a glass fiber filter. The small RNAs were immobilized on the filter and larger RNAs were washed off. The analyte was recovered by washing the filter with deionized water. However no recovery data was reported for the extraction of the oligonucleotides from these samples.

With increasing demands being made on bioanalytical methods, the need for high sample throughput has become a significant criterion in method development. Many kits are now available for DNA and RNA extraction. These are mostly geared toward PCR applications, but several are also being marketed for small length oligonucleotide applications, especially miRNA. A substantial need exists for simple, high throughput methods for extracting small oligos. The

methods must be amenable to a variety of biological matrices including cells and tissues. The primary need is for robust recovery of small amounts of RNA and DNA for PK/PD analysis, as well as, for the study of cellular mechanisms of oligonucleotides.

Chromatographic Separations:

The development of analytical methods for sensitive, quantitative analysis of therapeutic oligonucleotides will be a hallmark for the advancement of this field. Analytical methods must be able to robustly separate, quantify and characterize these biomolecules and their metabolites at levels low enough to allow for intracellular analysis and terminal end-phase kinetics. Another significant challenge lies in separating and identifying the metabolites of DNA aptamers and antisense, as well as siRNAs. siRNAs are double stranded molecules that can be metabolized by nucleases on the 3' and 5' end of either strand. This presents a significant separation and characterization challenge. In addition, methods are needed to separate and characterize synthetic DNA used for primers and microarray analysis. For these applications, the samples may contain DNAs that are the same length, but have different base pair combinations. This also applies to pooled siRNAs used in cell culture experiments[57]. Most of the methods reviewed here are amenable to both single stranded DNA (ssDNA) and double stranded RNA (dsRNA). The methods will be divided based on the mechanism of separation. Since DNA and RNA have a strong absorbance at 260 nm, most of the methods are amenable to UV detection and a few methods use fluorescence detection. In addition, there is a dedicated section on mass spectrometric detection focusing on the power of this detection method for low-level quantitation as well as for its ability to identify and characterize oligonucleotides. Specific features that are more amenable to DNA, RNA, or single versus double strand will be highlighted within the individual sections.

HPLC Methods

HPLC represents a platform that is amenable to high throughput analysis, is relatively inexpensive, easy to use, and robust. It is for these reasons that much work has been done to find separation methods to analyze therapeutic oligonucleotides and their fate in biological matrices. These techniques include ion-exchange chromatography [41] and ion-pair reversed-phase HPLC (IP-HPLC)[58].

Ion-exchange Chromatography

Ion-exchange chromatography with UV and fluorescence detection is an excellent method for separating charged molecules, and as such, is amenable to large multiply charged oligonucleotides[59]. The separation mechanism for oligonucleotides is achieved with positively charged stationary phases that exchange negatively charged analytes through competition with an increasing gradient of anions in the mobile phase. Typical mobile phases include sodium chloride and sodium perchlorate in Tris or sodium phosphate buffers. These offer excellent selectivity for separating oligos based on length via the number of charges on the phosphate backbone. Sodium perchlorate has been shown to offer selectivity for nucleobase composition. This appears to be due to increased hydrophobic interaction with the stationary phase support[60]. The capability of ion-exchange to separate oligonucleotides from other polar molecules in biological matrices, and its high tolerance for salts, significantly simplifies sample extractions when compared to many other techniques. These unique advantages confer ion-exchange chromatography an indispensable role in the study of the modifications, purification and kinetic analysis of oligonucleotides.

Both strong and weak anion-exchange supports have been used successfully for the separation of DNA[43, 44, 46, 50, 60, 61] and larger RNAs (> 30mer)[62]. Along with selectivity, ion-exchange allows for various modes of detection, including UV and fluorescence. Arora et al. used a unique fluorescence detection method for quantitative analysis of an antisense morpholino oligomer using a 4 x 250 mm column[46, 50]. Biological samples extracted from plasma and liver tissues were reconstituted with a 5' fluorescently tagged DNA that was complementary to the analyte. The fluorescently tagged DNA was annealed with the sample. The annealed sample was separated from a 15mer internal standard and the excess tagged DNA, allowing for quantification of the drug in the biological matrix. This method would not likely be specific for truncated metabolites, because they too, would anneal with the fluorescing primer. The mechanism of separation for this paper is intriguing since it is unclear that morpholino-type oligonucleotides would carry negative charges at the pH of 8 used in this study. Therefore, the separation appears to be employing a significant amount hydrophobic interaction. The paper indicates a UV limit of detection of 40 ng/mL, but no chromatograms showing the LOD or validation data were provided.

Another study used a unique lithium hydroxide, lithium chloride mobile phase with UV detection at 254 nm on a 4.6 x 150 mm column for separating analytes. The pharmacokinetics of a phosphorothioate bcl-2 antisense oligonucleotide were determined using a method originally published by Raynaud et al. [43, 44, 63]. An 18mer antisense DNA was extracted from liver, kidney, plasma and urine. The analytes were separated using a weak anion-exchange column and a gradient of lithium chloride. The chromatography separated the 18mer analyte from a 16mer

metabolite and had a reported UV limit of detection of 250 ng/mL. Recoveries for the extraction were reported at 98%, although no validation data was presented.

Analysis of oligonucleotide therapeutics will require separation of the parent molecule from its n-1 metabolites with UV or fluorescence detection. In addition, analysis of pooled oligonucleotides that are the same length, but differ in the composition of base pairs is necessary. This would be useful for analyzing primers, siRNAs and even miRNAs. In Thayer et al., base pair resolution of 21 – 25 nt unmodified ssDNA is demonstrated using a 4 x 250 mm column[60]. They also show selectivity for oligonucleotides between 21 and 25 base pairs that have different compositions. They demonstrate that the retention time increases with the pH of the mobile phase as the percentage of guanine and thymine increases in the single stranded oligonucleotides. The changes in retention are based on the ionization of the tautomeric oxygen on guanine and thymine. Higher mobile phase pH is also beneficial for denaturing oligonucleotides allowing for separation as fully linear species. The addition of acetonitrile reduced the retention time of oligonucleotides and altered the pH dependent retention time shifts. Overall, these retention differences can be exploited to separate both same length pooled oligos, as well as parent oligos and their metabolites. Although this is not a biological application, this work shows the selectivity and robustness of the glycidoxyethyl methacrylate based strong anion exchange columns that are specifically designed to be used for oligonucleotide separations. Figure 4 shows a chromatogram using the same stationary phase on a 2 x 150 mm column with a NaCl gradient in a Tris buffer at pH 9.0. The chromatogram shows the separation of four double-stranded siRNA 21mers that have different base pair combinations.

Buncek et al. analyzed sequence isomers using two different 4.6 x 150 mm ion-exchange columns with UV detection and a NaCl gradient at pH 8[61, 64]. They found that both columns resolved the sequence and positional isomers of a 21mer polyT and polyC modified with A, C, T and G bases in the middle or on the 3' or 5' end. They postulate that the orientation of ssDNA and the basicity of the nucleobases in the ion-exchange column affect retention. The negatively charged phosphate groups will be attracted to the stationary phase, while the positively charged bases will be repelled. At pH 9-10, deprotonation of guanine and thymine will contribute to the overall negative charge of the oligo. In addition, secondary conformational changes caused by A – T, G – C base pairing may affect retention. With these mechanistic findings, it is likely that in a true heterooligonucleotide, differences in protonation and hydrophobicity of the bases would contribute to the separation of sequence isomers or oligos that are the same length with different compositions. Neutral pH (6.5 - 8.0) using $NaClO_4$ as an eluting salt has been used for length dependent separations. The conditions were successfully applied to the quantitative bioanalysis of a 37mer ribozyme[45].

In considering the separation mechanism for oligonucleotides by ion-exchange chromatography, it is important to realize the complex nature of the interactions between the analyte and the stationary phase. Incomplete coverage of the particles of the stationary-phase by the ammonium groups provides the potential for significant non-ionic interactions to occur between the analyte and the stationary phase. These effects can especially be observed in phosphorothioates, where a chiral center is created when the oxygen atom is replaced by a sulfur atom, creating a mixture of 2^n diastereomers with a range of hydrophobicities[65]. This often causes peak broadening and subsequent loss of resolution due to the impact of these complex interactions with the column.

This likely provides the main reason for the less than successful applications of ion-exchange chromatography with phosphorothioate oligonucleotides. To date, there are no reports of resolution between peaks differing by only one base for this class of oligonucleotides.

Recently, Thayer et al. demonstrated a new column technology for purification of modified and unmodified synthetic ssDNA and ssRNA[66]. The stationary phase uses porous ion-exchange "nanobeads" that are attached to monolithic polymers on a 4.6 x 50 mm column. The 3 μm pore size is optimized to allow for mass transfer through convection rather than diffusion to improve column efficiency. They demonstrate the semi-preparative purification of 8.25 mg of a 25mer DNA using a larger version of the column in a single run.

UV is the most commonly used detector with ion-exchange chromatography. UV detection is compatible with the high salt content of ion-exchange mobile phases and provides reasonable detection limits in many methods. With very crude sample preparation, Chen et al. had detection limits of approximately 200 ng/mL from plasma and urine samples using a hand-packed 1 x 20 mm column[41]. Using more rigorous sample preparation methods, detection limits of approximately 50 ng/mL were achieved for a phosphorothioate 37-mer using a 4 x 250 mm column[45]. Since phosphorothioate oligonucleotides have greater band broadening than other types of oligonucleotides, it would be reasonable to expect LOQs as low as 10 ng/mL with unmodified oligos.

Fluorescence detection generally requires derivatizing oligonucleotides to improve their response. However, the hydrophobic nature of fluorophores such as fluorescein may compete with charge

density dependent separations like ion-exchange chromatography and alter the separation of labeled oligonucleotides. A recent study by Devi et al. separated a 20-mer phosphorodiamidate morpholino dsDNA, a 15-mer dsDNA (internal standard) and a ssDNA primer and found that the elution order of the fluorescein-labeled compounds was reversed relative to the unlabeled compounds[50]. Direct labeling of the 5' end of an antisense DNA with fluorescein was used in a study of inhibition of gene expression[50, 67]. Using fraction collection of the eluent followed by fluorometric analysis, the assay achieved a detection limit of 1 pM for a DNA 20-mer on a 4.6 x 250 mm, column.

A more recent development in oligonucleotide detection is the use of inductively coupled plasma mass spectrometry (ICP-MS). ICP sources do not tolerate high concentrations of organic solvents in the mobile phase; therefore, it would be considered optimal for interfacing with ion-exchange chromatography. ICP-MS provides elemental and isotopic information with a wide linear dynamic range that may exceed six orders of magnitude. Elemental phosphorus from phosphodiester linkages or both elemental phosphorus and sulfur from phosphorothioate linkages can be measured using ICP-MS with good sensitivity[68]. However, since ICP is a destructive ionization mode, it cannot provide additional information about the structure of the analyte.

These examples demonstrate the potential for ion-exchange chromatography in the analysis of oligonucleotide therapeutics. It is realistic to expect that a well-validated procedure with known metabolites could benefit from the high throughput and optimal selectivity of ion-exchange methods. Ion-exchange chromatography with UV detection provides a universal platform that allows for quantification of analytes and their metabolites. However, the identification of new

peaks would likely require fraction collection and characterization by MS. Interfacing ion-exchange chromatography with mass spectrometry using the electrospray ionization interface would allow for direct determination of molecular weight and possibly sequencing of oligonucleotides. However, there are significant barriers that will need to be overcome to successfully apply this approach. The electrolytes used for separating oligonucleotides make forming an electrospray plume difficult. The use of an eluent suppressor to remove interfering electrolytes has been used in the past to allow ion-exchange chromatography to interface with mass spectrometry[69]. However, eluent suppressors limit the selection of mobile phase to species such as hydroxide or carbonate that have not been successful for separating oligonucleotides.

Columns and Stationary Phases for Ion-Exchange Chromatography

Columns for ion-exchange chromatography use both strong anion and weak anion exchange as mentioned earlier. Most reported work was achieved on the 4 to 4.6 mm internal diameter (id) columns[43, 44, 50, 61, 64]. Early glycidyl methacrylate polymer based columns have been improved with a glycidoxyethyl methacrylate[60]. The new columns are more stable at higher temperature and pH and exhibit a much improved column lifetime. A monolithic stationary phase with porous ion-exchange "nanobeads" has recently been introduced[66]. Both the methacrylate and monolithic columns are now available in custom 1 mm and capillary sizes as companies move toward supporting nano chromatography applications that have already been so successful in proteomics. As with other column supports, the smaller id columns should provide improved resolution and detection limits although literature references are not available at this writing.

Ion-Pair Reversed-Phase Liquid Chromatography

Ion-pair reversed-phase liquid chromatography, especially if combined with volatile mobile phase additives, has the potential to be a tremendous advance in the analysis of oligonucleotides.

With adequate selectivity, this method could allow for universal UV detection for quantitative analysis, and could more easily be interfaced with mass spectrometry to provide exact mass data and MS/MS characterization. Numerous efforts have been made toward this goal since the first successful chromatographic separation of oligonucleotides in 1978[70].

Ion-pairing in a reversed-phase system is controlled by a number of factors. The degree of retention of the oligonucleotides is based on the chain length of the n-alkyl ion-pairing (IP) agent and its interaction with the hydrophobic stationary phase[71]. The number of available charges on the biomolecule as well as its secondary structure then governs the interaction with the IP agent. This allows for separations based on the length of an oligonucleotide. However the hydrophobicity of the individual bases can still affect analyte retention. Studies with ssDNA have found that the hydrophobicity of the bases follows the order C < G < A < T[71-73]. The retention difference between heteronucleotides of the same length appears to be based on the sum of the hydrophobicity of all of the base pairs in the sequence. These base pair dependent effects are less apparent with dsDNA because the bases are shielded by the charged backbone[71, 72]. Research in new ion-pairing agents has focused on two goals. The first, and most important goal is ensuring that the ion-pairing buffer is compatible with ESI-MS. These buffers are still useful for UV detection, but the addition of MS detection allows for further characterization of the analytes and potential metabolites. The second goal is to have an IP agent that allows for length-based separations with predictable contributions from the varying hydrophobicity of the bases.

The first chromatographic separations of nucleic acids used triethylammonium acetate (TEAA) because of its good separation efficiency[70, 74, 75]. Initially used to purify PCR products and primers, TEAA is an attractive ion-pairing agent because it can be evaporated from a purified sample[71, 72]. Typical TEAA concentrations are 100 mM at pH 7 with acetonitrile added to the eluting buffer. UV detection is most often used with this mobile phase. Its use in MS detection is discussed in detail in the Mass Spectrometry section.

In one of the few papers that provides linearity data, Huber et al. demonstrated an LOD of 8 ng/mL for a 14mer ssDNA with a wide linear dynamic range[71]. This separation used a TEAA mobile phase on a 4.6 x 50 column. This paper further showed n-1 separation of phosphorylated poly C and poly Ts ranging from 12 to 30 nucleotides. TEAA has also been used to separate synthetic primers up to 30mers, as well as long DNA sequences > 450mers [76]. Dickman et al. used a 4.6 x 50 mm column to show that TEAA could be used to separate curved or bent duplex DNAs that have poly A tracks[73]. These curved DNAs have longer retention times in ion-exchange LC that causes them to coelute with higher molecular weight fragments. This paper shows that the curved 378 bp DNA has no effect on analyte retention using TEAA so that dsDNAs can be separated based on length with no contributions from the conformation.

TEAA has also been successful in the separation of RNAs. In an excellent application for miRNAs, Dickman et al. used a commercially available capillary column with a TEAA mobile phase to concentrate miRNA from total RNA extracted from HeLa cells[77]. *Let-7* miRNA was spiked into total extracted RNA. The separation allowed for the miRNA to be enriched and separated from the total RNA fraction. This is an excellent proof of concept for research

applications involving miRNA research and one of the few oligo applications using a commercially available capillary column.

Gilar et al. did an extensive study of retention prediction with ssDNA using 4.6 mm id columns of varying length[76]. They presented a mathematical model to predict the retention of heterooligonucleotides using 100 mM TEAA at pH 7. Using 39 different heterooligonucleotides, they show the successful application of this model by predicting the mobile phase strength required to elute the oligonucleotides. The experimental data further showed that the hydrophobicity of C and G is less than that of A and T, confirming earlier findings[70-72, 76].

TEAB has also been used in a number of applications for the separation of single stranded and duplexed DNA. This mobile phase was developed because of its compatibility with MS detection. A thorough overview of this work is available in a review by Huber and Oberacher[78]. The first report of TEAB for the separation of oligos was in 1999 by Huber and Krajete using capillary columns[79]. They showed separation of 8 to 40mer ssDNAs. They also showed that TEAB showed improved resolution over TEAA for smaller oligonucleotides ($p(dT)_{12}$ and $p(dT)_{13}$), but similar resolution for larger oligos (> 30mer).

Oberacher et al. used capillary columns to successfully separate 21mer ssDNA primers of the same length and different composition, as well as, a dsDNA primer product[80]. They proposed that electrostatic interaction of the duplex was the retention mechanism for dsDNA, whereas the hydrophobic interaction of the bases were responsible for the separation of the ssDNA. The duplex could be analyzed for base substitutions by raising the column temperature above the

melting temperature (Tm) of the oligo. At this temperature, the single strands were easily resolved and characterized. This same paper introduced butyldimethylammonium bicarbonate (BDMAB) as an ion-pairing agent. They compared the separation efficiency of BDMAB to both TEAB and TEAA. Because BDMAB is a more hydrophobic IP agent, higher concentrations of acetonitrile can be used for elution allowing for greater sensitivity when interfaced with MS detection. Greater details on this mechanism are presented in the mass spectrometry section of this review.

Holzl et al. also used BDMAB for RNA separations on capillary columns. Interestingly, they added EDTA to the sample preparation to reduce the cation adduction that is often observed with MS detection. A synthetic unmodified 55mer RNA was prepared with a 900-fold molar excess concentration of EDTA. The chromatography easily separated the EDTA and allowed the detection of the desalted 55mer. They showed separation of synthetic 21mer RNAs from their failure sequences down to a 7mer, although coelution of some of the n-1 sequences was observed. This paper also gives the only example of on-line MS sequencing of a 32mer.

Bothner et al. used diisopropylammonium acetate and acetonitrile with a 0.5 x 150 mm column to separate modified oligos[81]. Both phosphorothioate oligos, and methylphosphonate oligos were investigated. Both of these modifications produce chiral centers creating enantiomers with varying hydrophobicities, which makes chromatographic separation more difficult. An oligo containing 50% methylphosphonates along with its n-1 and n-2 metabolites were analyzed. Although not fully chromatographically resolved with UV detection, MS detection with selected ion monitoring allowed for the determination of each metabolite. The same result was found

with a phosphorothioate oligonucleotide. This is an excellent early example of investigations of modified oligos that are very challenging to analyze.

Hexylammonium acetate (HAA) has also been used as an ion-pairing agent[82]. Introduced recently by McCarthy et al., HAA separation of unmodified duplex RNA from excess single strands was demonstrated using UPLC with a 2.1 x 50 mm column. They also used this mobile phase for semi-preparative purification of synthetic RNA. They noted minor on-column melting of duplex RNA at ambient temperatures and found that this IP agent afforded reasonable MS detection.

Hexafluoroisopropanol/TEA buffers:

Apffel et al. made a significant contribution towards oligonucleotide chromatography with the introduction of the buffering system containing HFIP and TEA[83, 84]. Methanol is used as the organic eluent because acetonitrile is only miscible in HFIP in very small amounts. This buffer allows for excellent selectivity and is compatible with MS detection. With HFIP in the mobile phase, TEA becomes less soluble and, therefore, more likely to bind to the stationary phase allowing for a more stable layer of ion-pairing agent. Overall this makes the separation mechanism predominately an ionic interaction [76]. This separation mechanism is highlighted in the determination of phosphorothioate oligonucleotides where the peak broadening effects caused by the different hydrophobicities of phosphorothioate enantiomers is eliminated[85]. Because TEA has far greater solubility in methanol, as the concentration of the methanol increases, TEA is desorbed from the stationary phase, and the ion-paired analytes are eluted[76]. The sensitivity of the adsorption of TEA to the stationary phase, and the elution with increasing methanol concentration is evident in the shallow gradients that are used in these applications.

The HFIP/TEA mobile phase is most effective at 400 mM HFIP and 16.3 mM TEA, a far lower concentration than the 100mM optimum TEAA concentration[85]. The lower concentration of TEA improves the ionization efficiency compared with TEAA. This lower concentration also lessens competition of TEA with other cations so increased adduction is possible with this mobile phase. These optimized concentrations of TEA and HFIP allow for increased separation efficiency and enhanced signal intensity for MS detection[85]. Additionally, this mobile phase allows for improved length based separation because there is less dependence on the hydrophobicity of the bases as compared with other IP agents[76].

There are a number of examples of HFIP/TEA mobile phase separations of both synthetic and biological oligo applications. Synthetic oligos are used in many applications ranging from primers for PCR and genotyping, to therapeutic DNA and RNA. Synthesis byproducts include failure and mismatch sequences, as well as, oligos with cyanoethyl groups that were not completely cleaved during the final deprotection reaction. HFIP/TEA has been used for quality control of SPE purified primers and Taqman probes with excellent separation of the synthetic byproducts[72, 85]. The separation also allows for purification and quality control of modified and unmodified therapeutic oligos and their failure sequences[85].

By far, the most difficult oligo separations are those from a biological matrix. Even with the extensive sample preparations described earlier, there are still impurities that must be separated from the sample. These impurities can have detrimental effects on reproducibility, column lifetime, and may cause significant ion suppression in ESI-MS.

There are a few examples where duplex RNA is separated from its single strands. This degree of separation allows for complex analysis of siRNAs in both formulations and biological systems. The RNA interference mechanism ends with the guide strand in the RISC complex, so ultimately, a method should be able to separate and detect this single strand. Beverly et al. separated the duplex from the single strands to determine the ocular metabolism of an siRNA directed against a VEGF receptor[49]. This siRNA was modified by end-capping the sense strand with abasic residues, and substituting two uracils with phosphorothioate linked thymines on the 3' antisense strand. Although the chromatography did not separate the n-1 duplex RNA, MS detection of the duplex allowed the identification of metabolites based on their molecular weight. Denaturing chromatography confirmed the identification by allowing MS detection of the single strands and their metabolites. This paper represents the most thorough analysis of siRNA and its metabolites *in vivo*. Two other papers by Beverly and coworkers also showed separation of the duplex from the single strands for siRNA modified with both 2' fluoro and 2' O-methyl groups on the ribose[48, 86]. siRNA and their metabolites were extensively characterized in urine, rat and human serum as well as liver microsomes[48, 86].

Validation of analytical methods is required for all assays that test therapeutics in humans. Validation describes the sensitivity, reproducibility and robustness of a method. In one of the few oligo papers to describe such data, Murugaiah et al. use an HFIP/TEA separation to analyze a liposomal formulation containing two different duplex siRNAs containing 2'-O-Me and phosphorothioate modifications[87]. The chromatography separates all four single strands. This allows for quantitative analysis of the dosage form with UV detection with no sample preparation. Validation data showed excellent linearity and reproducibility for all four single

strands. Although the LLOQ is very high at 10 μg/mL, this assay represents the first demonstration of the analysis of a liposomal formulation with two different siRNAs.

The HFIP-TEA mobile phase has been used for the quantification of oligonucleotides with varying success with respect to sensitivity and specificity. Dai et al. used 100 mM HFIP and 8.6 mM TEA as a mobile phase with a 2.1 x 50 mm column to successfully separate an 18-mer phosphorothioate antisense ssDNA from six metabolites, including a 3' n-1 sequence. 3' and 5' single nucleotide deletions were also clearly distinguished from each other by comparing product ion mass spectra. All of the metabolites were quantified together with the parent compound[52]. The sensitivity was limited (LOQ = 100 ng/mL) possibly due to the over-simplified sample clean-up. Several other studies have attempted to improve method sensitivity using the HFIP-TEA buffer system. In one study, the organic portion of the mobile phase with HFIP – TEA consisted of mixtures of acetonitrile and methanol instead of the more commonly used 50% methanol in water, for the determination of the liver metabolism of a phosphorothioate DNA oligonucleotides[52]. Possible rationale for this solvent choice is the reported higher ionization efficiency of acetonitrile when used as a sheath liquid when analyzing oligonucleotides by capillary LC-MS. Zhang et al. provided a comprehensive report on the sample preparation and quantitative analysis of a phosphorothioate oligonucleotide in rat plasma using a 2.1 x 50 mm column[56]. This paper has one of the lowest detection limits for quantitation of DNA or RNA at 5 ng/mL. Deng et al. followed this with a similar method that obtained a LLOQ of 4 ng/mL[53]. Both of these methods used tandem mass spectrometry for detection.

Columns and Stationary Phases for Reversed-Phase Applications

Most of the successful methods using reversed-phase liquid chromatography use either a porous C_{18} stationary phase or a pellicular or monolithic poly(styrene-divinylbenzene) (PS-DVB) stationary phase. Huber and coworkers use the PS-DVB stationary phase to make monolithic 0.2 x 60 mm capillary columns[78]. They reported extraordinary sensitivity for both UV and MS detection of oligos in the femtomole and attomole range[79, 88-90]. PS-DVB monolithic nanocolumns are now available commercially, although no literature reports on quantitative analysis are currently available. Other companies are also beginning to produce their oligonucleotide columns in capillary sizes. This will no doubt be important to increase separation efficiency and detection limits mirroring improvements seen in proteomics[91].

Silica based and polymeric based C_{18} columns have also been used for oligo separations. Selectivity for these large biomolecules appears to improve with smaller particle size[76]. Slow mass transfer of oligos in the stationary phase can contribute to peak broadening. A smaller particle size shortens the diffusion path and improves separation for these slowly diffusing molecules[73, 92]. Both elevated temperatures and lower flow rates also enhance the diffusion process. Polymeric columns appear to be more stable to higher temperatures than conventional silica based columns.

A recent paper by Easter et al. introduced oligo separations on a hydrophilic interaction liquid chromatography (HILIC) column (2 x 150 mm) with detection by inductively coupled plasma mass spectrometry (ICPMS)[68]. HILIC columns use water as the strong eluent and allow for separations without the use of IP agents. Although the chromatography could only separate the full-length unmodified oligo from the n-5 oligo, the novel use of ICPMS to measure the

phosphate backbone as phosphorous oxide (*m/z* 47) is noteworthy. This method could also be used for phosphorothioate oligos by measuring the sulfoxide and phosphorous oxide. Detection limits for a dT_{30} were 0.336 ng/mL. Further work on improving separations could make this technique an excellent choice for quantitative analysis of oligos.

Mass Spectrometry:

Mass spectrometry (MS) detection with electrospray ionization (ESI) provides the most accurate and thorough characterization of oligonucleotides. The easiest mass spectral measurement is the determination of molecular mass, which can be used to confirm the identification of synthetic by-products and metabolites. The molecular mass alone is a significant piece of information as demonstrated by Pomerantz et al. who applied a simple algorithm that determined the base compositions of oligonucleotides arising from T1 RNA digests[93]. While molecular weight can indicate composition and even highlight potential modifications in the oligonucleotide's structure, more detailed analysis is required for confirmation.

One approach to confirm the presence of modifications in an oligonucleotide is to digest the oligo into its nucleosides and then use LC-MS to determine the presence of any novel structures[94]. In source fragmentation of the glycosidic bond yields ions that are diagnostic of the base and sugar portions of the nucleoside and allows for further localization of the modification in to these portions of the structure. Sequencing of smaller oligonucleotides or partial sequencing of larger oligonucleotides can be accomplished using nuclease digestion. In this case, aliquots are taken from the digestion reaction at fixed time intervals and the molecular weight of the remaining oligonucleotide is measured. When used with either a 3' or a 5' specific nuclease, approximately 3-4 bases from each end can be determined using this approach[95]. In two recent

papers, Farand et al. used digestion of siRNAs for sequence confirmation of oligos modified with 2'-F, 2'-O-Me, and abasic residues. Their methods allow for the de novo sequencing of both strands of highly modified siRNAs[96, 97].

However, full characterization of oligonucleotides using MS/MS is often required to absolutely confirm sequence or to place a modification within a sequence. The nomenclature for sequencing oligonucleotides using MS/MS was proposed by McLuckey and co-workers[98, 99] and is patterned after the widely used Roepsdorf-Fulman nomenclature for peptide sequencing (Figure 6). The fragments arise from cleavages along the phosphodiester backbone resulting in a series of ions that can be used to determine the order within an oligonucleotide. In DNA, the major series of ions are the w ions and the a-Base ions, while in RNA the major ions are the c/y series. For DNA, w ions are used to determine sequence in the 3' to 5' direction while a-Base ions are used to determine the sequence in the 5' to 3' direction. In some cases, other specific ion series such as the y ions and the c ions may also be useful for sequencing DNA[100]. RNA sequencing uses the y ions to determine the sequence in the 3' to 5' direction while the c ions are used to determine the 5' to 3' direction. While oligonucleotides up to the 50-mer level have been successfully sequenced using FT-MS and MS^n [101], it is difficult to fully sequence oligonucleotides above the 25-mer level with only a single stage of MS/MS in most mass spectrometers. This size limitation is due to inefficient transfer of energy into such large molecules, and also to the secondary fragmentation and the resulting complexity of the mass spectrum. Modifications to the backbone or to the nucleobases can alter (sometimes radically) the fragmentation behavior of oligonucleotides[102, 103]. While this can sometimes make fully sequencing modified oligonucleotides challenging, it often highlights the positions of the

modifications. The modifications that have the greatest change in the fragmentation are those that occur in the nucleobases. These modifications alter the gas-phase acidity or electronegativity of the nucleobase, which is considered to be a critical factor in the formation of both the w and a-Base sequence ions[99, 104]. Since modifications of the backbone, sugar or nucleobase are intrinsic features of an oligonucleotide there is little, outside of derivatization that can be done to improve mass spectral fragmentation behavior[105]. Therefore, efforts should be turned toward maximizing signal intensity from the source; however this requires a thorough understanding of the factors influencing ionization of oligonucleotides during their transfer from the solution-phase into the gas-phase.

Electrospray ionization has emerged as the method of choice for the determination of oligonucleotides by mass spectrometry. Two theories have emerged to explain the mechanism of the transition of molecules into the gas phase by electrospray ionization.[106-110] Malcolm Dole originally proposed the charged residue model (CRM), which states that electrospray ionized droplets undergo a series of coulombic explosions as the solvent droplet evaporates and the charge repulsion within the droplet equals the surface tension (the Rayleigh limit). This ultimately leads to a droplet that contains one molecule of solute, which then becomes a free gas phase ion as the last of the solvent evaporates. Iribarne and Thomson proposed the ion evaporation model (IEM). In this model, the droplet evaporates and approaches the Rayleigh limit. As the Rayleigh limit is reached, the field strength of the ions on the surface of the droplet become strong enough to overcome solvation forces and ions are ejected into the gas phase. Desolvation continues and more ions are ejected as the Rayleigh limit is repeatedly approached until the droplet is completely desolvated.

Recently, Fenn and Nguyen explored the ESI of biomolecules in the presence of water vapor[110]. They found that adding a polar solvent vapor to the bath gas increased the abundance of desolvated ions. They proposed that this was due to the condensation enthalpy released as solvent vapor molecules bound to the droplet surface. This causes solute ions to be sputtered from the droplet surface. Their findings indicate that the IEM model more closely explains the ESI behavior of biomolecules.

The IEM also more fully supports the differences in ionization efficiency observed when different ion-pairing agents are considered. There are several significant factors that impact the transition of ionized oligos from charged electrospray droplets into the gas-phase. They include the concentration, volatility and density of the mobile phase components, the proton affinity of the ion and counter ion, and the surface tension of the droplet. In order for an ion to leave the droplet, there must be sufficient repulsion energy for the ion to break through the surface tension. Lower droplet surface tension is found with higher organic solvent concentrations. However, higher organic solvent concentrations will impair the chromatographic separation. The challenge is to find the appropriate ion-pairing agent, both ion and counter ion, and balance this with sufficient organic concentrations to lower surface tension to allow for sufficient separation while improving ionization efficiency.

Triethylamine is the most common ion-pairing agent used for oligonucleotides. Extensive studies have looked at the impact of the acidic counter ion on signal intensity. When considering the desolvation of the droplet, the boiling point of the ion and counter ion play an important role.

Experimental evidence has shown that TEAA gives lower signal intensity than HFIP/TEA or TEAB[79, 83, 84, 90, 92]. TEA has a lower boiling point than acetic acid (89 ℃ vs 118 ℃). When TEAA is used with ESI, the TEA evaporates more rapidly than the acetic acid. This raises the pH in the droplet leading to lower ionization efficiency likely due to competition for ionization between the acetic acid and the oligo. TEAA is also less hydrophobic than other IP agents. It requires less organic modifier so the surface tension of the droplet is higher, which again, causes lower ionization efficiency.

When TEAB or BDMAB are used for ion-pairing, the volatility of the counter ion, bicarbonate, is far greater than both ion-pairing agents. Huber et al. showed that, with the addition of an acetonitrile sheath liquid, TEAB gave up to sevenfold higher signal intensity than TEAA with equivalent chromatographic performance[79]. In a separate paper, Huber et al. found that a BDMAB mobile phase increased sensitivity even more than TEAB. BDMAB is significantly more hydrophobic than TEAA or TEAB. This allows for a higher concentration of acetonitrile to elute the oligo. This higher organic concentration reduces the surface tension providing up to 10 times greater signal intensity than TEAB[111].

The conductivity of the counter ion can also play a role in the ionization. Huber and Krajete did an interesting study in which they tested various counter ions with TEA. They used acetate, bicarbonate, formate, and chloride in solutions containing 20% acetonitrile at pH 8.9. They found that the volatility of the counter ion did not correlate with increased signal intensity[79]. The acetate counter ion gave the best signal intensity under these conditions. They inferred that the

increased conductivity of the counter ion suppressed the oligo signal through competition for ionization.

Ionization efficiency must also be balanced with charge state reduction. Charge state reduction allows for greater signal intensity over fewer m/z values, and this can increase detection limits. In general, the charge state is reduced when protons remain associated with the phosphodiester backbone leaving fewer negative charges. This process occurs during the transfer of ions to the gas phase as well as within the gas phase. Muddiman et al. proposed a mechanism for charge state reduction in which the hydrogen bound proton is shared in a dimer between the phosphodiester bond and the counter ion[112]. Because of the higher proton affinity of the backbone, the counter ion is lost as a neutral on entry, as well as, within the gas phase. In general, the charge state is reduced with the addition of acids with a lower pKa. Figures 7 and 8 illustrate the desorption and ionization of oligonucleotides in an HFIP/TEA mobile phase. These figures offer a visual model of how the mechanisms established by Muddiman, Fenn, and the IEM model might look with this mobile phase.

The HFIP/TEA mobile phase developed by Apffel et al. has been the most widely used in biological applications measuring oligos[23, 48, 49, 52, 53, 56, 113]. Gilar et al. optimized the buffer composition of HFIP/TEA to 400/16.3 mM to maximize separation efficiency and mass spectral signal intensity for ssDNA[92]. They found that HFIP/TEA gave superior signal intensity over TEAA. As stated earlier, Huber et al. found that TEAB with an acetonitrile sheath liquid increased the signal intensity up to sevenfold compared with TEAA[79]. The direct comparison of the signal intensity of HFIP/TEA and TEAB or BDMAB has not been published, but it is

apparent that these mobile phases all provide improved signal intensity over TEAA. The addition of HFIP also shifts the charge states to higher values and does not produce the charge state reduction seen with TEAB and BDMAB. This indicates that the proton affinity of HFIP is higher than bicarbonate[48, 89]. One advantage of HFIP is its very low boiling point (bp = 58.2 ℃). Apffel et al. proposed that this allows HFIP to evaporate in the gas phase, raising the pH of the droplet toward 10[83, 84]. As the pH increases, TEA dissociates from the backbone and the oligo is desorbed in to the gas phase. The lower pKa of HFIP (9.3) also allows for rapid evaporation because it is not significantly dissociated at the typical pH used with these buffers. Ionization of HFIP does cause background interference at low m/z values (< 500 m/z) as indicated in Figure 8, but this does not typically interfere with the charge state distributions from therapeutic oligos (19 – 24mers).

Recently, Ivleva et al. offered additional work using HFIP/TEA with the separation of phosphorothioate and LNA siRNAs using UPLC and a 2.1 x 50 mm column with MS^E characterization[114]. MS^E characterization is achieved by alternating between low and elevated (MS^E) energy in the collision cell allowing for on-line sequencing. The low energy data provide molecular weight information about the analytes. The elevated energy data provide ion fragmentation information for all of the analytes. This provides an advantage over MRM detection because all of the ions are fragmented and detected, not just selected ions. Therefore, if new peaks arose in a chromatogram, MS^E would be able to analyze these without alterations to the MS method. The authors used this method to characterize modified synthetic siRNA and its failure sequences and by-products as well as metabolites from an in vitro hydrolysis procedure. They showed that MS^E characterization of siRNAs gave superior and more rapid performance

over an existing MS/MS method for structural isomers as well as metabolites. They also noted that MS^E gave a superior signal to noise ratio, which may improve detection limits. Nikcevic et al. demonstrated separation and detection of low level impurities from the synthesis of phosphorothioate oligos[115]. In addition to separating critical synthesis impurities, this paper offers a comparison of reversed-phase columns as well as a detailed explanation of expected synthetic impurities and their origin.

Detection limits will continue to be the greatest challenge with MS detection. Zhang and co-workers have demonstrated validated detection limits of 5 ng/mL and 4 ng/mL for a single stranded phosphorothioate DNA oligomer using a 2.1 x 50 mm column with MRM detection[53, 56]. Castleberry et al. used a proteomics technique with a 1 x 150 mm column to determine the relative quantitation of tRNAs in a mixture[116]. In this novel quantitation method for oligos, tRNAs were isotopically labeled with nucleases in ^{16}O or ^{18}O water. The resulting mixtures were analyzed and the relative quantitation of the tRNAs determined with detection limits in the femtomole range. This technique might be relevant to the burgeoning field of miRNA biomarkers where relative quantities can be indications of disease states.

Still, these low detection limits and quantitation have not been reproduced with dsRNA. The ultimate challenge remains to obtain the simultaneous separation and determination of the siRNA single strands and the duplex while providing superior ionization efficiency and signal intensity. Investigation must continue to find the balance between chromatographic performance and solution conditions necessary to obtain high signal intensity. The advent of nanospray

technologies and smaller columns will likely play a role in increasing sensitivity along with further improvements in mass spectrometers.

Matrix Assisted Laser Desorption Ionization (MALDI)

MALDI analysis has also been used to characterize oligonucleotides for quality control purposes. Because of the negative charge of the backbone, a neutral matrix, 6-aza-2-thiothymine (ATT), has been used to analyze ssDNA and duplex RNA and DNA[117, 118]. Duplex DNA is more difficult to stabilize than RNA. Various additives such as cobalt (III) hexamine and ammonium citrate have been used to stabilize the DNA duplex during the desorption process[119, 120]. Duplex RNA appears to be more stable in the desorption process. Bahr et al. have used MALDI for quality control of synthetic siRNAs[121]. They analyzed the purity and correct annealing of siRNAs using ssRNA as an internal standard. They were able to determine the relative quantity of duplex and single strands using ATT and diammonium hydrogen citrate. Bahr et al. further explored the de novo sequencing of siRNA with MALDI[122]. They applied a simple fast method using acid hydrolysis followed by the characterization of the mass ladders to identify modified sequences containing both phosphorothioate and 2'-MOE modifications.

Metabolite Characterization Using Mass Spectrometry

In addition to the quantification of parent oligonucleotide drugs, structural characterization of unknown metabolites plays an important role in the development of therapeutic oligonucleotides. As with all drug classes, the quantification and characterization of metabolites and their potential activity is required for regulatory approval. Identification of the active metabolites of therapeutic oligonucleotides can provide more accurate information about their potency and potential side effects. Characterization of the metabolite profiles can shed light on the metabolic processing of the oligonucleotides. Comparison of the metabolism pattern of oligonucleotides with different

modifications facilitates an understanding of stability. The combination of liquid chromatography with mass spectrometric analysis of therapeutic oligonucleotides and their metabolites can provide extensive structural information. In general, the most common metabolites arise from exonuclease and endonuclease activity producing chain shortened metabolites of the parent compound. Chromatographic separation of these metabolites decreases the complexity of the mass spectrum that results from multiple overlapping oligonucleotides and also from potential interferences from the biological matrix. Separation of metabolites can also increase detection limits due to less competition for ionization among species. This section will provide a brief review metabolite characterization in the literature. Lin et al. have written a more extensive review[113].

Most of the metabolites of therapeutic oligonucleotides can be directly sequenced by gas-phase fragmentation due to their limited chain length. Sequencing oligonucleotides above 25 bases is difficult due to secondary fragmentation patterns that make interpretation of the mass spectrum difficult. Additional enzyme digestion may therefore be necessary before MS analysis[123]. In general, a-B and w ions are the most abundant in the MS/MS spectra of DNA oligonucleotides and c and y ions are the most abundant in the MS/MS spectra of RNA oligonucleotides. However, different modifications have various impacts on the fragmentation pattern of the analytes. For example, Ni et al. observed that b ions and y ions are significantly more abundant in phosphorothioate containing oligonucleotides relative to those with phosphodiester backbones. This suggests that these ions may be more useful for constructing “mass ladders” for phosphorothioates[124].

Computational algorithms greatly facilitate the use of mass spectral data to identify and confirm the sequence of a given oligonucleotide[125]. Initially oligonucleotide sequencing involved manual interpretation of mass spectra by comparing a table of calculated *m/z* values corresponding to expected peaks present in a given mass spectrum[54]. One useful algorithm was developed to generate lists of DNA and RNA base compositions for a given molecular mass, mass tolerance and backbone composition in a metabolism study of an antisense phosphorothioate[47]. With the wider application of oligonucleotides in laboratory and clinical settings, internet based computer programs have become available to assist with mass spectra interpretation. The MONGO algorithm developed by McCloskey and coworkers is available from the website of University of Utah. This algorithm is capable of calculating the molecular mass, m/z values of an electrospray series and the masses of potential fragment ions arising from a selected precursor ion. However, this program supports few modifications to the oligonucleotide structure. The SOS algorithm later developed by the same group, allows users to define any combination of base, sugar or backbone modifications to the parent structure and modify and rebuild alternate sequences[126].

Even though many of the instrumental and interpretation tools available today were not available in the early stage of oligonucleotide therapeutic discovery and development, a great amount of information on oligonucleotide metabolic pathways and degradation patterns has been generated and accumulated over the past twenty years. Antisense phosphorothioate DNAs have been the most extensively studied therapeutic oligonucleotides including their *in vitro* and *in vivo* metabolism as well as tissue distribution. The *in vitro* metabolism of a 21mer phosphorothioate oligonucleotide was studied following incubation with liver homogenate[8]. The 3'-exonuclease activity was found to be predominant while 5'-exonuclease activity and endonuclease activity

were also observed. The *in vivo* metabolism of a 20mer phosphorothioate DNA (Isis 2302) was studied in pig kidneys. The parent drug and its metabolites were fractionated by anion-exchange chromatography and desalted prior to LC-MS analysis. For most metabolites a-B ions and w ions are the most diagnostic ions observed from fragmentation. Only 3' chain shortened metabolites were observed in fraction B, which contained several metabolites (N+1 to N-3). For metabolites of shorter chain length, fragmentation of the parent ion provides useful information on how many nucleotides are cleaved and from which end. The fragment ions also helped to identify a metabolite with an adducted iron atom, which otherwise could have been misinterpreted. Small amounts of depurination from the 3'-end were also observed. Interestingly, N+1 metabolites were also observed in fraction B but no definitive structural information was obtained for these. Similar findings are also seen from the *in vivo* metabolism study of two other phosphorothioate oligonucleotides[54]. The most abundant metabolites resulted from 3' degradation. The loss of one adenine base was also observed in liver metabolism. The metabolite profile from kidney and liver tissues displayed distinctive patterns. 3' metabolites had relatively high peak signal areas when compared to 5' metabolites observed from kidney. The sum of the area of all metabolites from the kidney was significantly higher than in liver. Degradation from both the 3' and 5' termini were observed in the liver. A 5' N-2 metabolite showed nearly twice the area when compared to that of the 3' N-2 metabolite in the liver. No 5' N-1 metabolites were found in the liver tissue.

Dai et al. characterized the metabolites of a bcl-2 antisense phosphorothioate DNA G3139 in human and rat plasma and urine with the assistance of the SOS program[52]. Six major metabolites were generated from 3' metabolism. It is worth noting that since the base on both

the 3’ and 5’ ends were thymines, the deconvoluted mass spectra of the precursor ions did not provide enough information to rule out either one of the metabolites. The researchers obtained tandem mass spectra of the standard of both metabolites and compared the w series and a-B ions with the unknown metabolites and assigned it as the 3’ N-1 chain shortened sequence. All other metabolites were easily identified using deconvoluted mass spectra and their sequences were verified using the SOS program.

Metabolite characterization of double stranded oligonucleotides can be more complicated due to the presence of both single stranded and double stranded metabolites. In addition, the double stranded ion species can dissociate in the electrospray source, which will further complicate the identification process unless sufficient chromatographic separation was achieved prior to ionization. Beverly et al. studied the *in vitro* and *in vivo* metabolism of an siRNA in urine and from ocular samples[48, 49]. 5’ to 3’ degradation profiles were both observed in the urine sample spiked with the sense and antisense strands. This was likely due to the abasic protecting group on the 3’ end of the sense strand and the phosphorothioate linkages on the 3’ end of the antisense strand. Interestingly, there were no N-1, 2 or 3 cleavage products observed from the 5’ direction. The author proposed a mechanism by which the endonucleases jumped the protected abasic site to start the cleavage, and the exonucleases took over the degradation from there. Different cleavage sites on the phosphate backbone were also observed among different metabolites. The degradation of the antisense strand started later in the time course because of the O-methyl group at the 2’ location of the ribose on each purine base on this strand. This slower onset of degradation was important for therapeutic reasons as the antisense strand would directly interact with the mRNA. The duplex exhibited much better stability when compared to the single strand

in urine. Due to the denaturing effects of the ESI process, the signal of both sense and antisense strands were observed in the mass spectrum of the duplex. The author pointed out that single strands formed from the denatured duplex could reanneal, and give false information on duplex metabolites. This issue was addressed by spiking single stranded metabolites (5' N-3 sense strand) at twice the concentration of the duplex and analyzing the result. The mass spectrum did not contain a mass corresponding to a duplex containing the N-3 metabolite, indicating that annealing between the full length and metabolite strands had not occurred. In the *in vivo* metabolism study of the duplex, siRNA sequences shorter than N-2 on one strand are all unambiguously identified, including 3' N-3 and 3' N-5 antisense strands binding to a full length sense strand, respectively, and a 3' N-5 antisense strand binding to a 5' N-5 sense strand both containing terminal phosphates. However, a sequence of N-2 antisense strand binding to a full length sense strand could not be assigned as a 3' chain shortened metabolite or a 5' end one with a terminal phosphate with enough resolution (0.05 Da difference).

Conclusion:

The analysis of oligonucleotides will be a driving force behind the successful laboratory and clinical applications of these biomolecules. The work presented here highlights the successes and challenges in this area. Both RNA and DNA therapeutics will benefit from methods that can quickly and successfully isolate, separate, quantitate and characterize the parent drug and its metabolites. The validation of these methods will be critical for regulatory approval including precision, accuracy, and recovery from biological matrices. In addition, methods will need to be validated for the characterization of synthetic therapeutic oligonucleotides and for the identification and characterization of the metabolites formed *in vivo*. Research in RNomics continues to demand improved methods to identify and characterize RNAs. Approximately 90%

of the genome sequence is transcribed into RNA, and only 1.5% of this is translated in to proteins. This leaves a substantial number of non-coding RNAs that have not been identified[91, 127, 128]. There are considerable needs for precise, robust methods to identify miRNAs as biomarkers and to further define the mechanisms of siRNAs and other small RNAs.

In the future, research needs to focus on the development of more salt tolerant methods to allow ion-exchange chromatography to become more compatible with mass spectrometry. This may involve the development of two-dimensional separations such as ion-exchange chromatography followed by reversed-phase chromatography. Similar to trends in proteomics, studies need to be carried out using capillary LC and nanospray mass spectrometry to achieve better sensitivity for both ion-exchange and ion-pairing methods. Simpler sample extraction procedures with high recovery also need to be developed. Although biological approaches such as RT-PCR and hybridization methods are still the most prevalent methods used in pre-clinical and clinical studies of oligonucleotide therapeutics, the separation and identification ability of LC-MS methods provide obvious advantages, especially in support of pharmacokinetic and metabolism studies.

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Table 1
Oligonucleotide therapeutics involved in clinical trials. Information includes the drug name, company sponsoring the clinical trial, the status, phase, and route of administration.

Drug	Company	Status	Phase	Administration	Disease
siRNA therapeutics					
TD101	Transderm, Inc.	Completed	I	Local injection	Pachyonychia Congenita
AGN211745	Allergan/Sirna	Completed	I/II	Local injection	Age-related macular degeneration; Choroidal neovascularization
AGN211745		Terminated	I	Intravitreal injection	Age-related macular degeneration; Choroidal neovascularization
CALAA-01	Calando Pharmaceuticals	Completed	II	IV injection	Cancer; Solid tumor
Atu027	Silence Therapeutics AG	Recruiting	I	IV injection	Advanced solid tumors
Bevasiranib	Opko Health, Inc.	Completed	II	Intravitreal injection	Diabetic macular edema
	Opko Health, Inc.	Completed	II	Intravitreal injection	Wet age-related macular degeneration
QPI-1007	Quark Pharmaceuticals	Recruiting	I	Local injection	Chronic optic nerve atrophy; Non-arteritic anterior ischemic optic neuropathy
PRO-040201	Tekmira	Terminated	I	IV injection	Hypercholesterolemia
siG12D	Silenseed	Not yet recruiting	I	Local Drug EluteR	Adenocarcinoma of the pancreas
I5NP	Quark Pharmaceuticals	Recruiting	III	IV injection	Delayed graft function in kidney transplantation
	Quark Pharmaceuticals	Active	I	IV injection	Kidney injury, Acute renal failure
SYL040012	Sylentis, S.A.	Recruiting	I/II	Opthalmic drops	Glaucoma, Ocular hypertension
Aptamer therapeutics					
ARC1905	Ophthotech Corporation	Active	I	Intravitreal injection	Dry age-related macular degeneration
E10030	Ophthotech Corporation	Recruiting	II	Intravitreal injection	Neovascular age-related macular degeneration
ARC1905	Ophthotech Corporation	Active	I	Intravitreal injection	Neovascular age-related macular degeneration
EYE001	Eyetech Pharmaceuticals	Recruiting	II/III	Intravitreal injection	Neovascular age-related macular degeneration
REG1	National Heart, Lung, and Blood Institute	Completed	I	IV injection	Anticoagulation system
Pegaptanib sodium (Macugen)	Eyetech Pharmaceuticals	Completed	IV	Intravitreal injection	Exudative age-related macular degeneration
AS1411	Antisoma Research	Recruiting	II	IV injection	Acute myeloid leukemia
NOX-E36	Noxxon Pharma AG	Completed	III	IV and Subcutaneous	Chronic inflammatory diseases, Type 2 diabetes mellitus, Systemic lupus erythematosus
NOX-A12	Noxxon Pharma AG	Recruiting	III	IV injection	Hematopoietic stem cell transplantation
ARC1779	Archemix Corp.	Completed	II	IV injection	Von Willebrand factor-related platelet function disorders
Bevacizumab	Medical University of Vienna	Recruiting	III	Intraocular injection	Diabetic retinopathy
ARC19499	Archemix Corp.	Not yet recruiting	I/II	Subcutaneous injection	Hemophilia
Antisense therapeutics					
EGFR Antisense DNA	University of Pittsburgh	Not yet recruiting	I/II	Intratumoral injection	Head and neck squamous cell carcinoma
	University of Pittsburgh	Active	I	Intratumoral injection	Head and neck squamous cell carcinoma
AEG35156	Aegera Therapeutics	Recruiting	I/II	IV injection	Advanced hepatocellular carcinoma
	Aegera Therapeutics	Recruiting	I/II	IV injection	Chronic lymphocytic leukemia
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced pancreatic cancer
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced breast cancer
	Aegera Therapeutics	Terminated	I/II	IV injection	Advanced Non-small cell lung cancer
	Aegera Therapeutics	Terminated	II	IV injection	Advanced cancer
	Aegera Therapeutics	Active	II	IV injection	Leukemia

Table 1 (*Continued*)

Drug	Company	Status	Phase	Administration	Disease
XIAP	Aegera Therapeutics	Completed	I/II	IV injection	Acute myelomonocytic leukemia
OGX-427	OncoGenex Technologies	Recruiting	I	Injection	Prostate, Ovarian, NSCL, Breast or bladder cancer
	Vancouver Coastal Health	Recruiting	I	Intravesical instillation	Superficial bladder cancer
LErafAON-ETU	Neopharm	Active	I	IV injection	Advanced cancer
	Neopharm	Completed	I	IV injection	Advanced solid tumors
	Neopharm	Completed	I	IV injection	Advanced malignancies
AVI-4658	Imperial College of London	Completed	I/II	Intramuscular injection	Duchenne muscular dystrophy
TGFB2-Antisense-GMCSF	Mary Crowley Medical Research Center	Recruiting	I	Injection	Advanced cancer
G3139	University of Chicago	Active	I/II	IV injection	Recurrent small cell lung cancer
Genasense (Oblimersen)	Genta Incorporated	Completed	I/II	IV injection	Chronic lymphocytic leukemia
	Genta Incorporated	Active	III	IV injection	Advanced melanoma
	Genta Incorporated	Active	I	IV injection	Solid tumors
	M.D. Anderson Cancer Center	Completed	II	IV injection	Recurrent non-Hodgkin's lymphoma
	Genta Incorporated	Active	I/II	IV injection	Chronic lymphocytic leukemia
	Genta Incorporated	Active	III	IV injection	Melanoma
	Genta Incorporated	Active	III	IV injection	Relapsed or refractory multiple myeloma
	California Cancer Consortium	Completed	II	IV injection	Metastatic renal cell cancer
	Genta Incorporated	Active	III	IV injection	Chronic lymphocytic leukemia
	University of Wisconsin, Madison	Completed	I	IV injection	Advanced solid tumors
	University of Chicago	Completed	I	IV injection	Extensive-stage small cell lung cancer
	Genta Incorporated	Active	II	IV injection	Acute myeloid leukemia
	British Columbia Cancer Agency	Completed	I	IV injection	Diffuse large B-cell lymphoma
	M.D. Anderson Cancer Center	Completed	I/II	IV injection	Metastatic or locally advanced breast cancer
	Jonsson Comprehensive Cancer Center	Active	I	IV injection	Advanced malignant melanoma
	Genta Incorporated	Active	II/III	IV injection	Lung cancer
	Genta Incorporated	Active	II/III	IV injection	Advanced melanoma
	Children's Oncology Group	Completed	I	IV injection	Relapsed or refractory solid tumors
	European Organization for Research and Treatment of Cancer	Active	II	IV injection	Hormone-refractory adenocarcinoma (cancer) of the prostate
	Memorial Sloan-Kettering Cancer Center	Completed	I/II	IV injection	Solid tumors
	San Antonio Cancer Institute	Completed	I/II	IV injection	Metastatic or recurrent colorectal cancer
	Cancer and Leukemia Group B	Completed	III	IV injection	Acute myeloid leukemia

Table 1 (Continued)

Drug	Company	Status	Phase	Administration	Disease
	Genta Incorporated	Completed	I	IV injection	Solid tumors
	Southwest Oncology Group	Active	II	IV injection	Large B-cell non-Hodgkin's lymphoma
G4460	University of Pennsylvania	Active	II	Bone marrow transplantation	Chronic myelogenous leukemia
IZN-2968	National Cancer Institute	Recruiting	I	IV injection	Advanced solid tumors
	Enzon Pharmaceuticals, Inc.	Recruiting	I	IV injection	Advanced solid tumors or lymphoma
BP-100-1.01	Bio-Path Holdings, Inc.	Recruiting	I	IV injection	Leukemia
AP 12009	Antisense Pharma	Completed	II	Intratumoral injection	High-grade glioma
	Antisense Pharma	Recruiting	I	IV injection	Pancreatic neoplasms, Melanoma, Colorectal neoplasms
	Antisense Pharma	Recruiting	I	Intratumoral infusion	Anaplastic astrocytoma
LY900003	Eli Lilly and Company	Completed	II	IV injection	Non-small cell lung cancer
ISIS 113715	Isis Pharmaceuticals	Completed	I	Subcutaneous injection	Dermatologic effects
c-myb AS ODN	University of Pennsylvania	Recruiting	I	IV injection	Advanced hematologic malignancies
SPC2996	Sanbaris Pharma A/S	Active	I/II	IV injection	Chronic lymphocytic leukemia
ISIS 104838	Isis Pharmaceuticals	Completed	II	Subcutaneous injection	Rheumatoid arthritis
Alicaforsen (ISIS 2302)	Isis Pharmaceuticals	Completed	III	IV injection	Crohn's disease
	Isis Pharmaceuticals	Active	III	IV injection	Crohn's disease
	Isis Pharmaceuticals	Completed	II	Enema	Mild to moderate active ulcerative colitis
	Isis Pharmaceuticals	Completed	II	Enema	Mild to moderate active ulcerative colitis
Lucanix™	NovaRx Corporation	Completed	II	Vaccine	Stages II–IV non-small cell lung cancer
Autologous Dendritic Cell Therapy	University of Pittsburgh	Recruiting	I	Vaccine	Type 1 diabetes
Mipomersen	Genzyme	Completed	I	Subcutaneous injection	Hypercholesterolemia
	Genzyme	Completed	I	IV injection	Cardiac repolarization
	Genzyme	Completed	I	IV injection	Assessment of blood clotting and thinning
GTX™ Drug Eluting Coronary Stent System	Cook	Terminated	I	Coronary Stent	Lesion in the coronary artery
VRX496-Modified Autologous T cells	VIRxSYS Corporation	Active	II	IV injection	HIV
Custirsen (OGX-011)	NCIC Clinical Trials Group	Completed	II	IV injection	Locally advanced or metastatic breast cancer
	NCIC Clinical Trials Group	Completed	I	IV injection	Metastatic or locally recurrent solid tumors
	NCIC Clinical Trials Group	Completed	I	IV injection	Prostate cancer
	University of British Columbia	Recruiting	II	IV injection	Localized prostate cancer
	OncoGenex Technologies	Recruiting	III	IV injection	Hormone-refractory prostate cancer
ISIS 2503	University of Alabama at Birmingham	Completed	II	IV injection	Colorectal cancer
	University of Alabama at Birmingham	Completed	II	IV injection	Pancreatic cancer
EGFRvIII peptide vaccine	Southwest Oncology Group	Active	I	Vaccine	Gastric, Prostate, or ovarian cancer
OGX-427	British Columbia Cancer Agency	Recruiting	II	IV injection	Hormone-refractory prostate cancer

Table 1 (*Continued*)

Drug	Company	Status	Phase	Administration	Disease
ISIS 3521/ISIS 5132	Eastern Cooperative Oncology Group	Completed	II	IV injection	Metastatic breast cancer
ISIS 3521	Isis Pharmaceuticals	Completed	III	IV injection	Non-small cell lung cancer
RevM10 gene	Systemix	Active	I/II	IV injection	HIV-related non-Hodgkin's lymphoma
Syngeneic Lymphocytes (CD4+) Cultured with OKT3 (Ortho) and Interleukin-2 (Chiro Miravirsen)	National Human Genome Research Institute (NHGRI)	Completed	I	IV injection	HIV infection
	Santaris Pharma A/S	Completed	I	IV injection	Hepatitis C
	Santaris Pharma A/S	Recruiting	II	Subcutaneous injection	Treatment-Naïve chronic Hepatitis C
	Santaris Pharma A/S	Active	I	IV injection	Hepatitis C
Aezea® (Cenersen)	Eleos, Inc.	Completed	II	IV injection	Acute myelogenous leukemia
	Eleos, Inc.	Not yet recruiting	II	IV injection	Acute myelogenous leukemia
ASM8	Topigen Pharmaceuticals	Completed	I/II	Inhalation	Asthma
GTI-2040	Lorus Therapeutics	Completed	I/II	IV injection	Renal cell carcinoma
RESTEN-MP	AVI BioPharma, Inc.	Terminated	I	IV injection	Coronary artery disease, Coronary stent restenosis
	AVI BioPharma, Inc.	Completed	I	IV injection	Coronary artery disease, Coronary stent restenosis

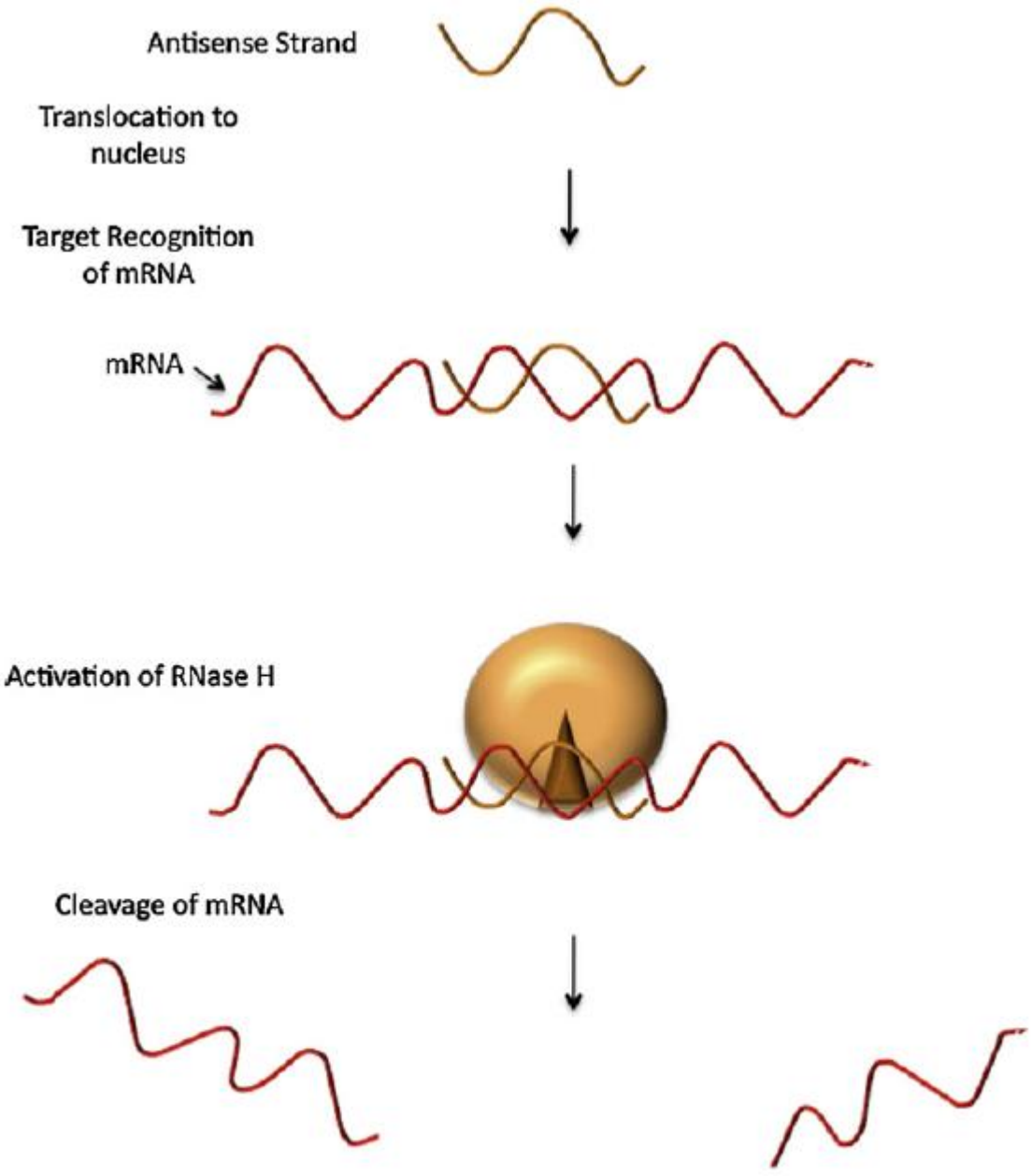

Fig. 1. Antisense DNAs enter the cell and are translocated to the nucleus. They bind with the complimentary mRNA sequence. The antisense DNA/mRNA duplex activates the nuclear enzyme RNase H, which then degrades the mRNA strand.

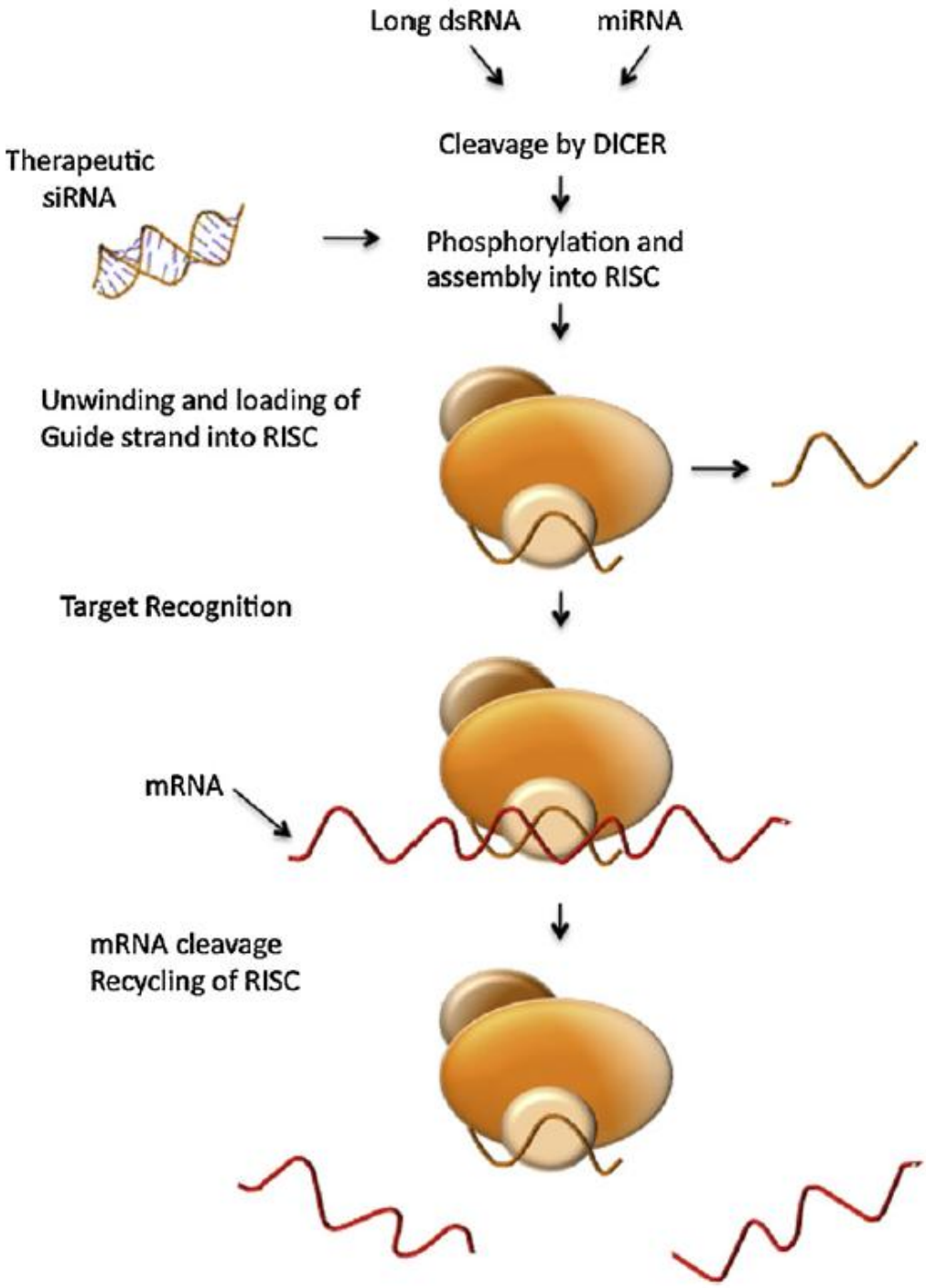

Fig. 2. RNA interference mechanism: The Dicer protein cleaves double stranded RNA in to 21–25 nt strands (siRNA). Dicer works with a double stranded RNA binding protein to guide siRNA in to the RNA induced silencing complex (RISC). Following phosphorylation of the 5′ ends, siRNA is incorporated in to RISC. The passenger strand is unwound, released and degraded leaving the guide strand bound to RISC. RISC recognizes mRNA through base pairing of a seed region that is 2–8 nt from the 5′ end. If the mRNA is perfectly complementary then it is cleaved. RISC is then recycled to perform further silencing.

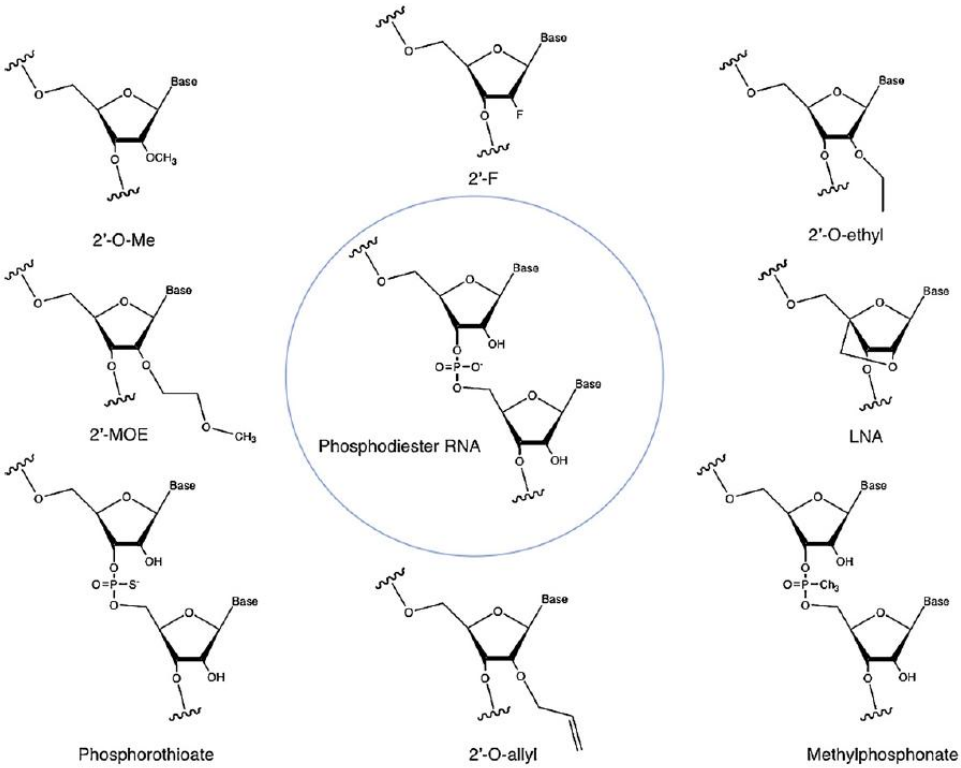

Fig. 3. Structures of RNA modifications. Unmodified RNA is shown in the center. DNA modifications are identical with the absence of the 3′ hydroxyl group on the ribose. Modifications of RNA and DNA include 2′-O-methyl (2′-O-Me), O-ethyl (2′-O-ethyl), O-methoxyethyl (2′-MOE), and fluorine (2′-F) substitutions as well as locked nucleic acids (LNA). Backbone modifications include phosphorothioate and methylphosphonate.

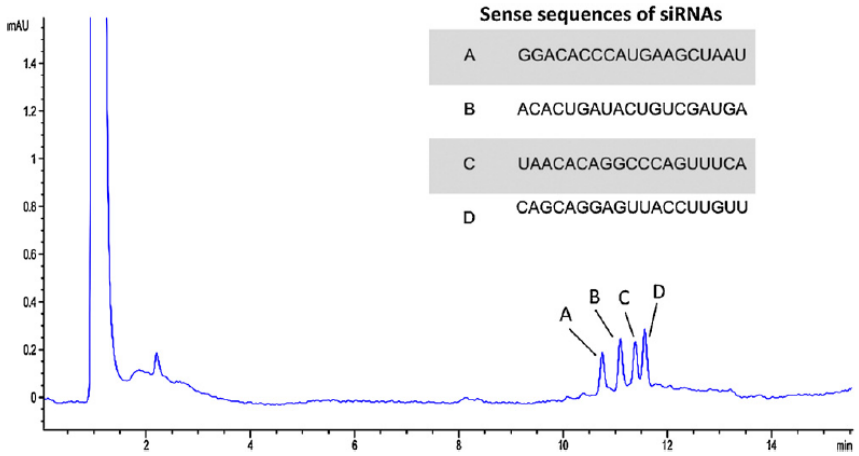

Fig. 4. Chromatogram demonstrating separation of heterooligonucleotides using ion-exchange chromatography with UV detection. Four 21-mer double stranded siRNAs with different base pair combinations were separated using a Dionex DNAPac PA200, 2 × 150 mm column. The sequences of the sense strand are shown. Both the sense and antisense strands also contain UU overhangs on the 3′ end. The mobile phase consisted of an increasing gradient of sodium chloride in a Tris buffer at pH 9.0.

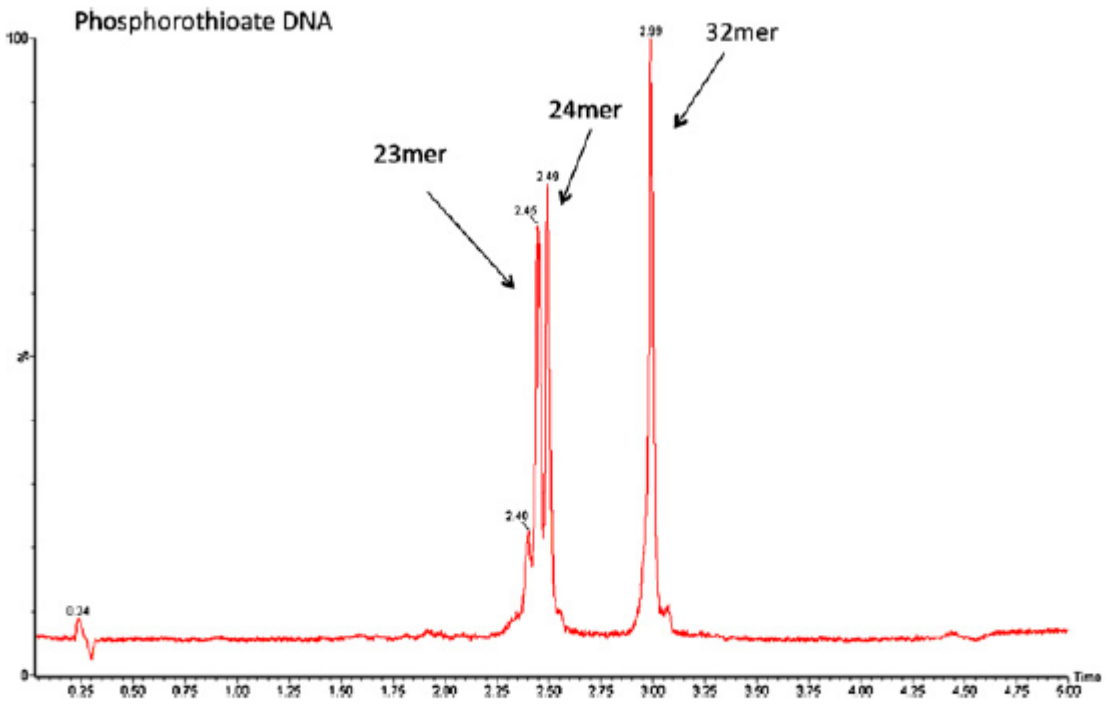

Fig. 5. Full scan total ion chromatogram showing the separation of a 23-mer and 24-mer phosphorothioate DNA and 32-mer poly dT internal standard on a Waters 2 × 50 mm BEH column. The mobile phase consisted of an increasing gradient of 50% methanol in a buffer containing HFIP and TEA.

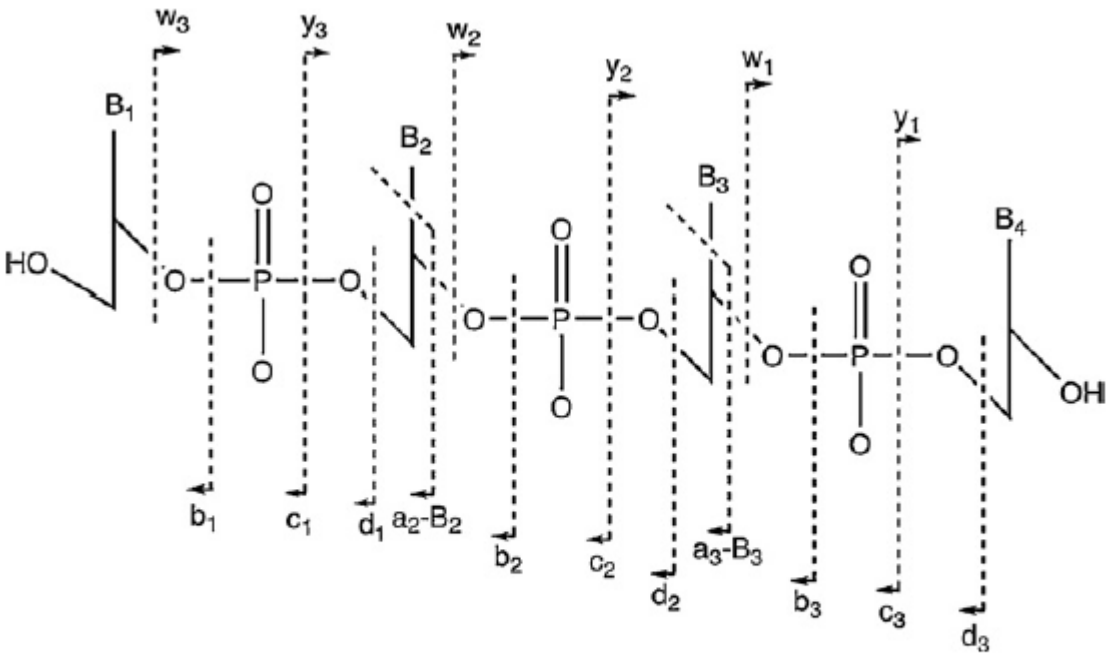

Fig. 6. The fragmentation of DNA produces predominantly a-Base (a-B), b, and d ions while RNA produces predominantly c ions from the 5′ end. Fragmentation from the 3′ end produces predominantly w and y ions for DNA while RNA produces predominantly y ions. The d and w ions are produced by loss of water [98].

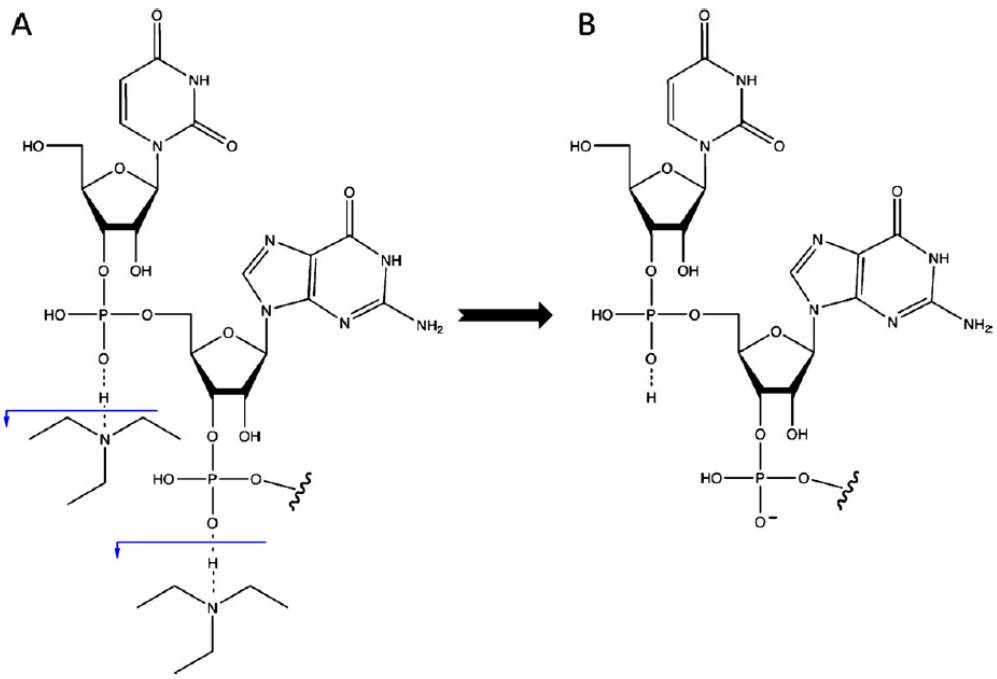

Fig. 7. Proposed mechanism for ionization of oligonucleotides in an HFIP/TEA mobile phase. A. The positively charged TEA molecules are hydrogen bound to the phosphate backbone in solution. B. As the ions are desorbed, TEA evaporates as a neutral, leaving the proton bound to the oligonucleotide backbone. TEA might also desorb as the protonated positive ion leaving a negative charge on the backbone.

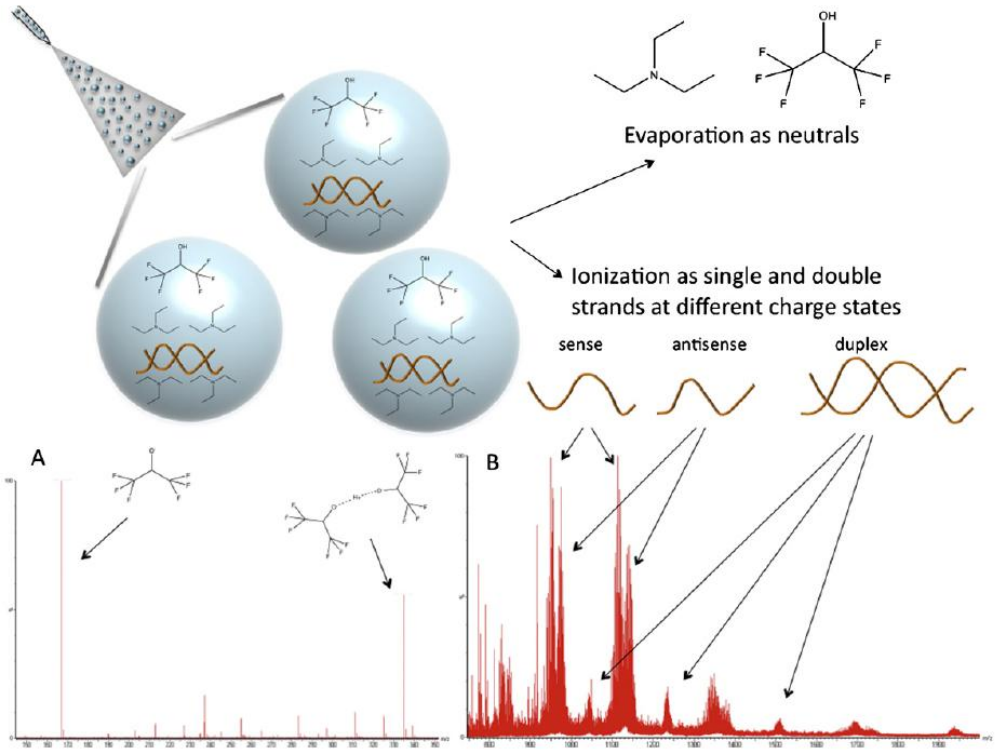

Fig. 8. Illustration of the ionization of siRNA in an HFIP/TEA mobile phase. As the ions are desorbed, both TEA and HFIP can evaporate as neutrals. Ionization of these molecules produces positively charged TEA, which is not detected. A. Panel A shows HFIP, plus an HFIP dimer, which are detected at *m/z* 167 and 335 respectively. B. The mass spectrum in panel B shows that siRNA can be detected as the duplex, or be denatured in the ESI process to produce the sense and antisense strands.

Chapter 3

The effect of mobile phase composition on electrospray ionization charge-state distribution and desorption efficiency for oligonucleotides

Chen B, Mason SF and Bartlett MG. Submitted to Journal of American Society for Mass Spectrometry

Abstract:

The chemical composition of the solution has a critical impact on the electrospray desorption efficiency of oligonucleotides. Several physiochemical properties of various organic modifiers were investigated with respect to their role in the desorption process of oligonucleotides. The Henry's Law Constant, which reflects the volatility of alkylamines, was found to have a prominent effect on both the electrospray charge state distribution and desorption efficiency of oligonucleotides. Alkylamines with higher k_H^{cc} (aq/gas) values such as hexylamine, piperidine and imidazole reduced the charge state distribution by forming complexes with the oligonucleotide and dissociating from it in the gas phase; while alkylamines with extremely low k_H^{cc} (aq/gas) values reduced the electrospray charge state distribution by facilitating ion emission at an earlier stage of the electrospray desorption process. Ion-pairing agents with moderate k_H^{cc} (aq/gas) values do not alter the electrospray charge state distribution of oligonucleotides and their ability to enhance oligonucleotide ionization followed the order of decreasing k_H^{cc} (aq/gas) values. The Henry's Law Constant also correlated to the impact of the acidic modifiers on oligonucleotide ionization efficiency. Trifluoroethanol was observed to cause signal suppression for oligonucleotide because of its low volatility. This also explains why carboxylic acids have been previously reported to suppress oligonucleotides ionization. Ionization enhancement effects were observed with hexafluoroisopropanol and this effect was attributed to its high volatility and moderate acidicity. The comprehensive effects of both alkylamine and hexafluoroisoproapnol on the electrospray ionization desorption of oligonucleotides were also evaluated and acid-base equilibrium was found to play a critical role in determining these effects.

Introduction:

Electrospray ionization (ESI) has rapidly become the technique of choice for the study of biotherapeutics. It allows large biomolecules like proteins and nucleic acids to be conveniently transferred into the gas phase for mass spectrometry analysis following a liquid chromatographic separation. This technological platform affords high throughput analysis and structural identification capabilities that traditional biochemical methods are unable to produce. Scientists have been slowly advancing the analysis of oligonucleotides by ESI since the early 1990s[1]. However, only recently has the sensitivity which allows the detection of clinically relevant concentrations been approached [2-4].

Among many of the previously published studies of factors affecting the electrospray ionization process and resulting mass spectra of nucleic acids, most focused on reducing cationic adduct formation and understanding alterations in the charge state distribution [5-8]. Only a few have focused on the factors determining the signal intensity of oligonucleotide by ESI-MS. Bleicher et al.[9] reported that increased solution pH and organic solvent percentage increased the signal intensity of oligonucleotides significantly. Gaus et al. [10] compared the signal intensity and adduct formation for oligonucleotides when using 7 different alkylamines as ion-pairing agents during optimization of their LC-MS method. No acid was used to neutralize the pH of the mobile phase. The highest signal intensity was observed when using tripropylamine. Huber et al.[11] later conducted a more systematic study on the impact of mobile phase composition on ESI-MS signal intensity and chromatographic separation of oligonucleotides. Four anionic counter ions were tested with triethylamine to observe their effect on ESI-MS performance. It was found that

the signal intensities decreased in the order acetate>bicarbonate>formate> chloride. Deguchi et al. [12] reported 10 to 30 fold signal enhancements for oligonucleotides when using a post column infusion of 0.1M imidazole in acetonitrile. These results all demonstrated that the composition of the mobile phase can dramatically affect the electrospray ionization efficiency of nucleic acid.

Despite the interesting findings on the impact of mobile phase composition, there are very few studies about the mechanism of these effects and how the physiochemical properties of each component of the mobile phase affects the desorption process for oligonucleotides. Bleicher et al.[9] attributed the higher signal intensity observed from oligonucleotides analyzed at higher organic solvent percentages and pH values to decreased surface tension and increased charges on the oligonucleotide respectively. Huber et al. [11] argued that organic acid additives with the lowest conductivity would suppress oligonucleotide ionization the least. Muddiman et al. [5] proposed a model for interactions between the oligonucleotide and the ion-pairing agents. In this model, acetic acid in the solution protonates piperidine and imidazole, enabling hydrogen bonding between them and the phosphate backbone of the oligonucleotide. A neutral loss of piperidine and imidazole would occur when the complex of oligonucleotide and the ion-pairing agent enters the gas phase, because the proton affinity of the phosphodiester moiety is estimated to be higher than that of the ion-pairing agents.

Apffel et al. [13] replaced acetic acid with hexafluoroisopropanol (HFIP) as the counter ion used in the LC-MS mobile phase, which was a milestone in the evolution of buffering systems for oligonucleotides. They proposed the mechanism of signal enhancement as the following: due to the extremely low boiling point of HFIP (49 ℃), when the eletrosprayed droplet was heated in the ion source HFIP will be evaporated quickly, increasing the pH of the droplet to

approximately 10. This enables enhanced ionization of the oligonucleotide in the droplet increasing the signal intensity. HFIP and triethylamine (TEA) have been the principle mobile phase for ESI LC-MS over of oligonucleotides the past 15 years. Many of the ion-pairing agents previously proposed as alternatives to TEA (e.g., butyldimethylamine (BDMA), diisopropylamine, and diisopropylethylamine (DIEA)) have seldom been used with HFIP. It is therefore necessary to conduct a systematic comparison of the physiochemical properties of each component of the mobile phase and its influence on the electrospray ionization desorption of oligonucleotides in order to both enhance understanding of the electrospray process and to optimize the mobile phase composition for LC-MS of this class of biomolecules.

Experimental Section:

Reagents. The 24mer (5'-TCGTGCTTTTGTTGTTTTCGCGTT-3'), 18mer (5'-TCGTGCTTTTGTTGCCCC-3'), pC18 and pT18 single stranded DNA were purchased from Integrated DNA Technologies (Coralville, IA). Chemicals such as 1,1,1,3,3,3-hexafluoro-isopropanol(HFIP), trifluoroethanol(TFE), hexylamine(HA), dipropylamine(DIPA), triethylamine(TEA), dimethylbutylamine(DMBA), di-isopropylethylamine(DIEA), tripropylamine(TPA) and tributylamine(TBA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). HPLC grade methanol was obtained from Fisher Scientific (Pittsburgh, PA). Each DNA sample was placed in solution with different concentrations of the organic modifiers and solvents listed above to achieve a final oligonucleotide concentration of 1.3μM.

Mass Spectrometry. All mass spectra were acquired in the negative-ion mode on a Waters Synapt G2 HDMS quadrupole time of flight hybrid mass spectrometer (Waters, Milford, MA) system. The capillary voltage was set at 2.3kV, the cone voltage was 20V, the extraction cone

voltage was 2V, the source temperature was 120 ℃, the desolvation temperature was 450 ℃, cone gas was 6L/h and the desolvation gas was 700L/h. The data were collected in full-scan negative ion mode over the mass range from 500-2000 m/z. All measurements were performed in triplicate.

Theory:

There are three major steps in the gas-phase production of ions by electrospray ionization from electrolyte containing solutions. All of these steps are affected by the physiochemical properties of the solution components. The steps are: (1) production of charged droplets at the electrospray capillary tip; (2) shrinkage of the charged droplets by solvent evaporation and repeated droplet disintegrations, leading ultimately to very small, highly charged droplets capable of producing gas-phase ions, and (3) the actual production of ions from charged droplets[14]. In the first step, the radius and the charge on the droplet formed at the ES capillary tip are described by the following equation[15]:

$$R = \left(\frac{3\varepsilon\gamma^{1/2} V_f}{4\pi\varepsilon_0^{1/2} KE} \right)^{2/7} \quad \cdots\cdots eq.1$$

where γ is the surface tension of solvent, ϵ is the permittivity of the solvent, ϵ_0 is permittivity of vacuum, K is the conductivity of the solution, E is the applied electric field at capillary tip and V_f is the flow rate. In the second step, the charged droplets shrink due to solvent evaporation while the charge remains constant [16, 17] until the electrostatic repulsion of the charges at the surface reach the Rayleigh limit[18]. The diameter of the subsequent droplets are predicted to be[19]:

$$d_m \approx \left[\frac{\gamma}{\rho}\left(\frac{\varepsilon}{K}\right)^2\right]^{1/3} \quad \cdots\cdots eq.2$$

where ρ is the density of the solvent. This equation indicates that the decrease of the subsequent droplets diameter is not directly the result of the decreasing diameter of the original droplet but rather the increase in the solution conductivity.

The droplet then undergoes jet fission until the second generation droplets reach the Rayleigh limit again. As for how gas-phase ions are formed from small and highly charged droplets, two mechanisms have been proposed: ion evaporation (IEM) and charged residue (CRM). The difference lies in whether the ions are directly emitted from the surface of the droplet[20] or formed as the residual following continuous Rayleigh fission events[21]. Low molecular weight, singly charged ions appear to be formed primarily from IEM, while large macromolecular ions, such as globular, nondenatured proteins, are believed to fit the CRM better [22]. However, explaining the electrospray behavior of macromolecules using both models has gained increasing popularity in recent years[22]. When the charge state of the macromolecule exceeds the maximum charge of a droplet based on the size of the macromolecule predicted by the Rayleigh limit (q_R), the molecule is considered to be generated through ion emission[14]. However, calculating q_R is challenging for oligonucleotides because molecular diameter is required for the estimation and they are linear shaped molecules. Our findings in this study more closely model the IEM. Therefore, the IEM is employed in the interpretation of the experiment results in this paper.

Fenn [23] proposed a rate equation that predicts ion evaporation from a charged droplet:

$$N_{iz} = 3AN_i / re^{-\frac{\Delta G_{iz}}{RT}} \quad \cdots\cdots eq.3$$

where A is the proportionality constant which depends on the analyte's surface activity, N_i is the number of moles, r is the droplet radius, ΔG_{iz} is the energy required for desorption of an ion, R is the Rydberg gas constant and T is the temperature. ΔG_{iz} is dictated by several properties of the droplet:

$$\Delta G_{iz} = \Delta G_{iz}^0 - zQ\Delta r^* / 4\pi\varepsilon_0 r^2 \quad \cdots\cdots eq.4$$

where G_{iz}^0 is the energy associated with transferring the ion from condensed phase to gas phase[7], z is the charge carried by the ion, Q is the excess charge on the droplet, Δr^* is the distance required for the ion to be free of the droplet, ϵ_0 is the permittivity of vacuum and r is the droplet radius.

Almost all the variables in the three major steps of the ESI process are dictated by the physiochemical properties and concentrations of the solvent and organic modifiers, which makes it extremely difficult to intuitively optimize the solution composition. In this report, several series of related ion-pairing agents and acidic modifiers at different concentrations were used to gain insight into how their properties influence the electrospray ionization desorption of oligonucleotides.

Results and Discussion:

The impact of ion-pairing agent volatility on the charge state distribution of oligonucleotides. As shown in **Figure 1a**, in the presence of HA, TPA or TBA the charge state distribution of a 24mer was reduced while DIPA and TEA did not affect the charge state distribution when compared to the mass spectrum obtained without the addition of any ion-

pairing agents (**Fig 1b**). Muddiman et al. [5] attributed the charge state reduction of an oligonucleotide to the neutral loss of organic base from the complex formed with the oligonucleotide, because the proton affinity of the base (piperidine and imidazole) was lower than the estimated proton affinity of the phosphodiester backbone (315 kcal/mol) [5]. However, as seen in Table 1, all the ion-pairing agents used in this study have proton affinities within a narrow range below 250 kcal/mol, even though not all of them have charge state reducting capabilities. In order to investigate the reason for this discrepancy, the assumptions made for the model proposed by Muddiman et al. have to be examined. There are three equilibriums in the liquid phase as shown in **Scheme 1**. The complex formed between the oligonucleotide and the ion-pairing agent (**Scheme 1c**) has to be the dominant ion species at the droplet surface for charge state reduction to occur after transmitting into the gas phase. This requires enough ion-pairing agents remain in the solution at the onset of the third stage of electrospray: ion emission. The concentration changes for solutes that are somewhat volatile during evaporation can be predicted by their Henry's law constant[14]: solutes with k_H^{cc} (aq/gas)>1 will enrich while k_H^{cc} (aq/gas)<1 will deplete as the droplet evaporates. As shown in Table 1, HA, piperidine and imidazole all have k_H^{cc} (aq/gas) values higher than 1 while those of the remaining ion-pairing agents are all below 1. Given this information, it is reasonable to conclude that HA reduced the charge state of the oligonucleotides via a similar mechanism as piperidine and imidazole. As the droplet evaporates, they are more likely to remain in the solution with the oligonucleotide and form the complex prior to desorption. When k_H^{cc} (aq/gas)<1 as DIPA and TEA, the ion-pairing agent is more likely to be removed from the droplet and shift the equilibrium of the protonation reaction (**Scheme 1b**) toward the left, resulting in a lower concentration of protonated ion-pairing agent ions. Consequently, the equilibrium shown in **Scheme 1c** will also shift to left,

leaving unconjugated oligonucleotide anions as the dominant species at the droplet surface prior to ion emission. As a result, the charge state distribution will remain the same in the presence of the ion-pairing agent.

However, attributing charge state reduction solely to high k_H^{cc} (aq/gas) values does not explain why TPA and TBA also shifted the charge state distribution of oligonucleotides since they have the lowest k_H^{cc} (aq/gas) values among all of these ion-pairing agents. Fenn [23] argued that the charge state of a particular molecule is also dependent on the charge density at the time of ion emission. At the early stage of droplet evaporation, the probability for an ion to carry a higher number of charges is low and an ion with less charges (smaller z) is less likely to desorb from a droplet. However, if the surface charge density (Q) is sufficiently strong from the early stage of droplet evaporation, ΔG_{iz} will become low enough that ions that carry fewer charges could be desorbed. For ion-pairing agents that will be depleted during droplet evaporation their surface concentration would be lower than in the bulk solution due to continuous evaporation and incomplete diffusion. If they diminish quickly, they can shift the equilibrium in **equation 1b** and **1c** to left at a high rate. The deprotonated oligonucleotide anion concentration and charge density would then increase rapidly at the droplet surface relative to the bulk solution with ion-pairing agents of lower k_H^{cc} (aq/gas) values. Desorption of these anions would therefore occur at an earlier stage during the evaporation process and lower charge states would be observed. This also explains why in the mass spectra obtained with TPA and TBA, the lowest charge state is more abundant than in mass spectra acquired with HA.

Mechanism of signal enhancement and suppression effect of ion-pairing agents. Figure 2 shows the base peak (m/z 640) intensity of a 24mer in 50% methanol with 1-30mM of three different tertiary amines as the ion-pairing agents. It should be noted that the charge state

distribution did not change with the type or concentration of ion-pairing agents in this figure. DIEA enhances the signal intensity the most, followed by DMBA then TEA. The signal was first enhanced then slightly suppressed with an increase in ion-pairing agent concentration. The data in **Figure 2** were obtained using the mass spectrometer settings described in the experimental section. However, it should be noted that the trends observed in this figure are the same even when the source conditions are optimized for each ion-pairing agent. For all three of these ion-pairing agents, the charge state distributions of the oligonucleotide are identical to mass spectra acquired without any ion-pairing agents added to the mobile phase (data not shown). The nature of the signal enhancing effects of alkylamines could be physical or chemical. Physically, the conductivity of the sprayed solution would increase with an increase in ion-pairing agent concentration, resulting in smaller droplet sizes and faster droplet fission as shown in **equation 1** and **2.** Chemically, the ion pairing agents could assist the oligonucleotide to approach the droplet surface by forming a concentration gradient from the surface to the interior of the droplet and shifting the equilibriums in **Scheme 1** as described in the previous paragraph. Higher surface concentrations of oligonucleotides would increase the analyte partitioned to the fissioned droplets and the fraction of surface charge carried by the analyte therefore increasing the ESI response [7].

The physical aspect of this enhancement effect is supported by an experiment where the impact of gas flow rate was evaluated using DIEA (**Fig 3**). For each concentration of DIEA, the obtained signal intensity was normalized to the percentage of the signal intensity obtained with 700L/hr desolvation gas flow rate. As seen in **Figure 3**, the lower the concentration of DIEA, the greater the increase in observed signal intensity as the gas flow rate is elevated. The role of the desolvation gas is to accelerate the evaporation of the sprayed droplets and to decrease the

droplet radius faster. When the concentration of the ion-pairing agent is high, the evaporation rate would be fast enough, even at low gas flow and would be less affected by the increase of desolvation gas rate.

The chemical aspect of this enhancement effect was investigated by studying the impact of oligonucleotide sequence composition with respect to the concentration of DIEA (**Fig 4**). For each sequence, the obtained signal intensity was normalized to the percentage of the highest signal intensity. As seen in **Figure 4**, the signal intensity of T18 is least affected the by the concentration changes of DIEA, followed by a mixed base 18mer and C18 was affected most significantly by the concentration change of DIEA. Muddiman et al.[7] pointed out that the more hydrophobic bases in a nucleic acid sequence would increase its surface concentration and therefore increase its ESI response. By this rationale, the surface concentration of the three oligonucleotide would decrease as T18>mixed base 18mer>C18 because the hydrophobicity of the bases decreases as T>A>>C>G [7]. Therefore, T18 would be least reliant on the assistance of the ion-pairing agent, which increases with the concentration, to get on the surface of the droplet while C18 would be most reliant on it. The same mechanism could be used to explain the difference in signal enhancing capability of the three alkylamines (**Fig 2**). The k_H^{cc} (aq/gas) decreases as TEA>DMBA>DIEA. The ion-pairing agent with the lowest k_H^{cc} (aq/gas) would generate the steepest concentration gradient from the surface to the center and increase the surface concentration of oligonucleotide most.

It is worth noting that due to the formation of ammonium ions from protonation and the alkyl chain, all of these ion-pairing agents are somewhat amphiphilic and tend to accumulate on the surface of the droplet at higher concentrations. When the surface excess of the alkylamines is too high, they could prevent the analyte from desorbing because the analyte cannot gain sufficient

access to the surface, making charging more difficult and lowering ESI response [24]. This is also supported by the fact that the signal intensity of T18 is suppressed by the increase of DIEA concentration to the lowest extent while C18 and 18mer were suppressed more significantly. When more hydrophobic ion-pairing agents such as TBA were used the signal suppression at higher concentrations were even more pronounced (data not shown). Given these effects mentioned above, an ideal ion-pairing agent should have a relatively low k_H^{cc} (aq/gas) but moderate hydrophobicity although it would be difficult to change one parameter without altering the other. An ion-pairing agent with a k_H^{cc} (aq/gas) that is too low will reduce the charges carried by the oligonucleotide ion (smaller z) and lower the signal intensity. An ion-pairing agent that is extremely hydrophobic would prevent oligonucleotides from reaching the droplet surface and therefore suppress its ionization.

The "wrong way around ionization" effect of HFIP. Before Apffel et al. introduced HFIP as the acidic modifier, various organic carboxylic acids were most commonly used to adjust the mobile phase pH, [5, 11, 25]. Signal suppression was observed for oligonucleotides when using carboxylic acids due to the competition for ionization[5]. Apffel proposed that the advantage HFIP offered for ESI of oligonucleotides derived from its lower boiling point relative to TEA. This statement assumes that HFIP is merely an acidic modifier used to adjust the pH of the mobile phase to achieve better chromatographic peak shape and needs to be depleted for efficient ionization of oligonucleotides to occur. Cech et al.[26] suggested another fluorinated alcohol, trifluoroethanol(TFE), could be used as an alternative to HFIP for ESI of oligonucleotides although no details of the study were published. Considering the boiling point of TFE is also lower than TEA, we compared it to HFIP to assess its impact on the ESI of oligonucleotides. As shown in **Figure 5**, the signal intensity of the base peak (m/z=640) increased with higher

concentrations of HFIP but decreased with higher concentrations of TFE. Although neither HFIP nor TFE changed the number of charges of the base peak over the concentrations range tested, the relative abundance of the adjacent peaks to the base peak was shifted slightly in the presence of HFIP and TFE (**Figure 6**).

Given this result, HFIP does not appear to compete with oligonucleotides for ionization but rather facilitates its ionization instead. In negative ion ESI, the dominant reaction is electrochemical reduction. In solutions without any acidic modifier, protons provided by water and other solvents are presumably reduced to form hydrogen. The additional protons provided by an acidic modifier facilitate this reduction, making it easier for the sprayed droplets to carry excess negative charge[24]. Although both HFIP and TFE are weak acids with boiling points below TEA, the difference in their pKa (9 for HFIP and 12 for TFE) resulted in a dramatic difference in the amount of protons they can provide at the capillary tip. This is also supported by the increased signal intensity of the lower charge states observed with increases in the HFIP concentration and the nearly unaltered charge state distributions at all TFE concentrations (**Figure 6**). However, TFE is a weak acid which still provides more protons than a solution without it. Furthermore, stronger acids such as formic acid and acetic acid reportedly suppress the ionization of oligonucleotides [5, 11]. Suppression of oligonucleotide ionization does not correlate with the boiling point of the acidic modifier, which bracket the values of TEA (TFE=73 ℃, FA=100 ℃, HAc=118 ℃), because boiling point is more of a reflection of the interaction between the molecules of the acidic modifiers than with solvents. The k_H^{cc} (aq/gas) values of HFIP, TFE, FA and HAc are 0.96, 2.38, 216, and 208, respectively. For the reasons explained in the previous section, all other acidic modifiers would form a reservoir of intact molecules to be deprotonated at the droplet surface where the electrochemical reduction of the

protons drives the equilibrium $HA \rightarrow H^+ + A^-$ to the right and competes with oligonucleotides for ionization. HFIP, on the other hand, was depleted fast enough not to compete with oligonucleotides while providing more protons to facilitate the ionization of oligonucleotides. It is the rare combination of moderate acidicity and volatility (k_H^{cc} (aq/gas)) that enables HFIP to be such an optimum acidic modifier and this combination has yet to be reproduced by any other compounds.

Comprehensive Effect of ion-pairing agent and HFIP concentration. In addition to evaluating the impact of ion-pairing agents and HFIP on the ESI of oligonucleotides separately, different concentrations of ion-pairing agents and HFIP were also used to study the possible interactions between them and the corresponding impact on the ESI desorption of oligonucleotides. As shown in **Figure 7a**, as the concentration of DIEA increases, the signal intensity first increases then decreases at all HFIP concentrations. The optimum concentration of HFIP became lower at higher DIEA concentrations.

The free proton concentration in basic solutions was much lower than that in water[24]. As a result, the addition of small amounts of HFIP dramatically increased the signal intensity due to the free protons provided for electrochemical reduction. Meanwhile, a small amount of deprotonated HFIP ions also competed with the oligonucleotides for ionization but not to the extent of outweighing the signal enhancement effect or even causing signal suppression until the concentration was higher. In the presence of ion-pairing agents, the pH of the bulk solution was higher (**Figure 7b**), causing more HFIP to be ionized and to compete with the oligonucleotide at the droplet surface versus the absence of the ion-pairing agent. Although the solution composition at the electrospray droplets surface was usually different then in the bulk solution, the changes in bulk solution could still be reflected in the electrospray behavior of the analytes.

This hypothesis was in agreement with the signal intensity changes observed in **Figure 7a**. Even with only 1mM DIEA, signal suppression was observed at 100mM of HFIP, which did not occur in the absence of DIEA. The signal enhancement effects peaked at 10mM of DIEA and were attenuated as the concentration of DIEA increased. This was a reflection of the process of the signal enhancing effect caused by the higher free proton concentration gradually being offset by the signal suppression effect caused by the increasing concentration of HFIP anions. This process could also be attributed to the drop of optimum concentration of HFIP from 50mM to 20mM when the concentration of DIEA was above 20mM. The results from this experiment suggest that the interactions of ion-pairing agents and the acidic modifier should also be taken into consideration when optimizing the mobile phase composition.

Conclusion:

In this study, we established a new model for the electrospray desorption behavior of oligonucleotides that explained their interaction with various ion-pairing agents. We found that the Henry's law constant more accurately predicted the behavior of ion-pairing agents at the interphase between the electrospray droplet and the gas phase, which was critical in explaining the differences in oligonucleotide ionization with different types and concentrations of ion-pairing agents. Compared to previously proposed mechanisms, this model explained both charge state distribution and ionization efficiency of oligonucleotides with a wider range of organic bases. Furthermore, we found several ion-pairing agents that demonstrated improved performance when compared to traditional TEA modifiers.

This study also reported on the "wrong way around" ionization phenomenon for oligonucleotides. This signal enhancement effect was also dependent on the low Henry's law constant of HFIP and its moderate acidity. The previously prevalent belief that the lower boiling point of HFIP was

responsible for its superiority over carboxylic acids was shown to be untenable. The signal enhancement effects of HFIP should be carefully evaluated with the presence of ion-pairing agents as their basicity can increase the magnitude of the ionization of HFIP in bulk solution and result in competition with oligonucleotides for ionization.

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Scheme 1. Equilibriums in the liquid phase (IP=ion-pairing agent)

$$Oligo \ + nH_2O \Leftrightarrow Oligo^{n-} + nH_3O^+ \quad \text{.....(a)}$$

$$IP + H_2O \ \Leftrightarrow HIP^+ + OH^- \quad \text{.....(b)}$$

$$HIP^+ + Oligo^{n-} \Leftrightarrow \left[Oligo \Lambda \ H \Lambda \ IP\right]^{(n-1)-} \quad \text{.....(c)}$$

Table 1.

Compounds	hexylamine	diisopropylamine	triethylamine	dimethylbutylamine	diisopropylethylamine	tripropylamine	tributylamine
k_H^{cc} (aq/gas)[a]	1.57	0.42	0.35	0.27	0.19	0.10	0.08
Boiling point(℃)[b]	131	105	88	93	127	156	216
proton affinity(kcal/mol)[b]	221	232	234	231	237	236	238

[a] k_H^{cc} (aq/gas) values were calculated using using the US Environmental Protection Agency's EPISuite™

[b] b.p and proton affinity were obtained from NIST chemistry webbook---NIST Standard Reference Database Number

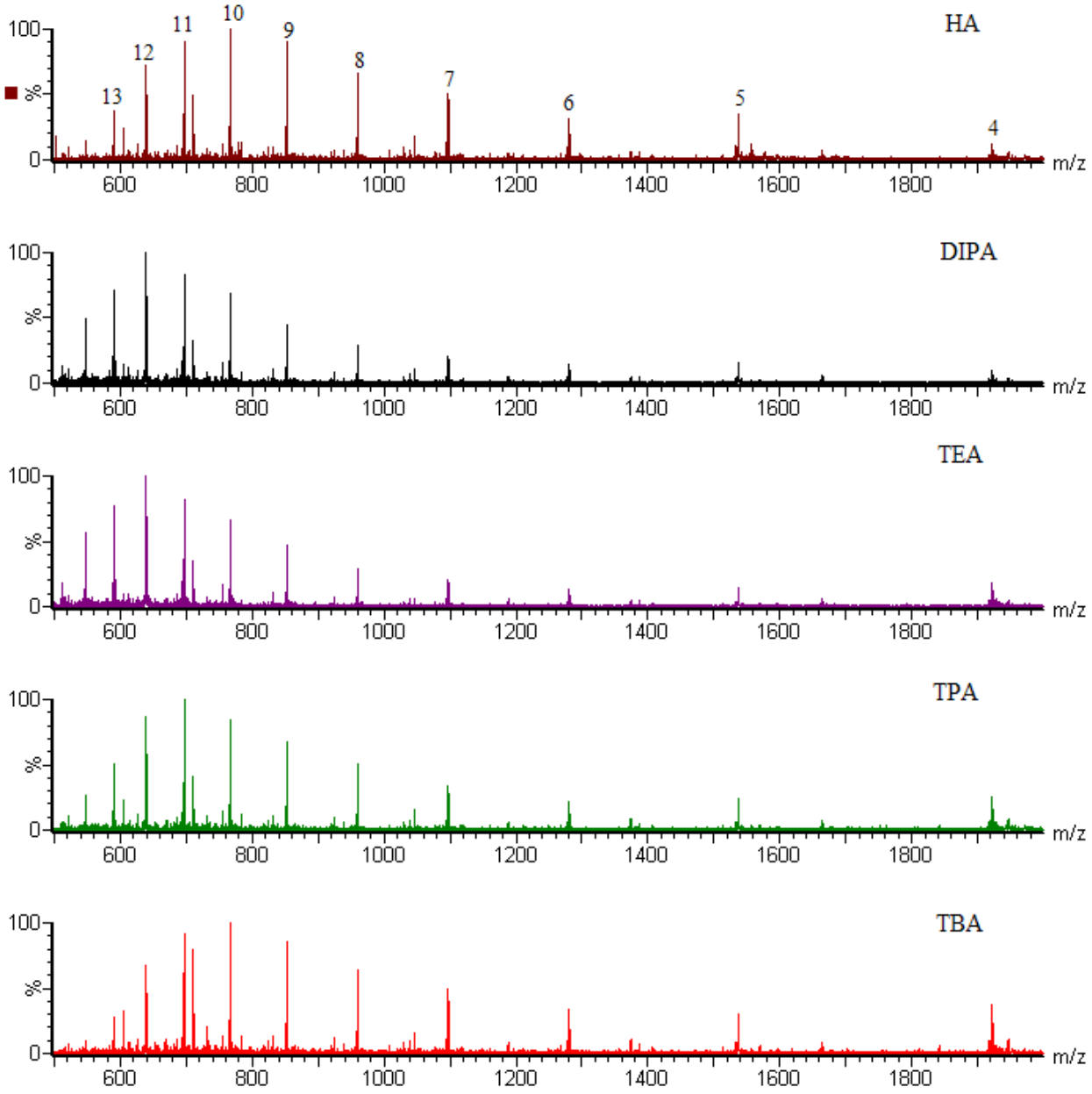

Figure 1a. Charge state distribution of the 24mer oligonucleotide in 15mM of different alkylamine ion-pairing agent in MeOH: H_2O=50:50

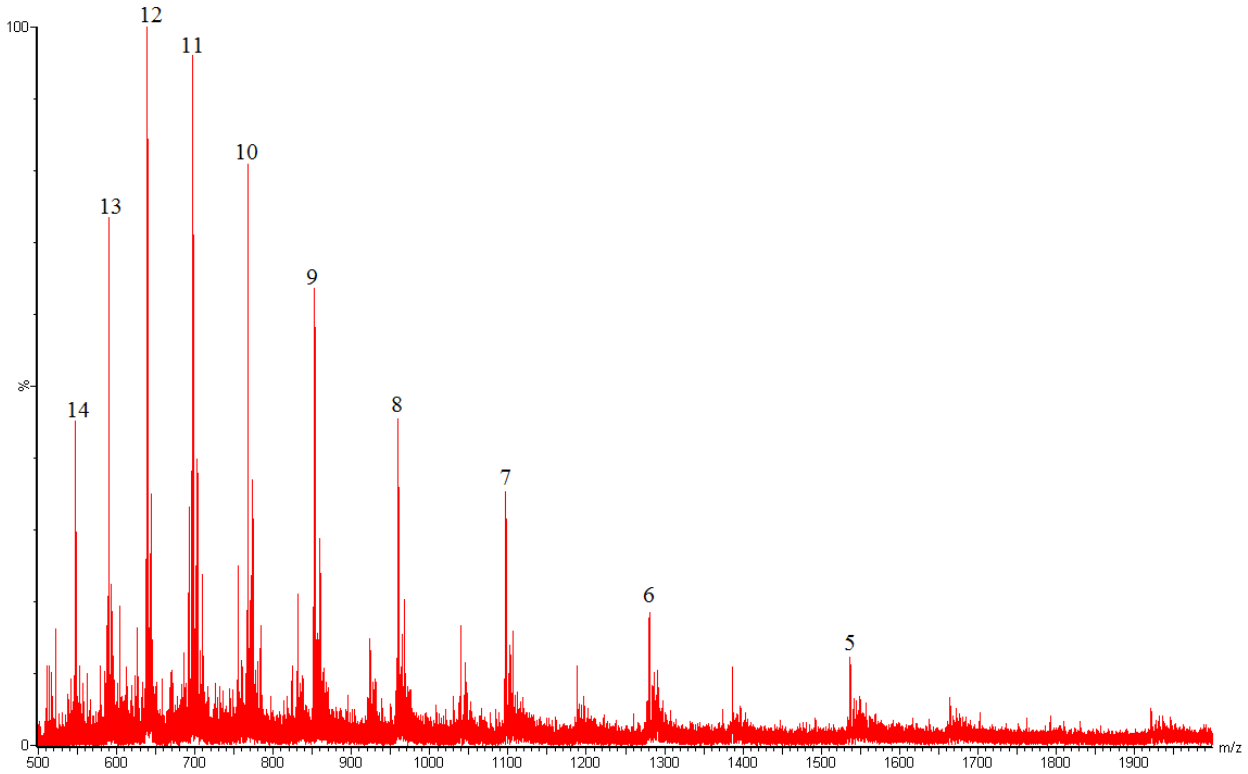

Figure 1b. Charge state distribution of the 24mer oligonucleotide in MeOH: H_2O=50:50

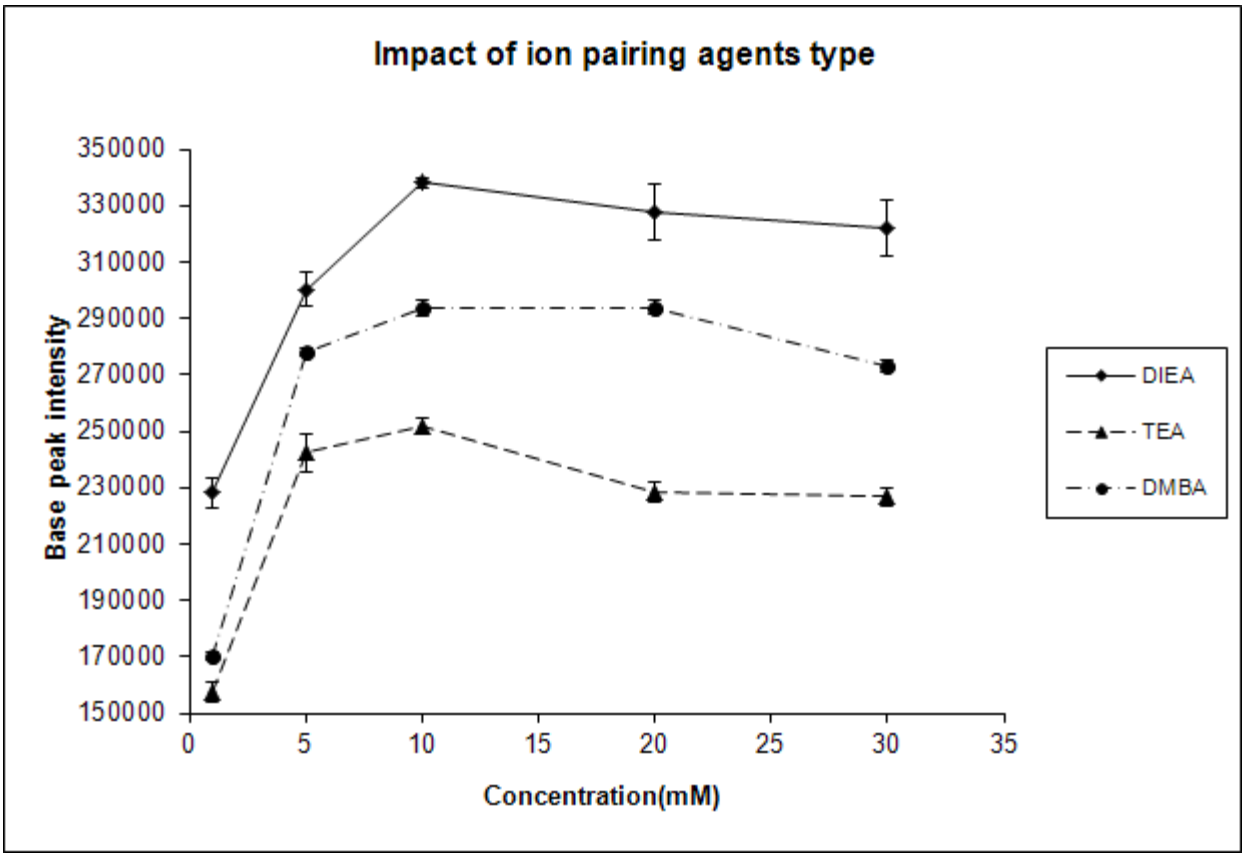

Figure 2. Signal intensity of the 24mer oligonucleotide in 15mM of different alkylamine ion-pairing agent in MeOH: H_2O=50:50

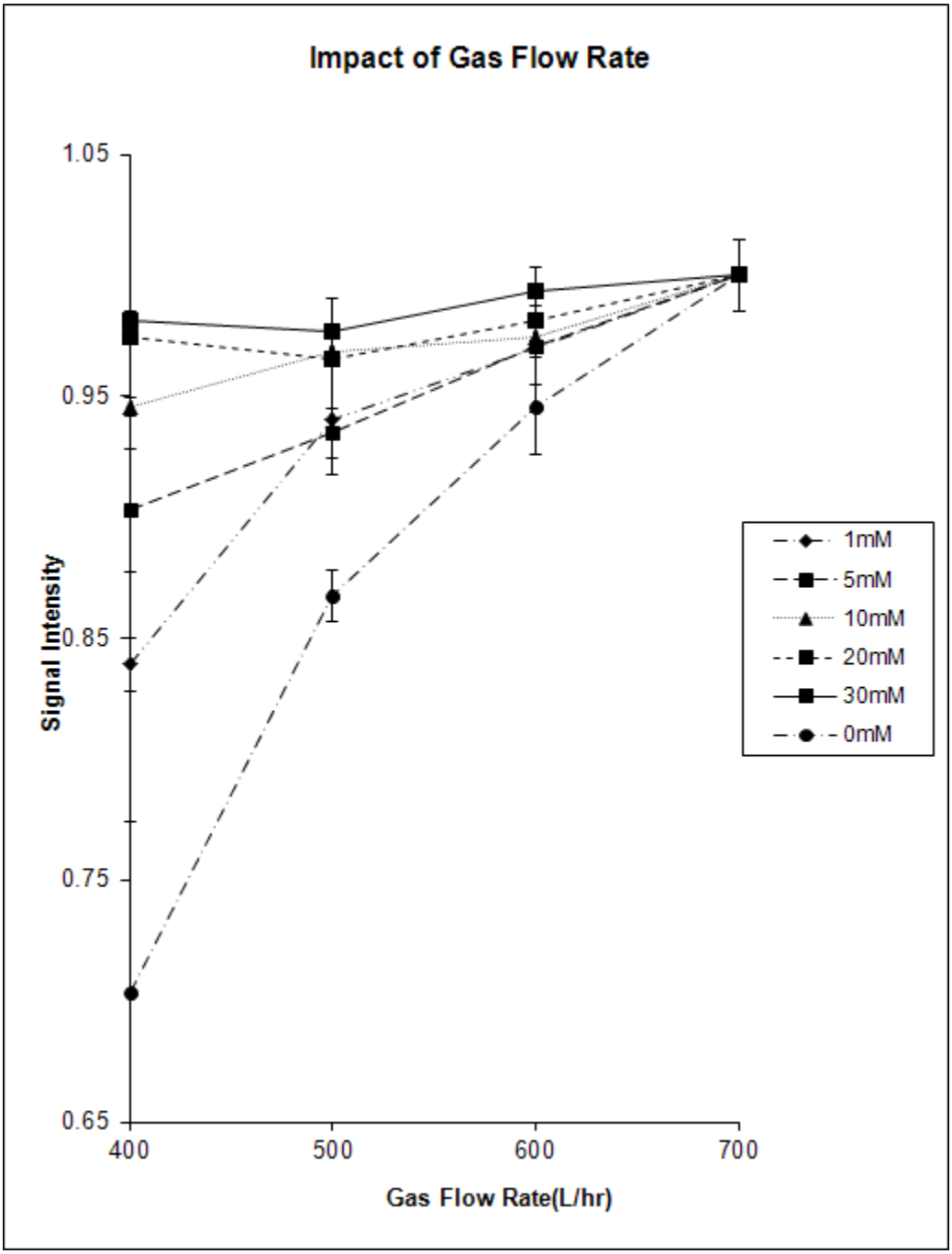

Figure 3. Signal intensity ratio of the 24mer oligonucleotide in 0-30mM of DIEA in MeOH: H_2O=50:50 at different desolvation gas flow rate (The signal intensity of 24mer at 700L/h desolvation gas flow rate is used as 100%)

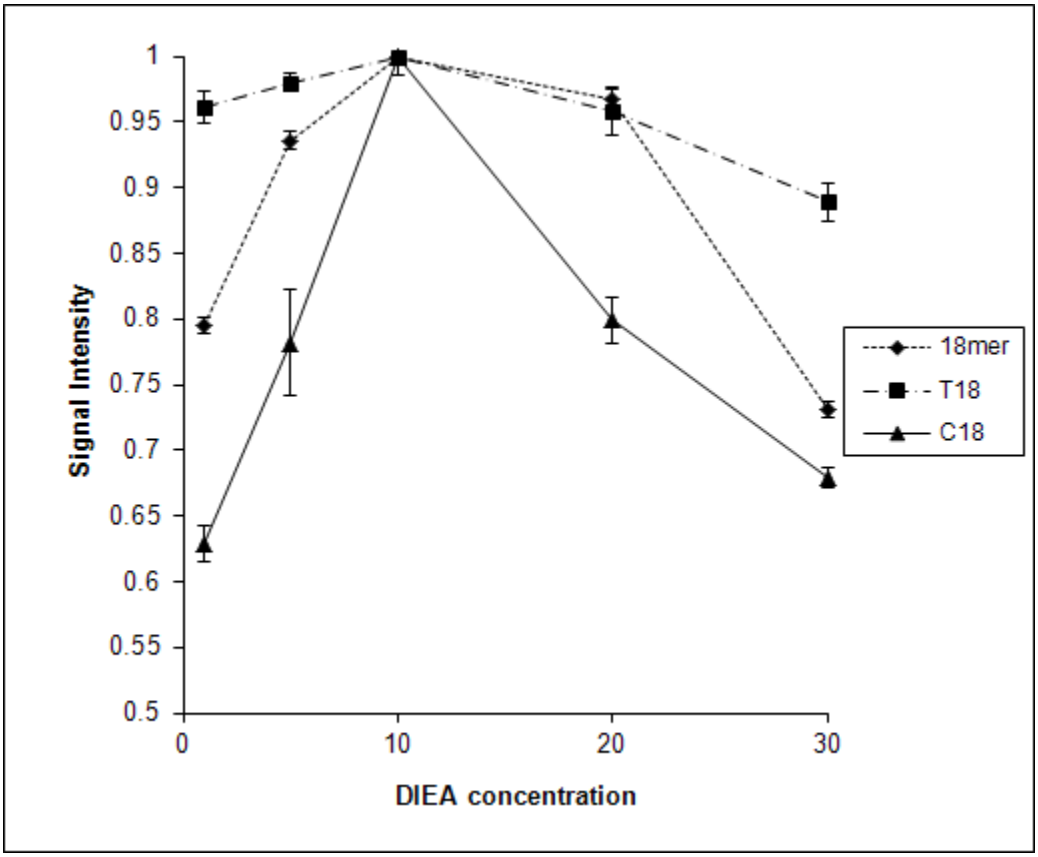

Figure 4. Signal intensity ratio of a poly C18, a poly T18 and a mixed base 18mer oligonucleotides in 0-30mM of DIEA in MeOH: H_2O=50:50 (The signal intensity of at 10mM DIEA is used as 100%)

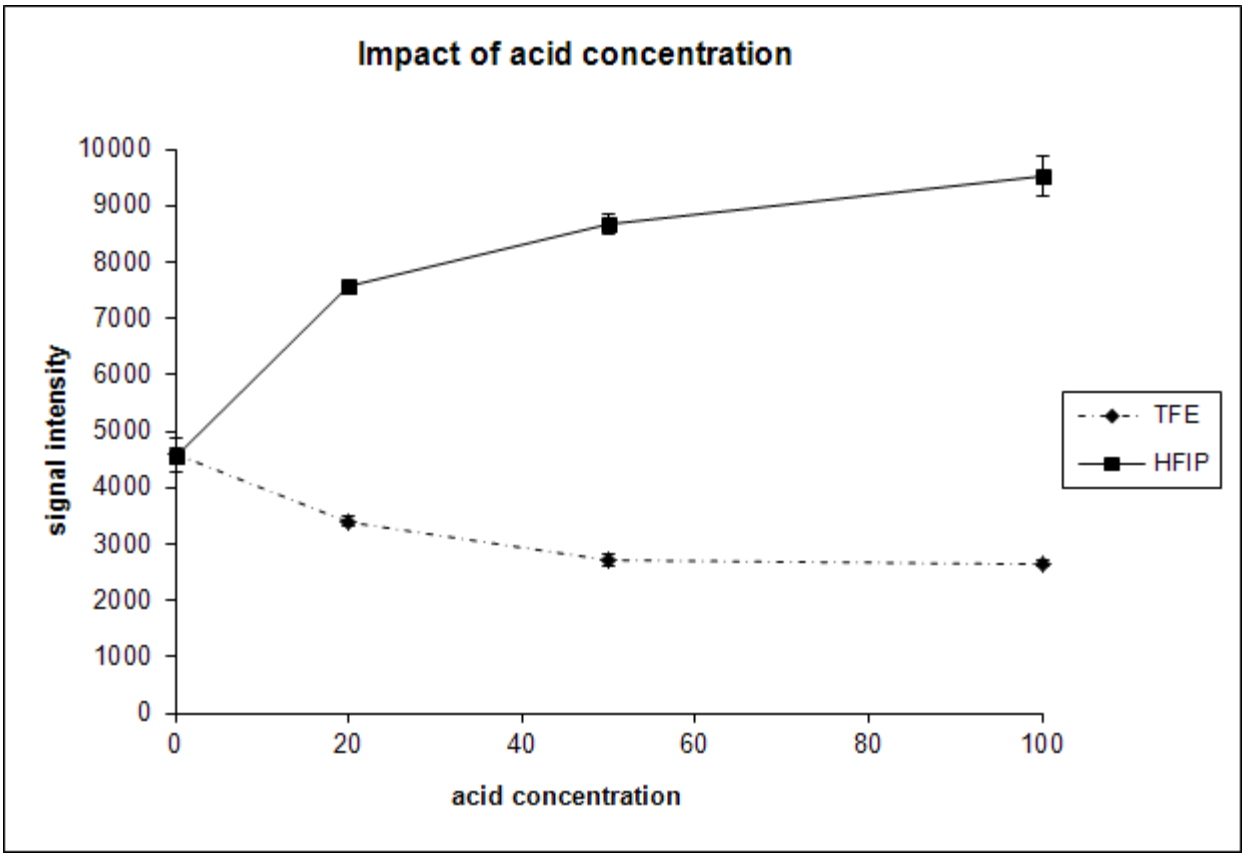

Figure 5. Signal intensity of the 24mer oligonucleotide in 0-100 mM of TFE or HFIP in MeOH: H_2O=50:50

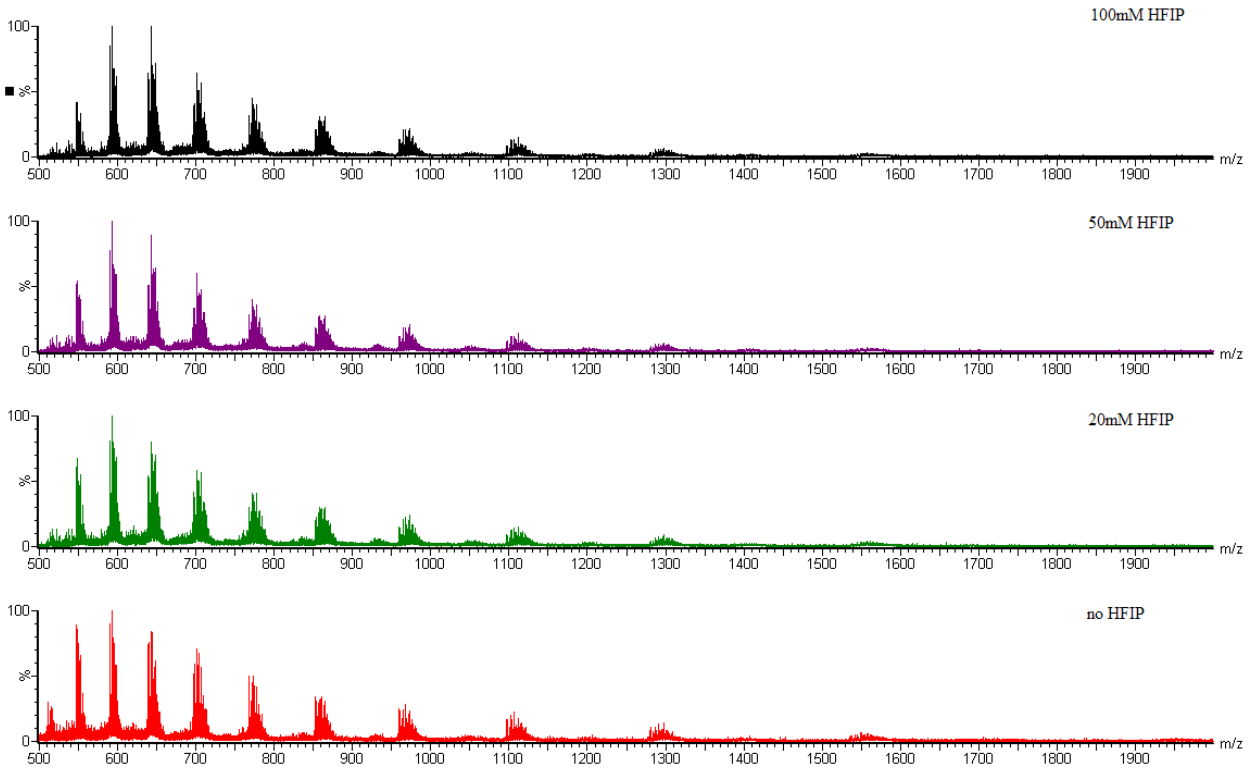

Figure 6a. Charge state distribution of the 24mer oligonucleotide in 0-100 mM of HFIP in MeOH: H_2O=50:50

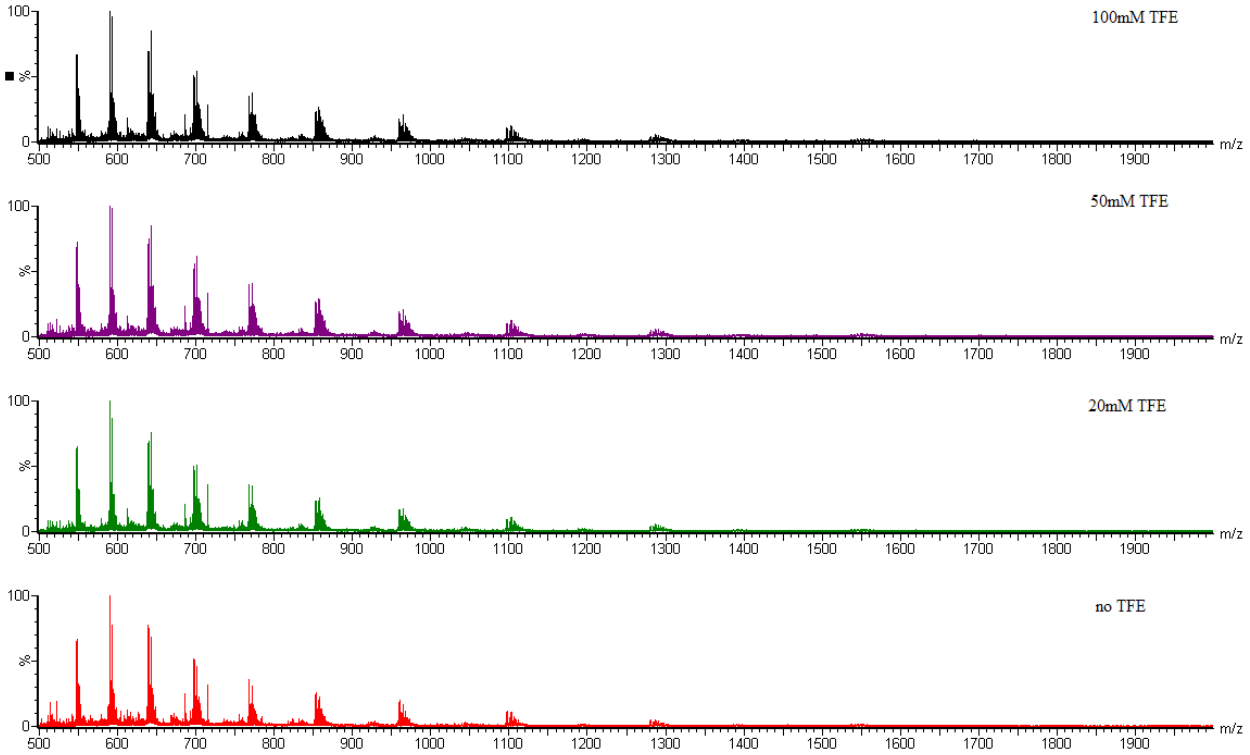

Figure 6b. Charge state distribution of the 24mer oligonucleotide in 0-100 mM of TFE in MeOH: H_2O=50:50

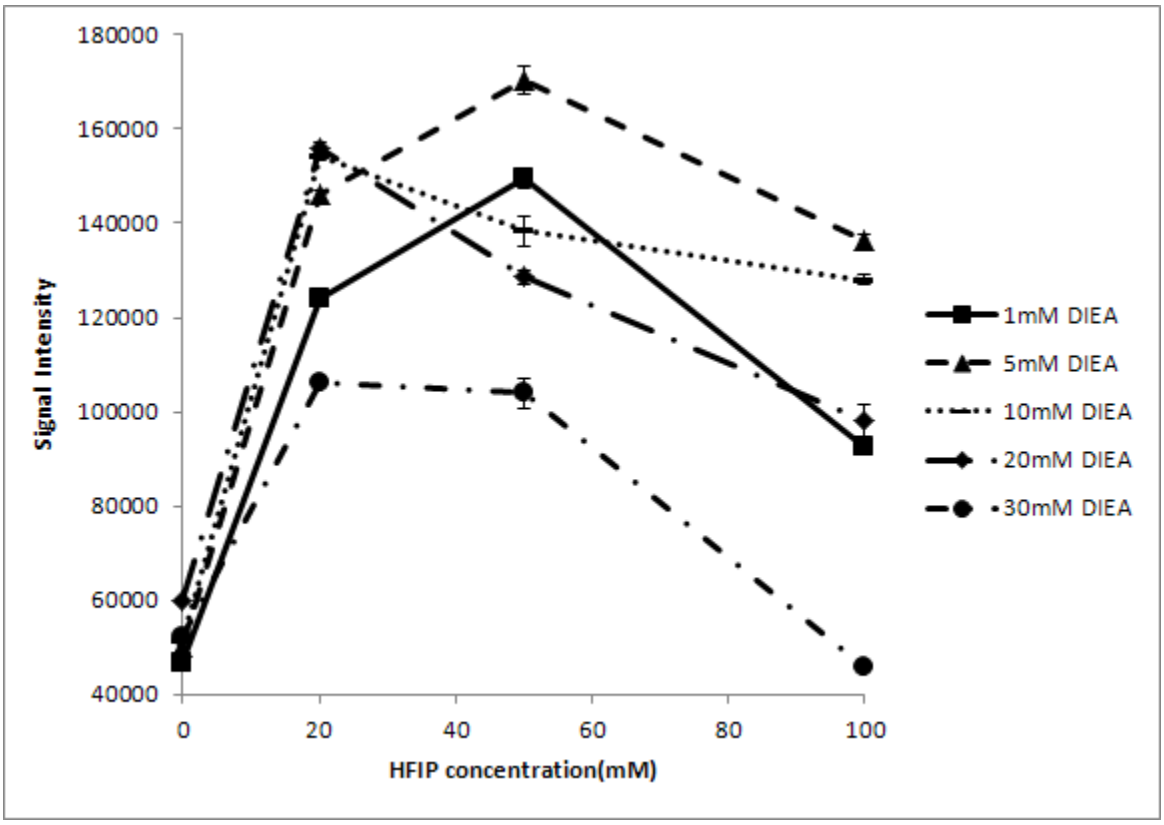

Figure 7a. Signal intensity of the 24mer oligonucleotide in 0-100 mM of HFIP and 0-30mM DIEA in MeOH: H_2O=50:50

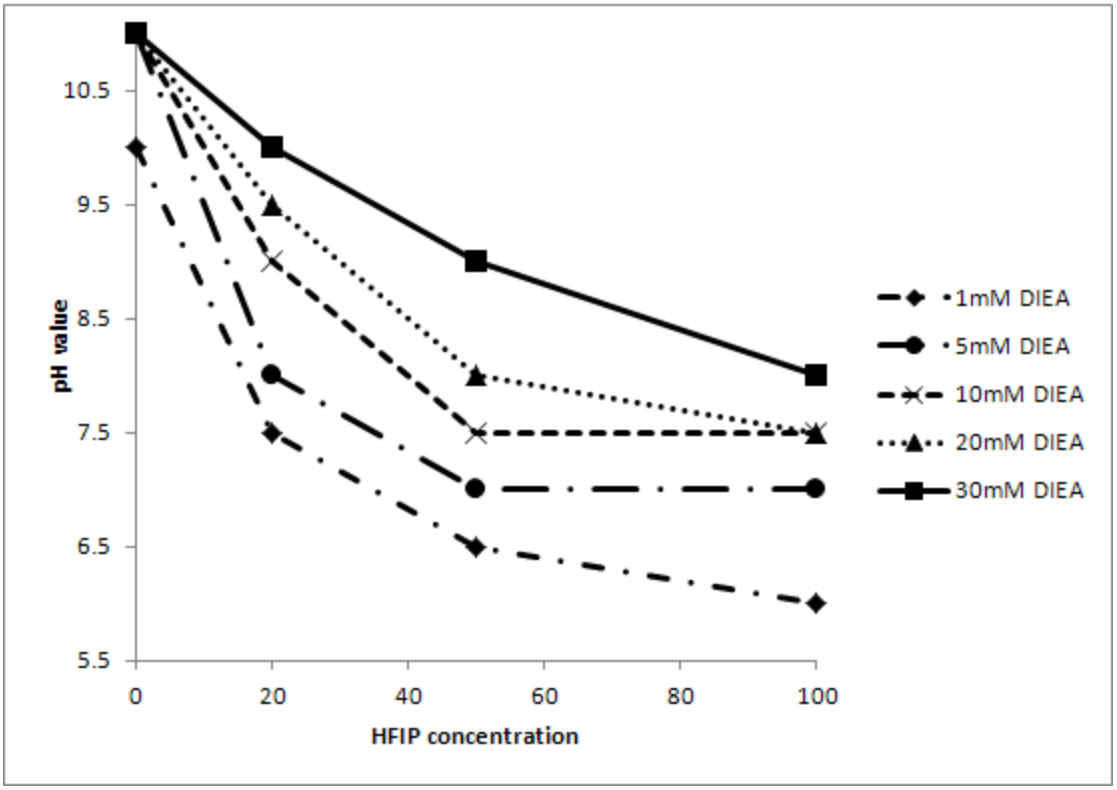

Figure 7b. pH value of different combinations of 0-100 mM of HFIP and 0-30mM DIEA in water

Chapter 4

A One-Step Solid Phase Extraction Method for Bioanalysis of a Phosphorothioate Oligonucleotide and its 3'n-1 Metabolite from Rat Plasma by uHPLC-MS/MS

Chen B and Bartlett MG. Submitted to AAPS Journal

Abstract:

Oligonucleotide therapeutics have emerged as a promising class of drugs to treat a wide range of diseases caused by genetic abnormalities. Replacement of the phosphodiester linkage with a phosphorthoate is one of the most successful modifications made to oligonucleotides to enhance their *in vivo* stability. The longer elimination phase of phosphorothioates and other modified oligonucleotides requires sensitive and selective methods to quantify the parent drug and their metabolites simultaneously. Liquid chromatography tandem mass spectrometry (LC-MS/MS) has excellent selectivity between the parent drug and its metabolites and a wide dynamic range. However, the biological sample extraction remains a formidable challenge in developing quantitative LC-MS methods for oligonucleotides. Protein precipitation, protein digestion, liquid-liquid extraction (LLE), reversed phase solid phase extraction (SPE), strong anion exchange SPE and combinations of them have been reported to extract oligonucleotides from biological matrices. Unfortunately, these methods either have low recoveries or present potential problems for applications with chromatography due to the large amount of matrix substances in the resulting solutions. In this study, a weak anion exchange (WAX) SPE method was optimized. The recovery ranged from 60% to 80% depending on the concentration. This is the first report of a one step SPE method with recoveries greater than 60% across the method dynamic range. This sample extraction procedure was used in combination with ultrahigh performance liquid chromatography tandem mass spectrometry. The lower limit of quantitation was 10 ng/mL (1.3 nM) and the dynamic range was 10-1000 ng/mL. The intra-and inter-day precision and accuracy were within 8.4% and 10.5%, respectively.

Introduction:

In recent years, more and more diseases have been found to have a significant genetic component. Oligonuleotides (OGN) designed to silence certain disease causing genes have emerged as a class of versatile therapeutic agents. They have been used to treat a wide range of diseases including cancer, AIDS, Alzheimer diseases and cardiovascular disorders. [1,2] Natural oligonucleotides are susceptible to nuclease degradation and have poor biostability. Thus various modifications have been introduced to enhance the stability of oligonucleotide therapeutics. The phosphorothioate (PS) backbone was one of the first modifications used for OGN and are still commonly used alone or in combination with other modifications. The phosphorothioate linkage increases nuclease resistance and significantly prolongs the plasma half life of OGN and the elimination phase when compared to phosphodiester linked OGN therapeutics[1]. Therefore, a sensitive and selective bioanalytical method is necessary for quantitative analysis. The hybridization method is very sensitive and has remained the gold standard for quantifying oligonucleotide therapeutics for clinical studies. However the selectivity is not as desirable as traditional LC-MS methods. [3, 10]

One of the major shortcomings of LC-MS methods are the extensive sample clean-up procedures required to achieve the necessary reproducibility and recovery. Due to the extensive binding to proteins by OGNs, direct protein precipitation and reversed phase solid phase extraction were reported to have low recoveries (<10%).[3] Strong anion exchange (SAX) extraction has been reported to extract PS-OGNs from plasma and urine. [4] Although the extraction procedure was fairly quick (5min), the high salt concentration used in the method requires further clean-up steps to be applied in LC-MS methods, which may result in recovery loss. Dai et al. [5] employed a one-step ion-pair reversed phase solid phase extraction using triethylammonium bicarbonate (TEAB) as an ion-pairing agent. However the recovery at lower concentrations was reported to be only 43% due to irreversible binding to the SPE cartridges. Proteinase K digestion combined with liquid-liquid extraction (LLE) was reported to have high recoveries (98%). [6,7] However, proteinase K digestion involves long incubation times (>10hrs), which would affect the throughput in preclinical and clinical studies. In addition, we have observed both proteinase K digestion and phenol-chloroform LLE produced samples with complicated matrix backgrounds.

These endogenous substances could cause interferences during LC separations and blockage of small particle uHPLC columns. Zhang et al. [3] reported a sample extraction procedure that combines phenol-chloroform LLE and ion-pair reversed phase SPE and resulted in relatively high recovery across the dynamic range (72-85%). Nevertheless, the multiple extraction and sample transfer steps in this method are arduous and time consuming.

In our study, we used a 24mer phosphorothioate oligonucleotide as a model compound and developed a one-step solid phase extraction method. The sample extraction procedure resulted in equivalent recovery and similar lower limit of quantitation (LOQ) from rat plasma as the LLE-SPE method reported by Zhang et al. [3] and is much simpler. Because of the use of a lysis buffer which dissociates the OGN from plasma proteins before the SPE step, sample transfer steps were eliminated. The various conditions were optimized to improve the recovery and specific issues related to this sample preparation method were investigated.

Materials and Methods

Materials

The 24mer PS-ODN (5'-TCGTGCTTTTGTTGTTTTCGCGTT-3') its 23mer 3'n-1 metabolite and internal standard (IS) pdT40 (PAGE purification, purity>90%) were purchased from Integrated DNA Technologies (Coralville, IA). Chemicals such as 1,1,1,3,3,3-hexafluoro-isopropanol (HFIP), di-isopropylethylamine (DIEA), tetrahydrofuran (THF), ammonium acetate, ammonium bicarbonate and tris (2-carboxylethyl)phosphine (TCEP) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). Clarity OTX lysis buffer and SPE cartridges were obtained from Phenomenex Inc. (Torrance, CA).

Instrumentation and LC-MS conditions

uHPLC-MS/MS analysis was performed on a Waters Acquity uHPLC system coupled with a Waters Synapt G2 qTOF mass spectrometer with electrospray (ESI) source(Milford, MA). Chromatographic separations were performed at a flow rate of 0.15 mL/min on a 1.7 μm Waters Acquity BEH C18 column (Milford, MA) 100 mm×1.0 mm column. Mobile phase A consisted of 15.7 mM DIEA and 50 mM HFIP in water, and mobile phase B consisted of the same

concentration of DIEA and HFIP in water: acetonitrile (50:50). A 20 µL injection of each sample was loaded onto the column and separated using the following gradient conditions [time (min), % mobile phase B]: (0, 5) (1,5) (2, 29) (4, 29) (5, 40) (5.01, 5) (9, 5). The column temperature was maintained at 60 ℃. The column eluent from 0-1.5 min was diverted to waste. The weak and strong washes for the autosampler needle were 600 µL water and 200 µL 5% methanol respectively. The system was operated in the negative-ion MS/MS mode with a 1 second scan time. The MS/MS transition optimized for the 23mer, 24mer and internal standard were m/z 590→319 (0-4.5 min), 667→319 (0-4.5min) and 604→319 (4.5-9 min) respectively with targeted mass enhancement at m/z 319. The capillary voltage was set at 2.3 kV, the cone voltage was 20 V, the extraction cone voltage was 2 V, the trap collision energy was 20 eV, the transfer collision energy was 0 eV, the source temperature was 120 ℃, the desolvation temperature was 450 ℃, cone gas was 6 L/h and desolvation gas was 700 L/h.

Preparation of stock solutions, working solutions, calibration and QC samples

23mer and 24mer solutions were prepared in deionized water to give a final concentration of 1 mg/mL. IS stock solution concentration was 200 µg/mL. Work solutions of the 23mer and 24mer were prepared by spiking 2 µL of their stock solutions respectively into 196 µL of plasma. The calibration samples with concentrations of 10, 20, 50, 100, 200, 500 and 1000 ng/mL and QC samples with concentrations of 10, 30, 150, 750 ng/mL were prepared by spiking appropriate amount of work solution of 23mer and 24mer. 100 µL of these solutions were then spiked with 1 µL of IS work solution before extraction.

Sample preparation

The equilibration buffer was made by dissolving 0.79 g ammonium acetate with 200 mL deionized water (50 mM NH_4OAc) and adjusting the pH with acetic acid to 5.5. The washing buffer was made by mixing 100 mL of equilibration buffer with 100 mL acetonitrile. The elution buffer was made by dissolving 0.78 g ammonium bicarbonate and 0.028 g TCEP (100 mM NH_4HCO_3, 1 mM TCEP) in 50 mL of water, adjusting the pH with ammonium hydroxide to 9.5 and mixing with 40 mL of acetonitrile and 10 mL of THF. The SPE cartridges were conditioned using 1 mL of methanol and 1 mL equilibration buffer sequentially. Plasma samples were then mixed with 200 µL of Clarity OTX buffer and loaded onto the column. The cartridges were

washed by 3 mL of washing buffer and the analytes were eluted by 0.5 mL×2 of elution buffer. The collected solutions were evaporated to near dryness under vacuum and reconstituted with 50 μL of deionized water. 20 μL of the reconstituted solution was injected into the uHPLC-MS/MS system for analysis. A schematic is shown to illustrate this process (Figure 1).

Method Validation

Plasma calibration curves were constructed using peak area ratios of the 23mer and 24mer to the IS and applying a weighted ($1/x^2$) linear regression. Precision (% RSD) and accuracy (% error) were calculated for the four QC samples (concentrations of 10, 30, 150, and 750 ng/mL). Five replicates of each QC point were analyzed each day to determine the intra-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. The absolute and relative recoveries and matrix effects were determined for spiked plasma samples (n =5) at concentrations of 10, 30, 150, and 750 ng/mL. Absolute recovery was calculated as the peak area for 23mer and 24mer in the plasma sample spiked before extraction, divided by the peak area of a water solution of the same concentration. Relative recovery was calculated by dividing the peak area for a sample spiked with 23mer and 24mer before extraction by the peak area for an equal concentration sample in the same matrix spiked after extraction. Matrix effects were calculated by dividing relative recovery by absolute recovery.[8] Stability of 23mer and 24mer during freeze-thaw (3 cycles), at room temperature (8 h), and in an autosampler (24 h) (n =5) were also determined and reported.

Method application

The validated analytical method was applied to an animal experiment where a single i.v. administration of 1.0 mg/kg 24mer to a male Sprague Dawey rat weighing ~160 g. 24mer in 0.9% sterile saline was given as an i.v. bolus dose at 1.0 mg/kg through the neck catheter. The dose volume was 1.25 mL/kg. 0.5mL of blood was withdrawn from jugular vein according to a schedule of 0 (predose), 5, 15, 30, 60, 120, 180 minutes after dosing. The blood samples were centrifuged at 11,000 × g for 5 min and plasma were collected and kept frozen at −20 °C until analysis.

Results

Selecting the optimum mobile phase composition and obtaining precursor and product ion mass spectrum

Different infusion solutions were evaluated for their ability to enhance the MS sensitivity of the 24mer. DIEA and acetonitrile displayed the highest signal enhancing capability (data not shown) and were chosen as the ion-pairing agent and organic solvent used for the mobile phase. Molecular ions were distributed between the 6-14 charge states with $[M\text{-}13H^+]^{13-}$ at m/z=590 being the most abundant ion. The most abundant fragment ion of $[M\text{-}13H^+]^{13-}$ is $w_1\text{-}H_2O$ at m/z=319 (Fig 2a and b). [11]

Optimization of SPE conditions

Various conditions were attempted to improve the recovery of the oligonucleotides including changing the plasma-buffer ratio, the equilibration buffer pH, the washing volume and the elution buffer composition. The results are summarized in Figure 3. Increasing the elution buffer pH from 8.8 to 9.5 increased the recovery of the 24mer from about 50% to over 70%. Higher elution buffer pH values were tested but did not result in any higher recovery (data not shown). Decreasing the wash buffer volume did not further improve the recovery

Evaporating the eluted solution under vacuum generated numerous additional peaks that eluted before the major peak in the chromatogram (Fig 4a). By adding 10 mM of TCEP in the elution buffer, these smaller peaks were substantially reduced (Fig 4b).

Choosing the appropriate concentration of internal standard

Different concentrations of internal standard were tested to achieve the best reproducibility and recovery for this one-step SPE procedure. When 10 μg/mL of internal standard was used, there was a significant amount of interference observed in the MS/MS channel for the 24mer at 10 ng/mL while the 23mer was unaffected (Fig 5c). When the concentration of the IS was decreased to 1 μg/mL, neither 24mer nor 23mer was detected at 10 ng/mL. This indicated that non-specific binding to the SPE cartridge was not negligible and the higher concentration of the IS protects the 23mer and 24mer from these losses. Increasing the elution buffer volume and organic solvent percentage did not retrieve any more analyte from the cartridge, which indicated that the binding was irreversible. 2 μg/mL of internal standard was therefore chosen as the concentration used for

validation. At this concentration level of IS, the recovery of the 23mer and 24mer was acceptable across the dynamic range and no significant interference was observed (Fig 5b).

Assay Validation

Assay Selectivity

Different lots of rat plasma (n>6) were extracted and analyzed. No significant peaks were observed in either the analyte or the IS MS/MS channels for these matrix control samples, indicating that the method was highly selective. The 23mer, 24mer and IS were all analyzed individually and no cross-talk was observed between their MS/MS channels.

Precision, Accuracy, Recovery, Matrix Effects and Stability

The dynamic range of the current method is 10-1000 ng/mL. The calibration curves for 23mer and 24mer have R^2 values of 0.9961 and 0.9973, respectively. The intra-day and inter-day precision and accuracy for both 23mer and 24mer are summarized in **Table 1.** The autosampler, bench top and freeze-thaw stability are summarized in **Table 2.** The absolute recovery, relative recovery and matrix effects of the method are summarized in **Table 3.**

Method application

Because high plasma concentrations were expected, the rat plasma samples at 5, 15, 30, 60 min were diluted 50 fold with blank plasma and then processed based on the proposed extraction protocol. The plasma concentration versus time profile is presented in Fig.6. Plasma samples were spiked with 23mer and 24mer at 50,000 ng/mL and diluted with blank rat plasma (1 in 50) prior to analysis (n = 5). All of the back-calculated values were within ±15% of the nominal concentrations. The precision was less than 9.0%, and accuracy was in the range of −3.8% to 7.3%, respectively, for 1 in 50 dilution.

Discussion

Optimization of SPE conditions

Clarity OTX SPE cartridges extract oligonucleotides using a weak-anion-exchange (WAX) mechanism. The WAX particles were first charged at low pH (5.5) to enhance binding with the

negatively charged phosphorothioate backbone. When the pH increased, the particles were neutralized and the oligonucleotides were released from the cartridge.

This SPE method eliminated the phenol-chloroform extraction step by disrupting the OGN-protein binding with the lysis buffer. The original extraction protocol provided by the manufacturer is as follows: plasma and lysis buffer are mixed in a 1:2 ratio then loaded to the column pre-equilibrated with equilibration buffer at pH=5.5. The column is then washed by 3mL of washing buffer at pH=5.5 twice and the OGN is eluted by elution buffer at pH=8.8. Due to the high concentration of detergent in the lysis buffer, a lower buffer/plasma ratio was attempted to reduce possible interferences or ion suppression when using MS detection. However, the experimental results showed that a 1:1 ratio of plasma and lysis buffer gave a lower recovery than when a 1:2 ratio was used. This indicated that the detergent concentration was important in the extraction procedure because if the OGN was still bound to plasma proteins and the complex did not carry a net negative charge when loaded on the cartridge, it would pass through the cartridge during loading or elute during the washing steps.

WAX SPE avoided the use of high concentrations of MS-incompatible salts from Strong Anion Exchange (SAX) SPE. Instead of eluting the analyte with a high concentration of involatile salts, WAX elutes the analyte by changing the pH and hence the charge on the solid support. Therefore, the shift from acidic pH to basic pH was critical for successful recovery of the OGN when using WAX SPE. This could be achieved in three different ways: a) applying higher vacuum after the washing step to drain the residual washing buffer and prevent neutralization of the elution buffer; b) increasing the washing buffer pH to avoid severe neutralization of the elution buffer once it is loaded onto the column; or c) increasing the elution buffer pH to ensure the neutralization of the solid support during the elution step. Without changing the pH of the washing or elution buffer, applying high vacuum after washing step only resulted in a 40% recovery of the analyte and IS. Increasing the equilibration and washing buffer pH to 6 decreased the recovery from 40% to 30% instead of increasing it, indicating that the high pH of the equilibrating and washing buffers resulted in a partial charge of the solid support particles and compromised the binding efficiency of the OGN to the solid support. Increasing the elution buffer from 8.8 to 9.5 significantly increased the recovery of both the analyte and the IS, so a pH of 9.5 for the elution buffer was used in the final protocol for extraction.

The phosphorothioate backbone can be oxidized to a phosphodiester during prolonged exposure to oxygen. Mcginley et al. [9] reported that under low vacuum and high temperatures, phosphorothioates underwent such severe oxidation that half of the analyte was oxidized species in the chromatogram. They proposed that using lyophilization intead of vacuum evaporation would eliminate the oxidation and improve the recovery. However, due to the high percentage of organic solvent in the elution buffer, at least 15 minutes of pre-evaporation is necessary to eliminate all of the organic solvent in the sample solution to better freeze the sample and prevent agitation during lyophilization. By adding 10 mM of TCEP, an MS-compatible antioxidant for sulfur containing compounds, to the elution buffer the oxidation of the backbone phosphorothioate was effectively prevented during the 3 hours of evaporation using vacuum centrifugation. Slight signal suppression ranging from 5-12% was observed in the matrix effect study from the addition of TCEP to the elution buffer but this did not affect the method accuracy and precision.

The washing step of the SPE method is the most time consuming step. More than 30 min was needed when no vacuum was applied which equaled more than half of the total extraction time. Decreasing the washing volume from 6 mL to 3 mL did not reduce the recovery, so in the optimized protocol only 3 mL of washing buffer was used.

Choosing the appropriate type and concentration of internal standard

Non-specific binding of OGNs to containers and SPE cartridges has been widely reported as one of the major challenges in bioanalysis of OGNs, especially at lower concentration levels[3]. We addressed this issue by increasing the concentration of the internal standard, which is used as a "sacrificial OGN" to saturate all binding sites on the SPE cartridges and containers during the sample extraction procedure and thus enhance the recovery of the 23mer and 24mer at low concentrations. However, this could cause some issues with MS/MS selectivity.

As seen in Figure 2a, at each charge state the parent ion of the oligonucleotide expands to an m/z window of almost 10 Da. Although the most abundant m/z value was selected and no interference would be observed when the concentration of each analyte were at similar concentrations, when the concentration of one OGN is overwhelmingly above the others, some of its resulting signal may be high enough to form interferences in the MS/MS channel of other

analytes. This is problematic since the product ion m/z 319 could be produced by any oligonucleotide with a 3' terminal T, making the MS/MS transition more susceptible to such interference. In this concern, overly high concentrations of internal standard should be avoided.

At higher concentrations of IS, "cross-talk" of some of the partial phsophrothioate or chain shortened impurities of the IS that elute close to the analyte are no longer negligible. **Figure 5a** is the chromatograms of the LOQ sample when IS concentration was 10 µg/mL. The background noise near the retention time of the 24mer was so high that the peak of the 24mer was obscured when 10 µg/mL of IS was used. However, at 1 µg/mL the concentration of the IS was not enough to saturate the binding sites on the SPE cartridges and the containers so the sensitivity of the 23mer and 24mer was compromised and the peak area of the IS became erratic (data not shown). Therefore, a concentration of 2 µg/mL was used for all studies to balance these two concerns.

By the same token, shorter polyT strands that elute after the analytes have higher propensity of "cross-talk" than longer polyT strands at high concentration. Compared to T40, T32 showed more interference in the analyte channel (data not shown). Therefore T40 was chosen as the internal standard. As shown in **Table 3**, the recovery of IS is slightly lower than 23mer and 24mer because the higher hydrophobicity of the longer poly T sequence causes it to have more extensive non-specific binding than the analyte. This characteristic of T40 makes it a better "sacrificial oligonucleotide" as an internal standard.

Application of the method

The $T_{1/2}$ is 30 minutes, which is similar to what was reported before (29 min)[12]. Longer sampling period could capture a more complete pharmacokinetic profile. However, the earlier report [12] has indicated that 4ng/mL LOQ would still not be enough to capture the elimination phase. Therefore, only 180 minutes sampling period was employed considering the body weight and blood volume of the subject.

Conclusions:

We optimized a one-step solid phase extraction (SPE) procedure for oligonucleotides with high recovery (70-80%) and short processing time (4 hours). We also developed a sensitive and fast (9 min) uHPLC-MS/MS assay for simultaneous quantitation of a test oligonucleotide and its

metabolite from rat plasma (10-1000 ng/mL). This is the first report of a single step solid phase extraction method with higher than 60% recovery. Specific issues related to this type of sample extraction procedure were investigated. An animal experiment was conducted as a proof of concept study for the validated analytical method and similar pharmacokinetic parameter was obtained when comparing to an earlier report[12]. However, lower LOQ still needs to be achieved to capture the complete terminal elimination phase of 24mer at clinically relevant doses. It is possible that other instruments such as a triple quadrupole may be capable of reaching these limits with the current method. Another possible method to obtain lower method limits of quantitation would be exploring capillary liquid chromatography. However, the low flow rates from capillary liquid chromatography will likely lead to a significant decrease in sample throμghput. Great strides have been made in LC-MS/MS methods for the quantitation of oligonucleotides over the past several years with these improvements LC-MS/MS is currently approaching the performance required to determine full pharmacokinetic profiles with specificity that is unrivaled by other applicable methods.

Acknowledgement:

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Table 1. Intra-day and Inter-day precision and accuracy of the method

Conc. (ng/mL)	Intra-day(n=5)						Inter-day(n=15, 3days)					
	Obsd conc. (ng/mL)		RSD(%)		Error(%)		Obsd conc. (ng/mL)		RSD(%)		Error(%)	
	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer
10	9.75±0.52	9.88±0.88	5.30	8.89	2.5	1.2	9.90±0.88	10.1±0.9	8.85	9.11	1.0	0.7
30	29.4±2.5	29.8±2.1	8.67	7.08	2.1	0.7	29.4±2.6	30.3±3.2	8.84	10.5	2.1	0.8
150	148±12	139±5	7.79	3.94	1.2	7.6	143±9	148±11	6.08	7.53	4.9	1.7
750	813±41	774±17	5.09	2.17	8.4	3.2	777±40	797±48	5.14	5.99	3.6	6.2

Table 2. Stability of 23mer and 24mer in rat plasma

	freeze-thaw stability(-20。C) (three cycles)				room-temp stability(8 h)				autosampler stability(24hr, 5。C)			
Nominal conc.(ng/mL)	**30**		**150**		**30**		**150**		**30**		**150**	
	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer
Obsd conc. (ng/mL)	31.5±1.8	31.2±2.7	161±9	136±6	31.3±2.0	28.5±2.1	156±16	134±4	32.6±2.7	29.7±1.2	153±12	140±15
Accuracy(%)	105	104	107	90.4	104.2	95.1	104	89.4	109	99.0	102.2	93.1
RSD(%)	5.81	8.76	5.82	4.09	6.31	7.28	10.2	2.82	8.18	4.04	7.63	10.4

Table 3. Absolute, Relative Recovery and Matrix Effects of 23mer and 24mer

Conc. (ng/mL)	absolute recovery(%)		relative recovery(%)		matrix effect(%)		type of effect	
	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer
10	78.4 ±8.0	68.2 ±6.9	83.2 ±8.5	74.8 ±7.5	94.3	91.2	5.7%Suppression	8.8%Suppression
30	68.7 ±3.3	62.7 ±6.3	74.0 ±3.7	70.7 ±7.1	92.8	88.6	7.2%Suppression	12.4%Suppression
150	64.2 ±2.9	61.4 ±7.4	71.4 ±2.9	66.7 ±8.0	89.9	92.0	10.1%Suppression	8.0%Suppression
750	64.1 ±4.0	68.4 ±1.9	71.4 ±4.5	73.5 ±1.8	89.7	92.9	10.3%Suppression	7.1% Suppression
IS(2 µg/mL)	56.8 ±6.7		62.8 ±7.2		90.5		9.5% Suppression	

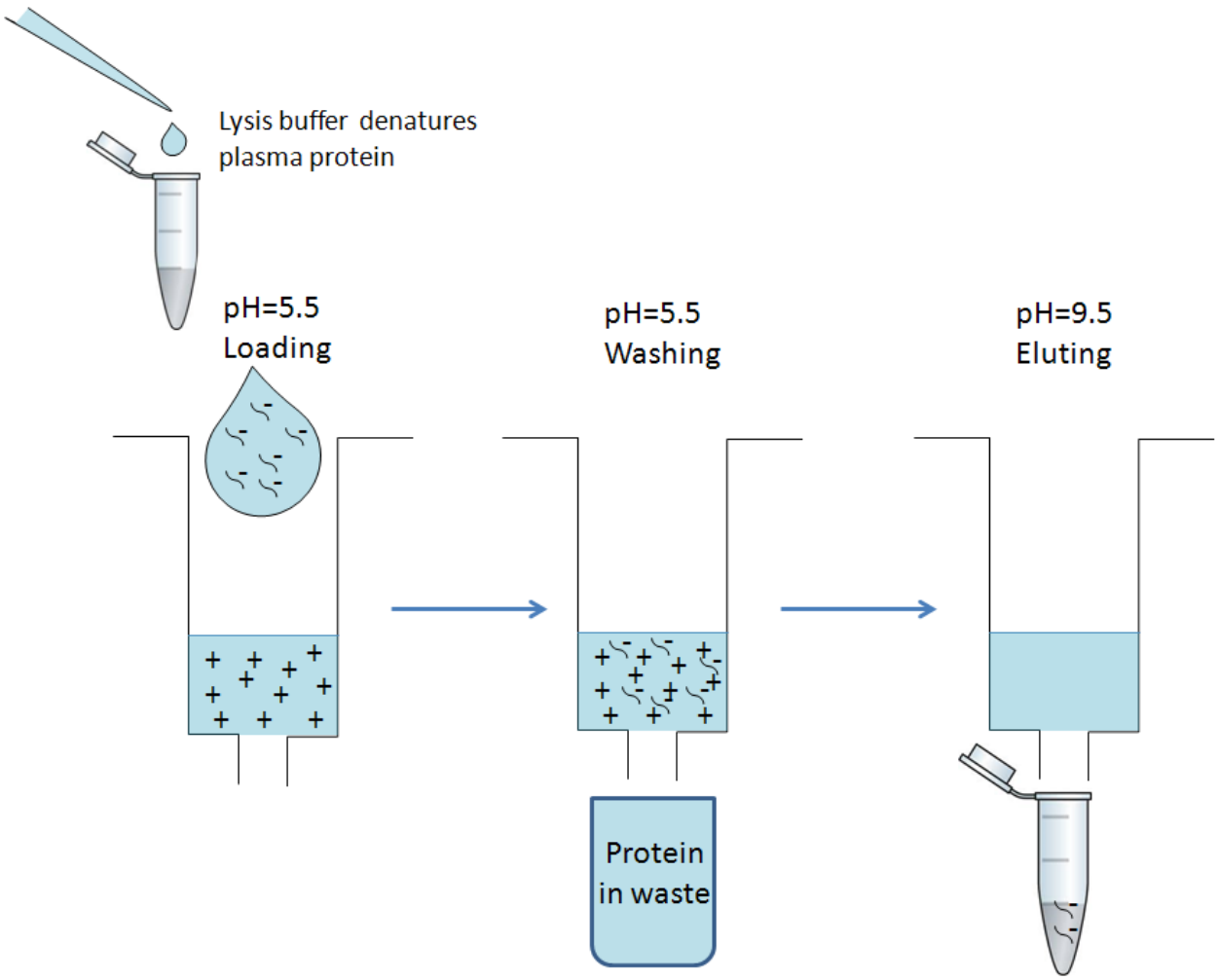


Figure 1. Sample extraction procedure

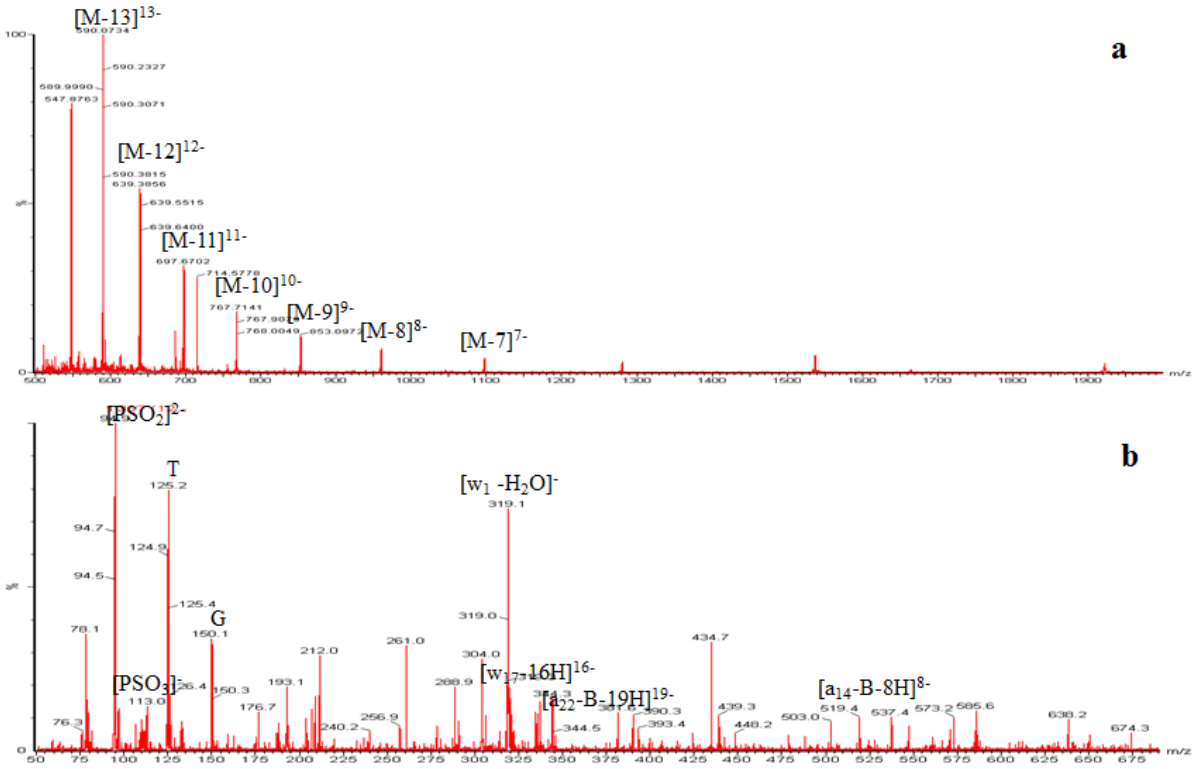


Figure 2. a. Full scan and **b.**Product ion mass spectra of 24mer

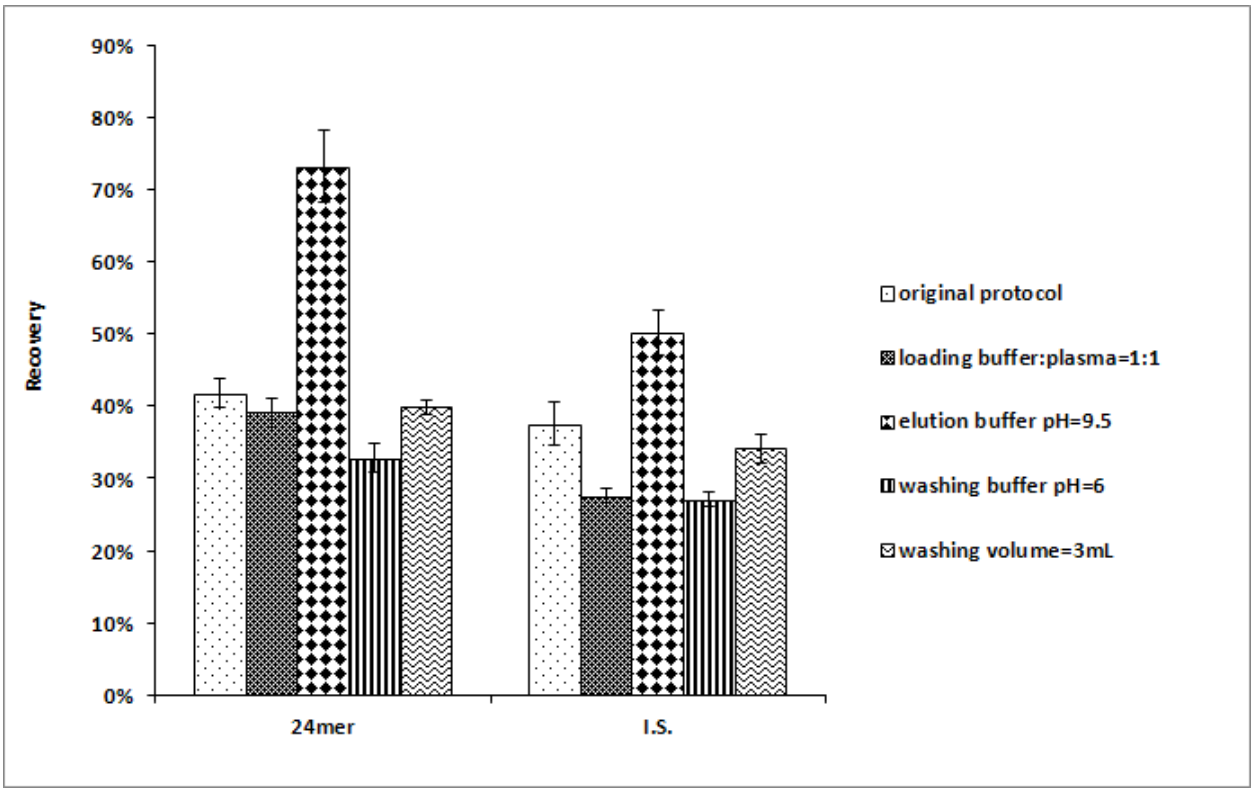


Figure 3. Recoveries obtained from different extraction conditions

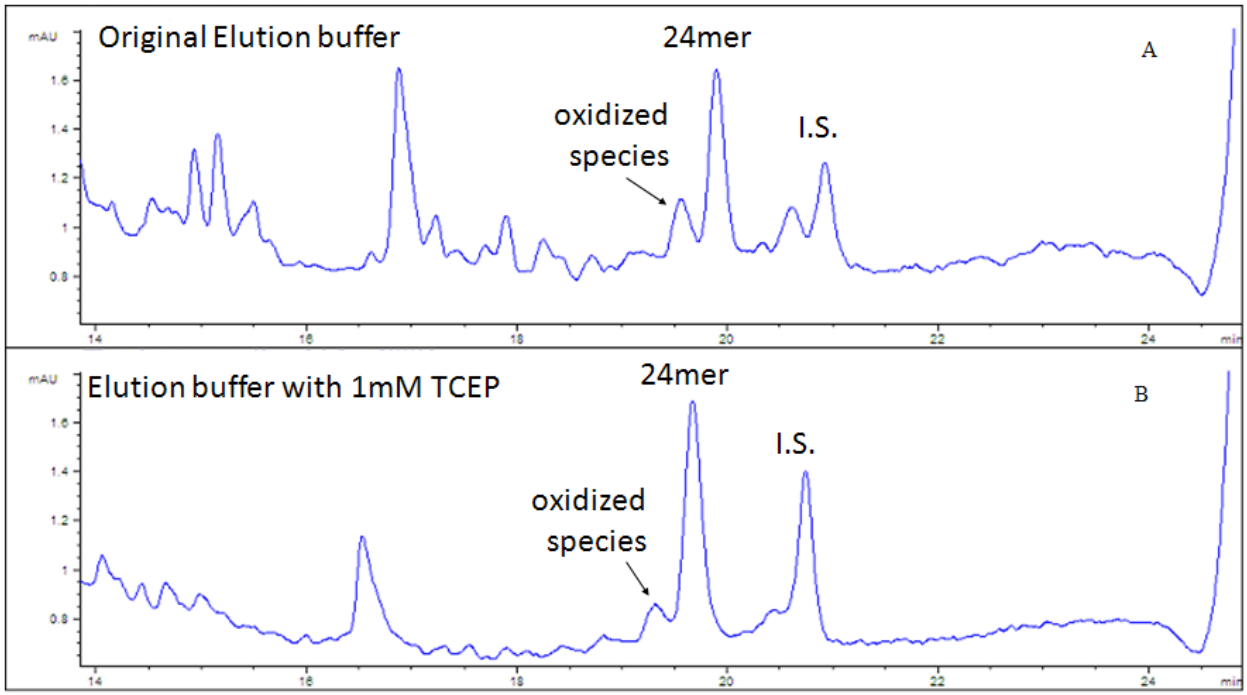
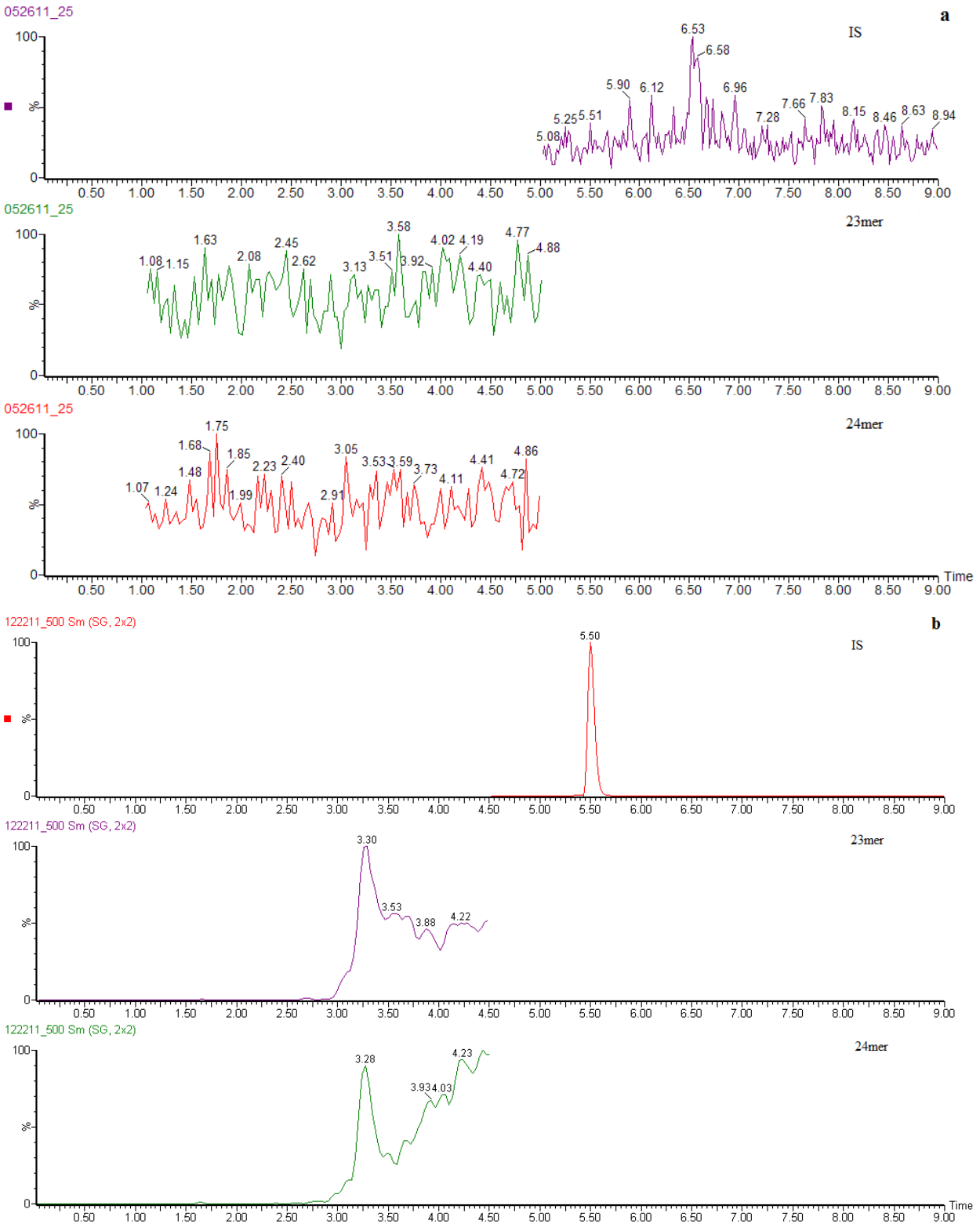


Figure 4. Chromatograms of samples eluted by buffers with and without TCEP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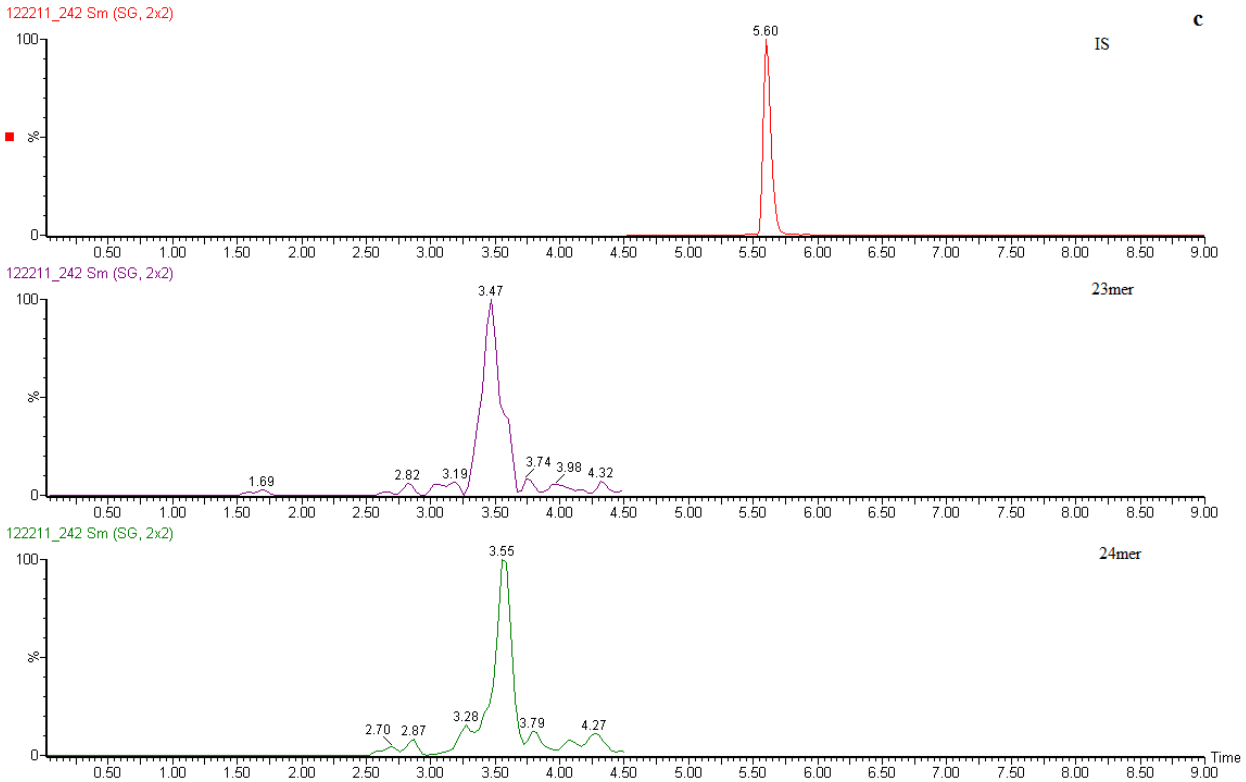

Figure 5. Representative chromatograms of **a.** extracted blank, **b.** 23mer and 24mer at LOQ (10 ng/mL) with IS at 10 µg/mL or **c.** with IS at 2 µg/mL

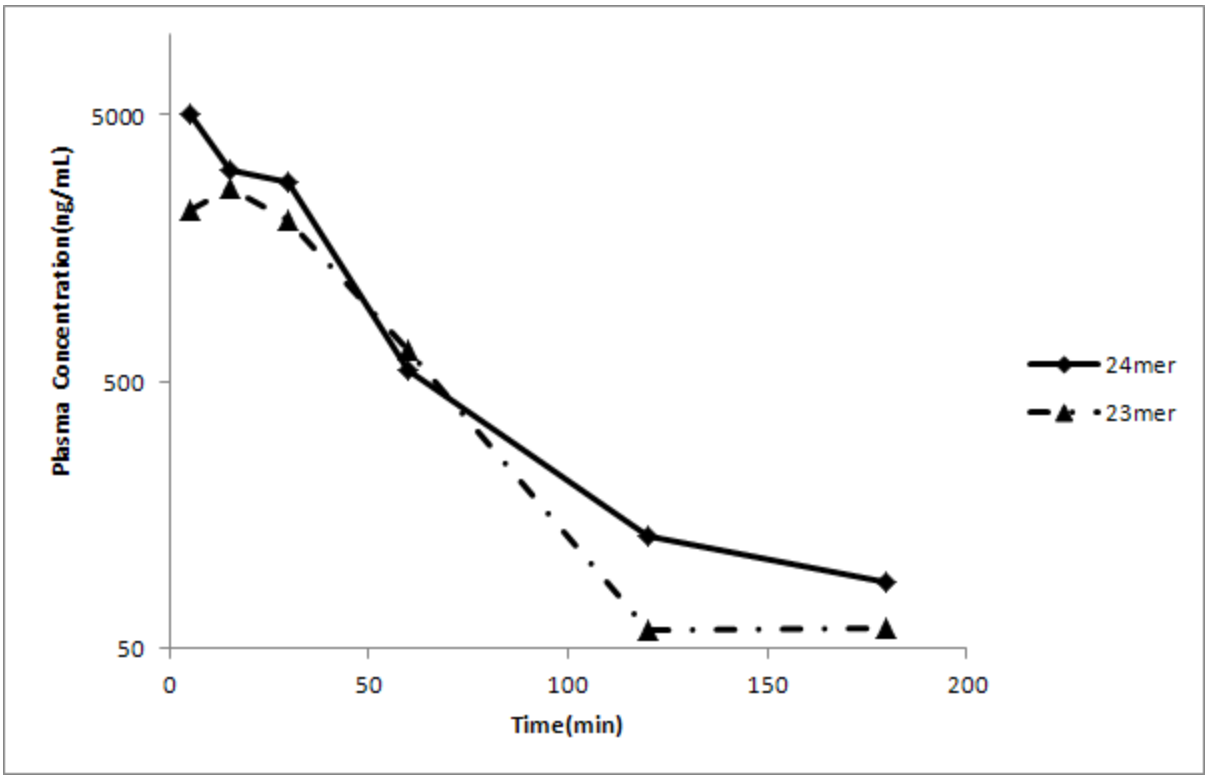

Figure 6. Plasma concentration curve from 0-180 min after i.v. bolus of 1mg/kg 24mer

Chapter 5

Strategies for enhancing assay sensitivity for the bioanalysis of oligonucleotide using ultra-high performance liquid chromatography and mass spectrometry (UHPLC-MS/MS): Application to phosphorothioate DNA

Chen B and Bartlett MG. To be submitted to Analytical Chemistry

Abstract:

LC-MS based assay is a promising approach for bioanalysis of oligonucleotide therapeutics due to its selectivity and structure identification capability. However, the lack of sensitivity and complicated sample preparation procedure remains to be the bottle neck for application of LC-MS based assays to preclinical and clinical studies. Numerous studies have shown that the mobile phase composition, especially organic solvent type has a significant impact on the MS sensitivity of oligonucleotides. In this study, we investigated the type of organic solvents and concentration of organic modifiers for their effect on electrospray desorption efficiency, chromatographic separation and LC-MS signal intensity. 25mM HFIP, 15mM DIEA and usage of ethanol as the organic solvent were observed to achieve up to two orders of magnitude increase in LC-MS signal intensity compared to the most commonly used mobile phase composition. Phenol-Chloroform LLE was commonly used in combination with ethanol precipitation for DNA purification in biochemistry research. However, this method was never used in quantitative bioanalysis of therapeutic oligonucleotides. Various conditions for ethanol precipitation were evaluated in respect of recovery and >75% absolute recovery was achieved using the optimized extraction procedure. Minimum residual substances were generated with this extraction procedure and no increase in column pressure or deterioration of separation was observed for >500 injections of biological samples. Other challenges such as non-specific binding, balancing LC separation and MS sensitivity, choosing appropriate anti-coagulant were also addressed in this study. The method run time was 5 minutes. The accuracy (% error) and precision (%RSD) are <5.09% and <10.56%, respectively, over a dynamic range of 2.5-

1000ng/ml. The assay was applied to a proof of concept animal study and similar PK parameter to previous study was obtained.

Since the approval of the first antisense drug Fomivirsen in 1998, tremendous efforts have been made to develop a sensitive and selective bioanalytical method for oligonucleotide therapeutics that would meet regulatory requirements. A variety of technique have been used for preclinical and clinical studies of oligonucleotides and their advantages and limitations have been extensively reviewed [1-6]. LC-MS has been considered a promising technique due to its increased specificity and structural characterization capability. However, LC-MS has suffered from a lack of sensitivity and has not been able to capture the terminal elimination phase of oligonucleotide therapeutics for pharmacokinetic studies. In addition, biological sample extraction remains a formidable challenge in developing quantitative LC-MS methods for oligonucleotides.

Various strategies has been attempted to enhance the ionization efficiency and detection sensitivity of oligonucleotides by ESI-MS. Bleicher et al.[7] reported that increasing the solution pH and organic solvent percentage significantly increased the electrospray signal intensity for oligonucleotides. Gaus et al. [8] compared isopropanol and methanol as organic solvents with 7 different alkylamines as ion-pairing agents for the mobile phase of their LC-MS method. The highest signal intensity was observed using tripropylamine in both organic solvents. 50% isopropanol was reported to provide a 5-fold increase in signal intensity compared to 50% methanol. Huber et al. [9] tested a variety of different sheath liquids including methanol, isopropanol, acetonitrile, hexafluoroisopropanol (HFIP), triethylamine, 10mM triethylamine in acetonitrile, and 400mM HFIP in methanol. Acetonitrile was found to be the most efficient at improving signal intensity. Deguchi et al. [10] reported 10 to 30 fold signal enhancement for

oligonucleotides when using post column infusion of 0.1M imidazole in acetonitrile. These results demonstrate that the composition of the mobile phase dramatically affects the electrospray ionization efficiency of oligonucleotides.

Another major hurdle for LC-MS methods is the extensive sample clean-up procedures required to achieve the necessary reproducibility and recovery needed for quantitation. Due to the extensive protein binding of oligonucleotides, direct protein precipitation and reverse phase solid phase extraction have been reported to have low recoveries (<10%)[11]. Proteinase K digestion combined with liquid-liquid extraction (LLE) was reported to have high recoveries (98%) but the incubation process is time consuming (>10hr)[12]. Ion-pair reverse phase solid phase extraction(SPE) has been reported but suffered from diminished recovery at lower concentrations due to irreversible binding to the SPE cartridge[13]. Strong Anion Exchange (SAX) SPE was also reported but the high salt concentration used in the method requires further clean-up steps to be applied prior to LC-MS analysis, which might result in further recovery losses[14]. Zhang et al.[11] reported a sample extraction procedure that combined phenol-chloroform LLE and ion-pair reverse phase SPE and resulted in relatively high recovery across a wide dynamic range (72-85%). Nevertheless, the multiple extraction and sample transfer steps are arduous and time consuming. A one-step weak anion exchange (WAX) SPE method was reported to have high recoveries [15] but the cost of this method was fairly high. Our preliminary experiment showed that direct phenol-chloroform LLE resulted in samples with complicated matrix background. These endogenous substances could cause interference for LC separation and blockage on small particle UHPLC columns.

Apffel et al. [16] replaced acetic acid with hexafluoroisopropanol (HFIP) as the counter ion used in LC-MS mobile phase, which was a milestone in the evolution of buffering systems for nucleic acid analysis. Due to the limited solubility of HFIP in acetonitrile, methanol has long been the organic solvent of choice for oligonucleotides. In this study we tested different organic solvents for their impact on the ionization efficiency of oligonucleotides and the interaction between the organic solvents and other organic modifiers in the mobile phase system. Phenol-chloroform LLE followed by ethanol precipitation is a classic biochemical approach to extract DNA from other cellular components but this assay has never been used in quantitative bioanalysis of oligonucleotides. In this study we optimized this assay and developed a sample extraction procedure from plasma with high recovery and low interference. In addition, the chromatographic conditions such as column length, diameter, and flow rate were optimized to ensure the highest sensitivity.

EXPERIMENTAL SECTION

Chemicals and Materials. The 24mer phosphorothioate DNA oligonucleotide (5'-TCGTGCTTTTGTTGTTTTCGCGTT-3'), its 23mer 3'n-1 metabolite and internal standard (IS) pdC18 (PAGE purification, purity>90%) were purchased from Integrated DNA Technologies (Coralville, IA). Chemicals such as phenol, 1,1,1,3,3,3-hexafluoro-isopropanol(HFIP), di-isopropylethylamine(DIEA) and glycogen(blue mussel) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Ammonium hydroxide solution (35%) was purchased from J.T. Baker (HPLC grade methanol, ethanol, isopropanol, acetonitrile and chloroform were obtained from Fisher Scientific (Pittsburgh, PA). DNA Lobind centrifuge tubes were purchased from Eppendorf (Hauppauge, NY).

Influence of mobile phase composition on mass spectrometric performance and chromatographic separation. 24mer stock solution was diluted using solution containing different ion-pairing agents and organic solvents(Detailed compositions shown in Table 1) to achieve final concentrations of 10 μg/ml and infused at a flow rate of 10 μL/min. The organic solvents tested included acetonitrile, methanol, ethanol and isopropanol. In the separation experiments the 24mer and C18 were dissolved in 15mM DIEA water solution at a concentration of 1μg/ml and 20μL was injected onto the LC-MS system.

Instrumentation and LC-MS conditions. UHPLC-MS/MS analysis was performed on a Waters Acquity UHPLC system coupled with a Waters Synapt G2 qTOF mass spectrometer (Waters, Milford, MA). Chromatographic separations were performed at a flow rate of 0.15ml/min on a 1.7 μm Waters Acquity BEH C18 column(Waters, Milford, MA) 100mm ×1.0mm column. Mobile phase A consisted of 15.7mM DIEA and 20mM HFIP in water, and mobile phase B consisted of the same concentration of DIEA and HFIP in water: ethanol (50:50). A 20 μL injection of each sample was loaded onto the column and separated using the following gradient conditions (time (min), % mobile phase B): (0, 5) (1,5) (2, 29) (4, 29) (4.01, 5) (5, 5). The column temperature was maintained at 60 ℃. The column eluent from 0-1.5min was diverted to waste. The weak and strong wash for the autosampler needle are 1000 μL water and 800 μL 5% methanol, respectively. The system was operated in the negative-ion MS/MS mode with a 1 second scan time. The MS/MS transition optimized for 23mer, 24mer and internal standard are m/z 590→319, 667→319 and 536→304 respectively. The capillary voltage was set at 2.3kV, the cone voltage was 20V, the extraction cone voltage was 2V, the source temperature was 120 ℃, the desolvation temperature was 450 ℃, cone gas was 6L/h and desolvation gas was 700L/h.

Preparation of stock solutions, working solutions, calibration and QC samples. 23mer and 24mer solutions were prepared in deionized water to give a final concentration of 1mg/ml. The IS working solution concentration was 20 μg/ml. Working solutions of 23mer and 24mer were prepared by serial dilution of their stock solutions with water to achieve concentrations of 0.05, 0.1, 0.2, 0.4, 2, 4, 10, 20 μg/ml. The working QC solutions with concentrations of 0.15, 1.5 and 15 μg/ml were also prepared in the same manner. The calibration samples with concentrations of 2.5, 5, 10, 20, 100, 200, 500 and 1000 ng/ml and QC samples with concentrations of 2.5, 7.5, 75 and 750ng/ml were prepared by spiking 10 μL of the working solution of 23mer and 24mer and 10 μL of the IS working solution to 180 μL blank rat plasma.

Sample preparation. Because of the non-specific binding of analytes to storage containers[11], all stock solutions, work solutions and samples are prepared in DNA Lobind tubes. To 200 μL plasma samples 100 μL 5% ammonium hydroxide and 40uL phenol-chloroform(w/v=2:1) were added. The mixture was vortexed for 1 min and centrifuged at 14,000 g for 10 min. The supernatant was transferred to a new tube and washed with 1mL isopropyl ether. The aqueous phase was then mixed with 2uL glycogen solution and 800uL ethanol before storage at -80 ℃ for an hour. The solution was then centrifuged at 14,000g for 30 min (0 ℃). The supernatant was removed and the precipitated DNA was air-dried in fume hood. 50 μL of 10mM DIEA in deionized water was used as the reconstitution solution and 20uL was injected into the UHPLC system.

Method Validation. Plasma calibration curves were constructed using peak area ratios of 23mer and 24mer to the IS and applying a weighted ($1/x^2$) quadratic regression. Precision (% RSD) and accuracy (% error) were calculated for the four QC samples (concentrations of 2.5, 7.5, 75, and 750ng/mL). Five replicates of each QC point were analyzed everyday to determine the intra-day

accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. The absolute and relative recoveries and matrix effects were determined for spiked plasma samples and neat solutions (n =5) at concentrations of 2.5, 7.5, 75, and 750ng/mL. Absolute recovery was calculated as the peak area for 23mer and 24mer in plasma sample spiked before extraction divided by the peak area of the neat sample of the same concentration. Relative recovery was calculated by dividing the peak area for a sample spiked with 23mer and 24mer before extraction by the peak area for an equal concentration sample in the same matrix spiked after extraction. Matrix effects were calculated by dividing the relative recovery by absolute recovery[17]. Stability of the oligonucleotides during freeze-thaw (3 cycles), at room temperature (8 h), and in an autosampler (24 h) (n =5) were also determined and reported.

Method application. The validated analytical method was applied to an animal experiment where a single i.v. administration of 1.0 mg/kg of the 24mer was given to a male Sprague Dawey rat weighing ~200g. 24mer in 0.9% sterile saline was given as an i.v. bolus dose through a neck catheder. The dose volume was 1.25 mL/kg. 0.5mL of blood was withdrawn from jugular vein according to a schedule of 0 (predose), 5, 15, 30, 60, 120, 180, 360 and 540 minutes after dosing. The blood samples were centrifuged at 11,000 × g for 5 min and plasma were collected and kept frozen at −20°C until analysis.

RESULTS AND DISCUSSION

Influence of solvent type on mass spectrometric performance. The effect of the physiochemical properties of organic solvents on the electrospray of ionic compounds in negative ion mode has been previously studied [18, 19]. Solvent polarity, dielectric constant and solubility of the analyte in the solvent were all considered to affect the electrospray ionization

process significantly. In this study we compared four HPLC-compatible organic solvents for their capacity to enhance oligonucleotide signal intensity and present a model for the observed behanvior. We studied the effect of the organic solvent on ionization of oligonucleotides beginning with only DIEA at first. As shown in Figure 1, the signal intensity of the oligonucleotide was highest when using an acetonitrile/water mixture as the solvent. The signal intensities obtained with methanol, ethanol and isopropanol were similar although they peaked at different DIEA concentrations. Acetonitrile, unlike the protic solvents such as methanol and ethanol, can only accept a proton from water to form a weak hydrogen bond but can not donate one[20]. As a result, the number of hydrogen bonds in acetonitrile/water mixture is lower than in water and the mixtures is microscopically heterogeneous[21]. By the same token, the number of hydrogen bonds formed between oligonucleotide molecules and the solvent would also be diminished in acetonitrile/water mixtures versus alcohol/water mixtures and therefore, the system entropy increases. Thermodynamically it is easier for the oligonucleotide to escape the solution and enter gas phase when in acetonitrile/water mixtures, resulting in higher signal intensities.

The signal enhancement effect of DIEA increased with the concentration of DIEA in acetonitrile but decreased at higher concentrations in methanol, ethanol and isopropanol. In order to understand this difference, it is critical to understand the mechanism of the signal enhancement effects of DIEA. An electrospray desorption model that depicts this mechanism was previously proposed by Chen et al[22]. As shown in scheme 1, when DIEA evaporates from the electrospray droplet surface, the equilibrium of equation 1b is driven to the left. The concentration of $HDIEA^+$ will decrease on the droplet surface and drive the equilibrium of equation 1c to left as well, increasing the concentration of $Oligo^{n-}$ near the droplet surface. Based

on this mechanism, desorption of oligonucleotide ions are dependent on the dissociation of DIEA-Oligo complex. Wang et al.[19] proposed that solvents with high dielectric constants are more capable of dispersing the attractive forces between formed ions and their parting counterions. This hypothesis was consistent with observations from this experiment. Isopropanol had the lowest dielectric constant (dielectric constant of acetonitrile, methanol, ethanol and isopropanol are 37.5, 33, 24.5 and 18, respectively[23] and the optimum concentration of DIEA was the lowest in isopropanol. This was because less dissociation of DIEA-Oligo complex shifted the equilibrium in 1b and 1c to right, leading to competition between the oligonucleotide anion and OH^-. Acetonitrile, on the other hand, sufficiently dispersed the complex, therefore no suppression of ionization was seen at higher DIEA concentrations.

The "wrong way around ionization" mechanism of HFIP was reported earlier[22]. The free protons provided by the weakly acidic HFIP facilitates electrochemical reduction thus enhancing oligonucleotide desorption. Meanwhile, the dissociation of HFIP molecules generates HFIP anions that will compete with oligonucleotides for ionization. Whether oligonucleotide ionization is enhanced or suppressed is determined by which process becomes the dominant one. As seen in Figure 2, increasing the concentration of HFIP in an acetonitrile/water mixture resulted in greater signal suppression of the oligonucleotide. In contrast, HFIP displayed signal enhancement effects in methanol, ethanol and isopropanol and the optimum concentration was 20mM for all three alcohols. The enhancement effect is more pronounced in ethanol and isopropanol. The difference could be explained by different extent of HFIP dissociation with different organic solvents. The dielectric constant of the acetonitrile water mixture is the highest so as low a concentration as 20mM of HFIP could generate enough HFIP anions to compete with the oligonucleotide. The same mechanism could be applied to the water mixture with methanol,

ethanol and isopropanol, where the signal enhancement effects were in the reverse order of the dielectric constants and the signal suppression effects were in the same order as the dielectric constants.

Influence of mobile phase composition on chromatographic separation. Both separation efficiency and signal intensity have to be taken into consideration when optimizing a LC-MS method. In our previous study[22] we have investigated the effect of different ion-pairing agents type, their concentration and the concentration combinations of HFIP and DIEA on the mass spectrometry signal intensity of oligonucleotides. Recently a study explored the impact of ion-pairing agent type on chromatographic separation of oligonucleotides[24]. In this section we further explored the effect of mobile phase composition on the chromatographic separation of oligonucleotides.

As shown in Table 1a, the selectivity of different mobile phase compositions were compared to each other. In order to make a direct comparison, a consistent gradient profile was used for all mobile phase compositions (time (min), % mobile phase B): (0, 5) (1,5) (3, 53) (5, 53) (5.01, 5) (8, 5). All the measurements were made in triplicate. The separation performance was evaluated by calculating the separation factor value for 24mer/C18 pair ($\alpha=t_2/t_1$, t_2 is the retention time of 24mer and t_1 is the retention time of C18). The results showed that the selectivity of the organic solvents decreases in the order of: isopropanol>ethanol>acetonitrile>methanol. Due to the significantly lower eluting power, methanol provided much better selectivity between the analyte and IS. This property of methanol could be of great use especially when extensive separation of parent drug and metabolites is the primary purpose of the method development. DIEA and DMBA both offered slightly better selectivity than TEA. The difference of selectivity between

the two isomers(TEA and DMBA) indicated that the longest alkyl chain, instead of the total alkyl chain carbon numbers, affects the ability of an ion-pairing agent to retain the analyte on column. Fountain et al.[25] studied the impact of different TEA and HFIP concentrations on chromatographic separation of oligonucleotides with special focus on the separation performance at HFIP concentration above 100mM. However, recent studies on bioanalysis of antisense DNA oligonucleotides commonly used mobile phases containing less than 100mM HFIP [11, 13, 26]. Our previous research also showed that HFIP concentrations of 100mM and above significantly suppressed the ionization of oligonucleotides[22]. Therefore, three different concentrations of HFIP below 100mM in combination with two commonly used ion-pairing agent concentrations [11, 13, 26] were compared to each other for selectivity. As shown in Table 1b, the selectivity of 15.7mM DIEA is higher than 1.7mM DIEA while the HFIP concentration did not affect the selectivity to a significant extent. This observation is consistent with the previous study where the authors concluded that the concentration of the alkylamine rather than the concentration of HFIP was the principal determinant of the separation[25]. Although it is worth noting that the ion-pairing agent type was different than the current study. This result indicated that for the purpose of separating short single stranded DNA, higher HFIP concentrations were not necessary to enhance the method selectivity.

Comprehensive effect of mobile phase composition on LC-MS sensitivity. The advantage of a certain mobile phase composition in enhancing ionization efficiency might be offset by its lack of retaining capability of the analyte on an LC column, because the analyte would elute when the organic solvent percentage was lower. Our direct infusion experiment showed that lower organic solvent percentages would decrease the signal intensity. The signal intensities of the 24mer in the same experiment described above were compared to each other to evaluate the comprehensive

effect of mobile phase composition on LC-MS sensitivity. The signal intensities of the IS (C18) were affected in a similar way so no details concerning the IS are shown here. As seen in Figure 3a, the signal intensities of the 24mer when using ethanol and isopropanol as the organic solvent were more than twice of that when using methanol and acetonitrile. This is consistent with the observation using direct infusion (Fig 2), which indicates that the impact of the organic solvent percentage difference upon elution did not offset the ionization efficiency enhancement significantly. Dramatic differences in signal intensities were observed when using different ion-pairing agents (Fig 3b). The signal intensity of the 24mer when using DIEA as the ion-pairing agent was almost 8 and 2 times of that when using TEA and DMBA, respectively. This difference probably reflected both the difference in the retaining capability and ionization enhancement effect of the three ion-pairing agents. As seen in Table 1a, the retention time difference of 24mer when using DIEA was 0.4 and 0.2 minutes longer than that when using TEA and DMBA, which equals to 4.8% and 2.4% of ethanol, respectively. In our previous study[22] the signal enhancement ability of DIEA was observed to be 50% and 10% stronger than TEA and DMBA respectively. In addition, these differences might be even be magnified in LC-MS experiments compared to direct infusion because the flow rate was 15 times higher.

The signal intensity difference was also explored using different concentrations of HFIP and DIEA. As seen in Figure 3b, 15mM DIEA provided a remarkable increase in signal intensity when compared to 1.7mM DIEA. 100mM HFIP suppressed the signal severely while no considerable difference between 25 and 50mM of HFIP was observed. Considering the lack of influence of HFIP concentration on the chromatographic selectivity, 25mM HIFP and 15mM DIEA were used in the optimized bioanalysis method. The optimized mobile phase system offered approximately two orders of magnitude of sensitivity increase compared to the

commonly used mobile phase system composed of 100mM HFIP, 1.7mM TEA and MeOH as the organic solvent when other conditions were identical.

Choosing optimum column dimensions and flow rate. There are several studies analyzing oligonucleotides using UHPLC with UV or PDA detection[27-29] and one study characterizing oligonucleotide using UHPLC-MS [30]. However, there are no reports on quantitative bioanalysis of oligonucleotides using UHPLC-MS so far. Previous research revealed that lower flow rates greatly favor sensitive detection of oligonucleotides by ESI-MS[11]. However the flow rate for sub-2 micron UHPLC columns to achieve optimum column efficiency is usually more than 0.5ml/min, which could be a major hurdle to hyphenate UHPLC with MS for oligonucleotide analysis. In this study we further explored the trade-offs that have to be made between MS sensitivity and chromatographic resolution. 20μL of 10μg/ml of 23mer, 24mer and polyT32 water solution containing 15mM of DIEA were injected onto an Acquity BEH C18 column (1.7μm, 2.1×50mm). It is worth noting that T32 was used here because its retention time difference from 23mer and 24mer is greater than C18 therefore better demonstrates the change of flow rate did not alter the retention time. In order to make side-by-side comparison, 0.5 second and 0.1 second scan time were used for 0.1ml/min flow rate and 0.5ml/min flow rate respectively so that the scan numbers per peak are approximately the same. As seen in Figure 4a, 0.5ml/min flow rate resulted in almost baseline separation of 23mer and 24mer while only partial resolution was achieved when the flow rate was 0.1ml/min. However, when the most abundant m/z peak of 24mer was chosen to evaluate the sensitivity, the signal to noise ratio of 24mer at the flow rate of 0.1ml/min was more than twice of that at the flow rate of 0.5ml/min (Figure 4b). Longer column with smaller internal diameter(ID) were tested to avoid band broadening caused by low flow rate needed for sensitive MS detection. Sharper peaks and reduced band broadening was observed

when increasing the column length from 50mm to 100mm and decreasing the column ID from 2.1mm to 1.0mm (details not shown). However, further increasing the column length to 150mm did not enhance the resolution and resulted in elevated column pressure. Therefore, a 1×100mm column and 0.15ml/min flow rate was chosen for UHPLC separation. Due to the high efficiency of the UHPLC column, the chromatographic separation time (5 minutes) was dramatically reduced compared to previously reported methods (>15minutes).

Optimization of biological sample preparation. In a recent review of oligonucleotide bioanalysis, the coupling of MS/MS with UHPLC was acknowledged to enhance method sensitivity and specificity by providing better chromatographic resolution and sharper peaks. However, they pointed out that these advantages were offset by the challenges for routinely analyzing oligonucleotides from biological matrices primarily due to the short life of UHPLC columns[31]. One of the major contributors to the short column lifetime in bioanalysis is the residual substances from the sample extraction. Therefore, it is critical to develop a sample preparation protocol that generates "cleaner" solutions of oligonucleotide for UHPLC-MS/MS assays to be successful. Liquid-liquid extraction using phenol and chloroform has been used in combination with SPE for bioanalysis of therapeutic oligonucleotide previously [11, 32-34], but a one-step phenol-chloroform LLE results in samples containing residual endogenous substances which could cause increased column pressure and selectivity issues. Ethanol precipitation of DNA following the LLE has long been used in microbiology, molecular biology and biochemistry for further purification of DNA[35]. However there are no reports using ethanol precipitation for the quantitative bioanalysis of oligonucleotides.

A standard protocol for ethanol precipitation of DNA is as follows: 1) add 1/2 volume of 5M ammonium acetate and 2-3 volumes of 100% ethanol; 2) mix and freeze overnight at -20℃; spin

at full speed in a microcentrifuge at 4 degrees for 30 minutes; 3) decant (or carefully pipet off) the supernatant; dry the pellet; 4) add desired quantity of water and vortex and spin down to resuspend[35]. There are some variations of this protocol for analyzing DNA fragments of different sizes such as using isopropanol instead of ethanol, adding ethanol without 5M ammonium acetate, freeze 1 hr at -80 ℃, washing the pellet with 95% ethanol, adding carrier molecule before precipitation, etc. We compared the impact of different conditions on the recoveries of oligonucleotides and optimized the precipitation protocol. 200μL of plasma was spiked with 100ng/ml 23mer and 24mer 2μg/ml IS (C18) in the different extraction protocols and the measurement of recovery under each set of conditions were made in triplicate. As seen in Figure 5, replacing ethanol with isopropanol resulted in lower recovery and adding 5M ammonium acetate dramatically reduced the recovery. Adding glycogen as the carrier molecule did not increase the recovery significantly but greatly enhanced the clarity of the DNA pellet. Therefore, in the optimized protocol ethanol was chosen as the precipitating solvent without addition of any salt and glycogen was used as the carrier molecule. Longer incubation times after the addition of ethanol have been reported to increase the recovery of smaller DNA fragments. So incubation times at -80 ℃ from 1hr to 8 hr were tested but no significant difference in recovery was observed (data not shown). Recoveries comparable to more complicated sample clean-up procedures[11] were achieved using this protocol (Table 4). Using this optimized protocol, no column pressure increases nor any noticeable separation deterioration was observed throughout this study (>500 injections of biological samples).

Elimination of non-specific binding and enhancing assay specificity. Non-specific binding to containers, LC columns and instrument surfaces is a major obstacle in the development of reproducible and sensitive quantitative methods for biomolecules[3, 5, 11]. In this study, all the

centrifuge tubes used for sample preparation were DNA Lobind tubes to minimize binding to the container. In addition, a high concentration (1μg/ml) of the internal standard was used as the "sacrificial oligonucleotide" to saturate all the surfaces where potential non-specific binding could occur. The effectiveness of this strategy was previously demonstrated[36]. However this study also observed that overly high concentrations of a polyT oligonucleotide (internal standard) could cause cross-talk between different MS/MS channels and compromise the method specificity, especially at the lower concentration range of analytes. Because the product ion selected for the 23mer and 24mer is [w_1-H_2O] which could only be generated by oligonucleotides with a terminal T, polyC18 was chosen as the internal standard so no cross-talk would occur irrespective of the IS concentration and excellent method specificity could be maintained.

Anti-coagulant reagents used in plasma can also cause assay specificity issues. Blank Sprague-Dawley rat plasma with di-sodium EDTA and heparin sodium were extracted using the optimized sample preparation respectively. As seen in Figure 6, no apparent peak around the retention time of the analytes and the IS were seen from the blank plasma with di-sodium EDTA while severe interference was seen from the sample containing heparin sodium. The sharp peak at 1.5min in Figure 6 was caused by diversion from waste to the MS. The interference was observed in all three MS/MS channels and choosing a more selective m/z ratio for the product ion or altering the gradient profile could not eliminate this interference. Special attention should be paid to this issue when developing bioanalysis methods for oligonucleotides.

Assay Validation. Different lots of rat plasma (n>6) were extracted and analyzed. No significant peaks were observed in either the analyte or the IS MS/MS channels for these matrix control samples, indicating that the method was highly selective (Figure 7). The 23mer, 24mer and IS

were all analyzed individually and no cross-talk was observed between their MS/MS channels. The dynamic range of the current method is 2.5-1000 ng/ml. The calibration curves for 23mer and 24mer have R^2 values of 0.9983 and 0.9967, respectively. The intra-day and inter-day precision and accuracy for both 23mer and 24mer are summarized in Table 2. The autosampler, bench top and freeze-thaw stability are summarized in Table 3. The absolute recovery, relative recovery and matrix effects of the method are summarized in Table 4. Plasma samples were spiked with 23mer and 24mer at 50,000 ng/mL and diluted with blank rat plasma (1 in 50) prior to analysis (n = 5). All of the back-calculated values were within ±15% of the nominal concentrations. The precision was less than 8.7%, and accuracy was in the range of −5.6% to 4.1%, respectively, for 1 in 50 dilution.

Method application. Because high plasma concentrations were expected, the rat plasma samples at 5, 15, 30, 60 min were diluted 50 fold with blank plasma and then processed based on the proposed extraction protocol. The plasma concentration versus time profile is presented in Fig 8. The $T_{1/2}$ was approximately 24 minutes, which was similar to earlier reports(29 min)[26]. The method was capable of detecting the pharmacokinetics profile of both the parent drug and its major chain-shortened metabolite up to 9 hours. The concentrations of 24mer and 23mer at 9hrs were 16.7ng/ml and 6.7ng/ml, respectively.

CONCLUSIONS

In this study we systematically optimized the mobile phase composition to enhance the sensitivity of the LC-MS method. The impact of mobile phase composition on ionization efficiency and chromatographic separation were investigated comprehensively. The optimized mobile phase system could offer as much as two orders of magnitude sensitivity enhancement when compared to the most commonly used LC-MS mobile phase systems. Column dimensions

and flow rates were optimized to balance chromatographic separation and MS sensitivity. The shortest reported method run time of 5 minutes was achieved after the optimization. An LLE protocol with ethanol precipitation sample preparation was optimized and first implemented for the quantitative bioanalysis of an oligonucleotide. The protocol offered high recovery and the extracted solutions contained minimum residual substances, which greatly enhanced the UHPLC column life time. The method was validated with an LOQ (2.5ng/ml) lower than the most sensitive LC-MS method reported(4ng/ml)[26] and was applied to a proof of concept animal experiment. The obtained pharmacokinetic parameters were similar to an earlier report. Therefore this method could be applied to pharmacokinetic or toxicokinetic studies of therapeutic oligonucleotides. The automatable and affordable sample extraction procedure, the fast chromatographic separation and the sensitive mass spectrometry detection of this method offers great potential for mitigating the limitations of LC-MS based assay for bioanalysis of oligonucleotide therapeutics. Substantial advances in LC-MS based assays for the oligonucleotides have been made over the past two decades. The substantial improvements have paved the way for LC-MS based assays to meet regulatory requirements and be fully applied to preclinical and clinical studies for therapeutic oligonucleotides.

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Scheme 1. Equilibriums in the liquid phase

$$Oligo \ + nH_2O \Leftrightarrow Oligo^{n-} + nH_3O^+ \quad \text{.....(a)}$$

$$DIEA + H_2O \ \Leftrightarrow HDIEA^+ + OH^- \quad \text{.....(b)}$$

$$HDIEA^+ + Oligo^{n-} \Leftrightarrow \left[Oligo \Lambda \ H \Lambda \ DIEA\right]^{(n-1)-} \quad \text{.....(c)}$$

$$H_2O + HFIP \Leftrightarrow HFIP^- + H_3O^+ \quad \text{.....(d)}$$

Table 1a

Chromatographic separation of IS and 24mer (15mM ion-pairing agent, 50mM HFIP in water as mobile phase A and in water:organic solvent(50:50) as mobile phase B)				
Organic solvent	Ion-pairing agent	C18 retention time(min)	24mer retention time(min)	Selectivity α (24mer/C18)
MeOH	DIEA	4.06	5.00	1.23
ACN	DIEA	2.99	3.26	1.09
EtOH	DIEA	3.24	3.41	1.05
IPA	DIEA	2.99	3.06	1.05
EtOH	TEA	2.89	3.00	1.03
EtOH	DMBA	3.04	3.21	1.05

Table 1b

Chromatographic separation of IS and 24mer (DIEA and HFIP in water as mobile phase A and in water:EtOH (50:50) as mobile phase B)				
DIEA concentration(mM)	HFIP concentration(mM)	C18 retention time(min)	24mer retention time(min)	Selectivity α (24mer/C18)
1.7	25	3.09	3.16	1.02
1.7	50	3.19	3.26	1.02
1.7	100	3.29	3.31	1.00
15.7	25	2.83	2.95	1.04
15.7	50	3.24	3.41	1.05
15.7	100	3.45	3.62	1.04

Table 2: Intra-day and Inter-day accuracy(%error) and precision(%RSD) of the method

Conc. (ng/ml)	Intra-day(n=5)						Inter-day(n=15, 3days)					
	Obsd conc. (ng/ml)		RSD(%)		Error(%)		Obsd conc. (ng/ml)		RSD(%)		Error(%)	
	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer
2.5	2.47±0.21	2.46±0.26	8.50	10.56	1.21	1.62	2.58±0.22	2.48±0.25	8.52	10.00	3.2	0.82
7.5	7.43±0.41	7.24±0.36	5.52	4.97	0.93	3.46	7.41±0.78	7.37±0.60	10.52	8.14	1.2	1.73
75	75.18±3.67	71.82±1.42	4.88	1.97	0.24	4.24	75.78±4.60	71.18±3.01	6.07	4.22	1.04	5.09
750	752.13±11.46	720.63±6.82	1.52	0.94	0.28	3.91	788.07±33.09	730.36±23.32	4.19	3.19	4.93	2.61

Table 3: Stability of 23mer and 24mer in rat plasma

	freeze-thaw stability(-80 ℃) (three cycles)				room-temp stability(25 ℃, 8 h)				autosampler stability(5 ℃, 24hr)			
Nominal conc.(ng/ml)	**7.5**		**750**		**7.5**		**750**		**7.5**		**750**	
	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer	23mer	24mer
Obsd conc. (ng/ml)	7.73 ±0.22	7.32 ±0.47	754.9 ±43.75	776.99 ±62.74	6.8 ±0.61	7.41 ±0.83	796.82 ±31.97	807.32 ±37.56	7.88 ±0.89	7.32 ±0.69	801 ±18.65	706.83 ±14.39
Accuracy(%)	103.1	97.6	100.0	103.60	90.66	98.8	106.2	107.6	105.0	97.6	106.8	94.24
RSD(%)	2.84	6.42	5.80	8.07	6.31	11.21	4.01	4.65	11.29	9.42	2.32	2.03

Table 4: Absolute, relative recovery and matrix effect of 23mer, 24mer and IS

Conc. (ng/ml)	absolute recovery(%)		relative recovery(%)		matrix effect(%)	
	23mer	24mer	23mer	24mer	23mer	24mer
2.5	76.66±8.06	76.78±6.68	83.33±8.33	79.48±7.30	91.99	96.60
7.5	79.07±5.78	78.32±4.41	81.81±8.39	82.82±3.01	96.65	94.56
75	78.73±2.46	79.38±2.93	84.75±3.10	88.88±2.36	92.89	93.66
750	80.43±3.27	78.92±0.92	88.38±1.64	87.07±2.82	91.00	90.63
IS(2 µg/ml)	75.39±8.90		78.86±3.34		95.59	

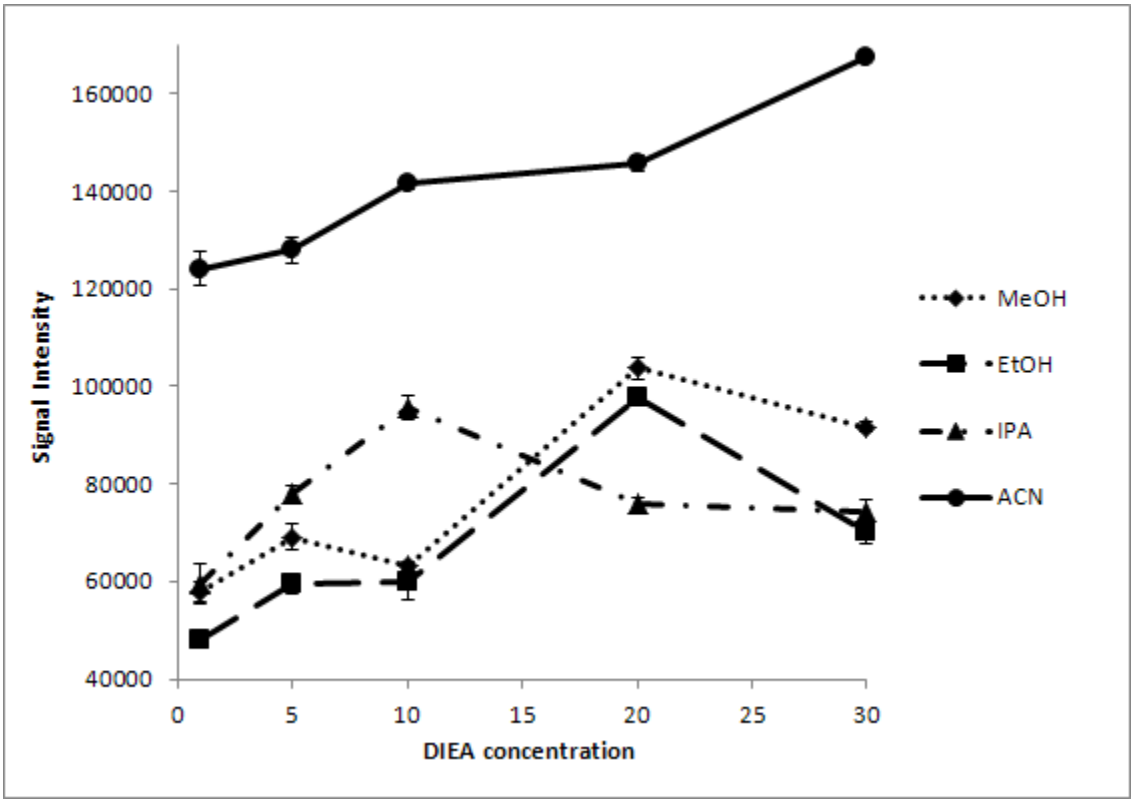


Figure 1: Impact of organic solvent type and ion-pairing agent concentration on signal intensity of the 24mer.

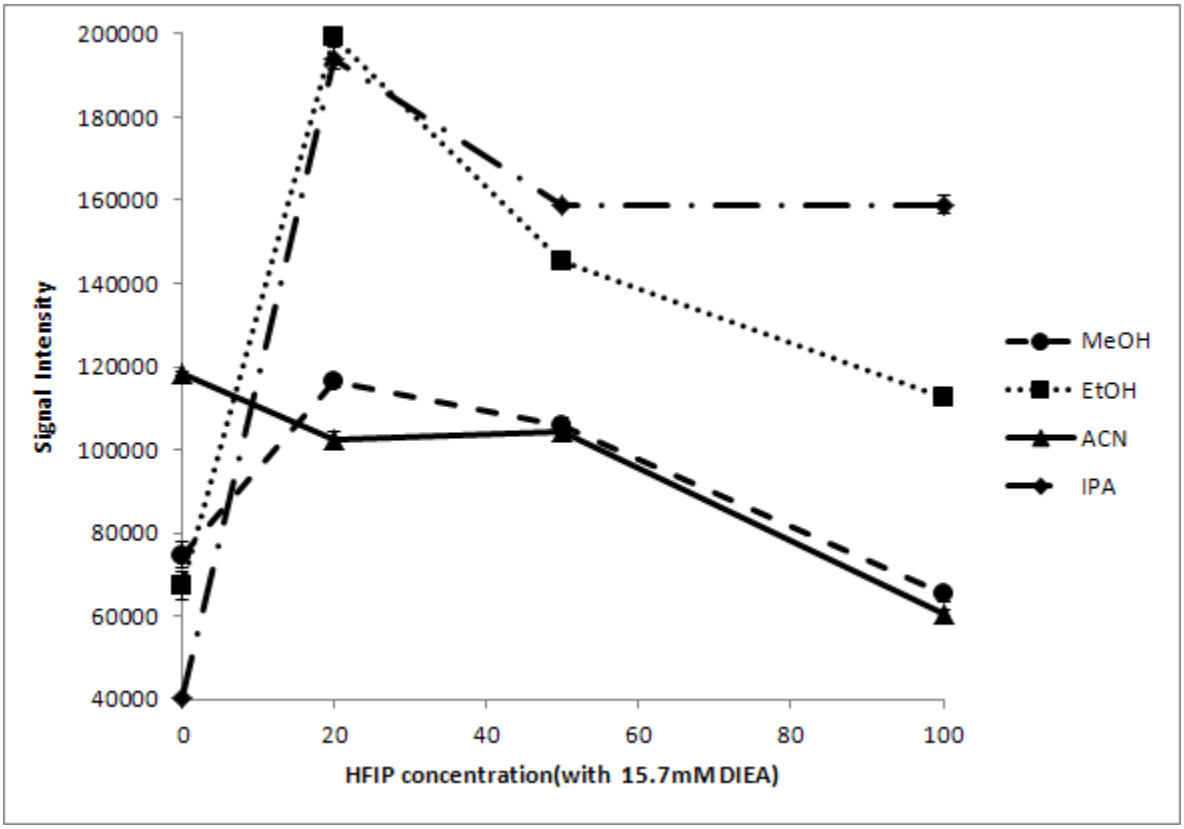
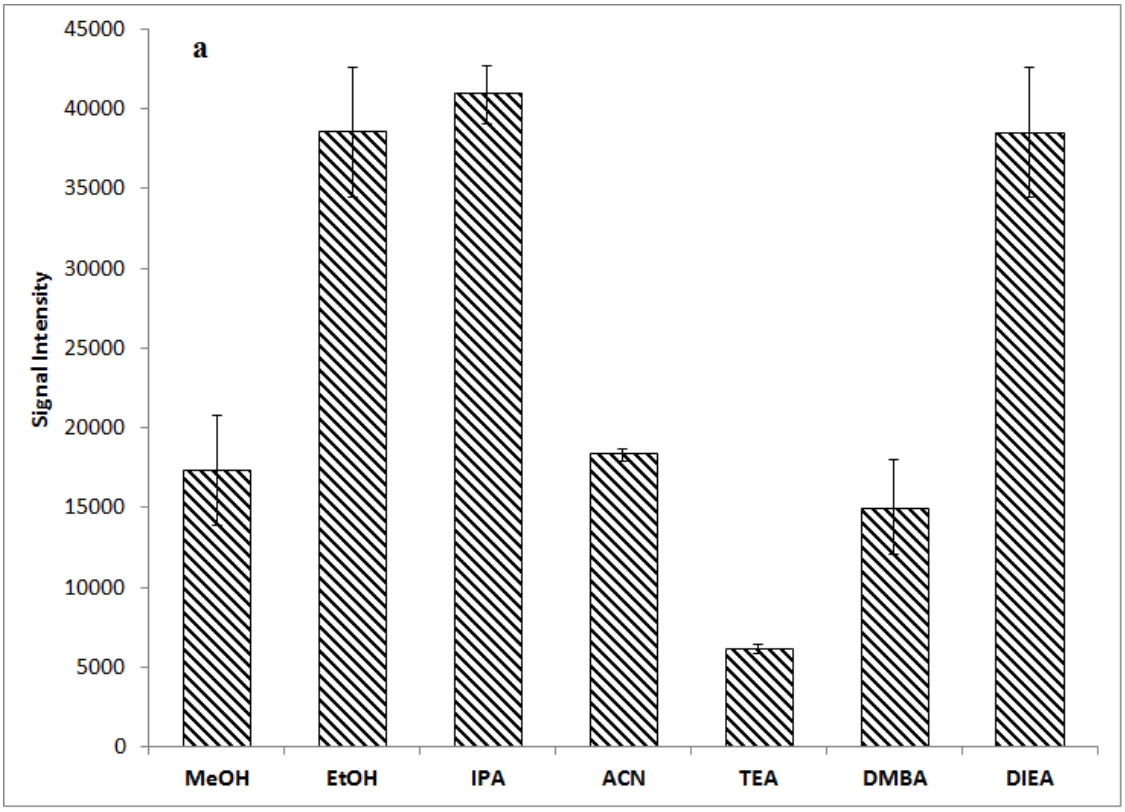


Figure 2: Impact of organic solvent type and HFIP concentration on signal intensity of 24mer



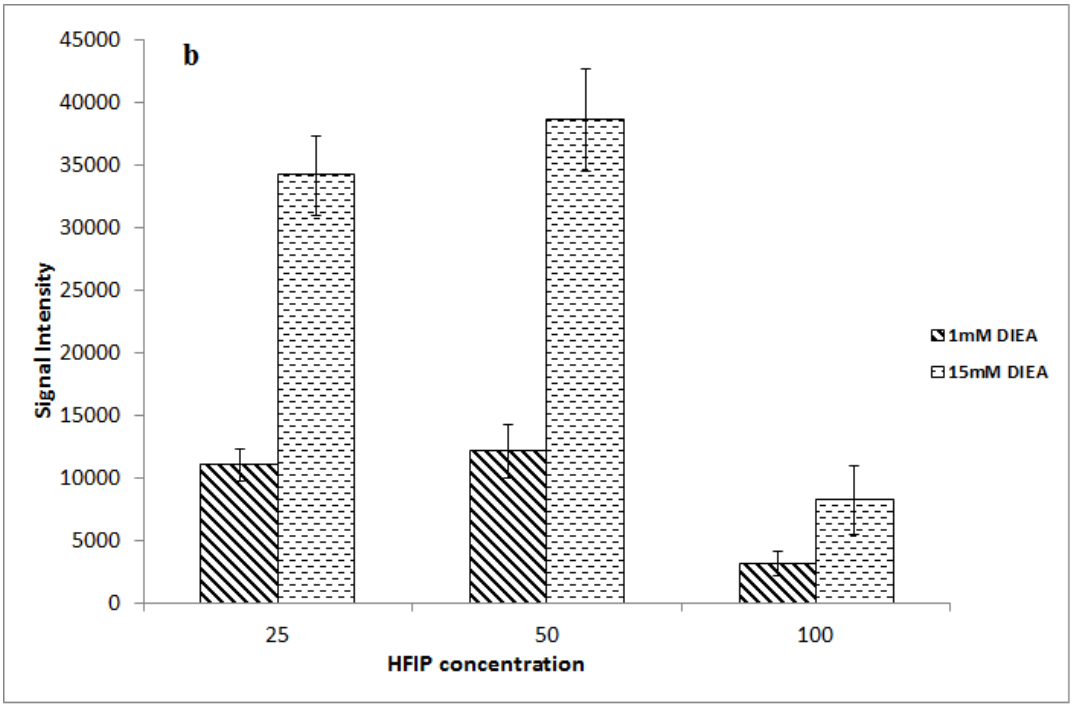


Figure 3: Impact of **a**: organic solvent and ion-pairing agent type **b**: DIEA and HFIP concentration on LC-MS sensitivity of 24mer

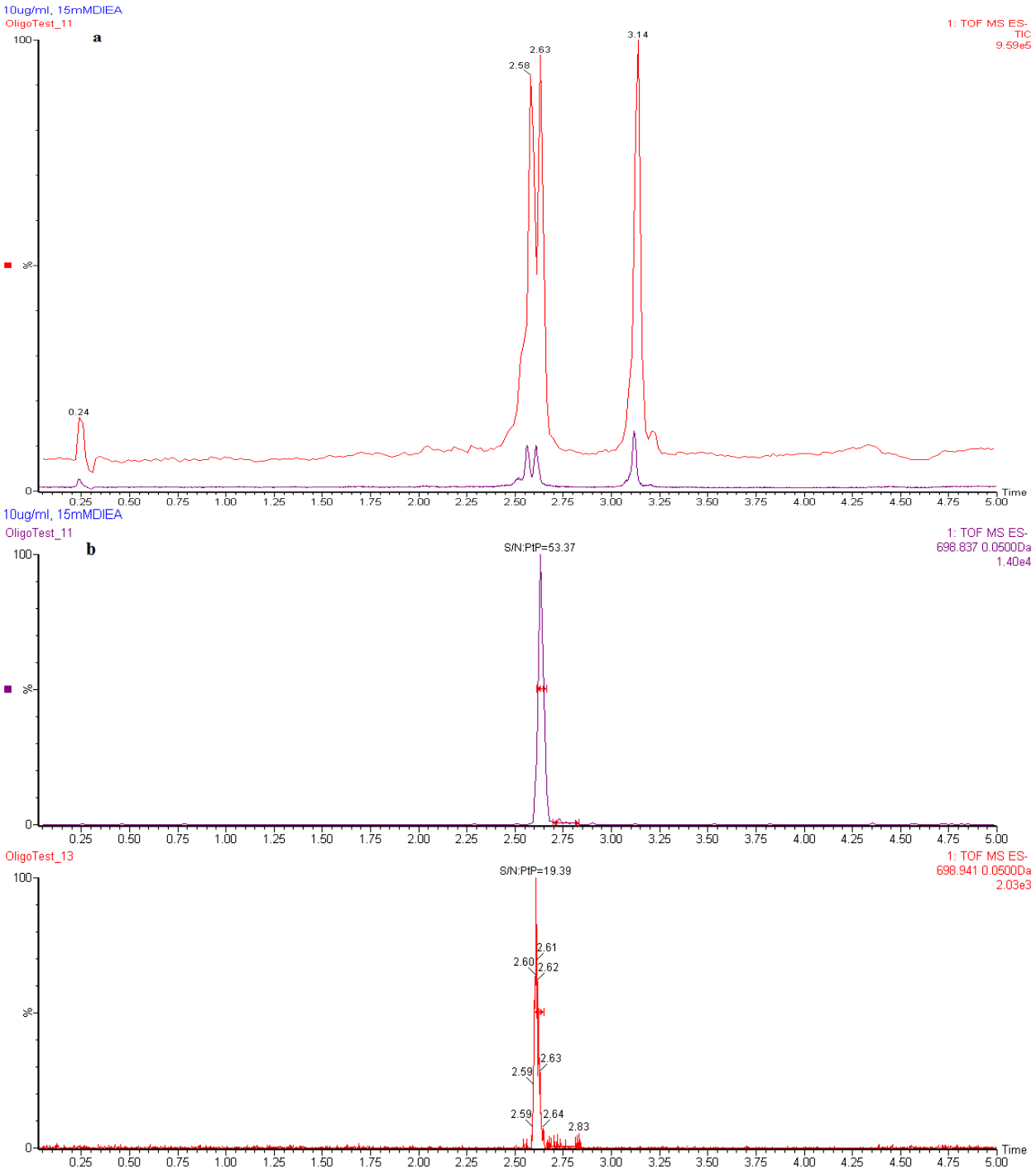


Figure 4: Impact of flow rate on **a**: chromatographic separation of oligonucleotides. Upper trace: flow rate=0.1ml/min, scan time 0.5s; lower trace: flow rate 0.5ml/min, scan time 0.1s. **b**: signal to noise ratio of 24mer. Upper panel: flow rate=0.1ml, scan time 0.5s; lower panel: flow rate=0.5ml, scan time 0.1s

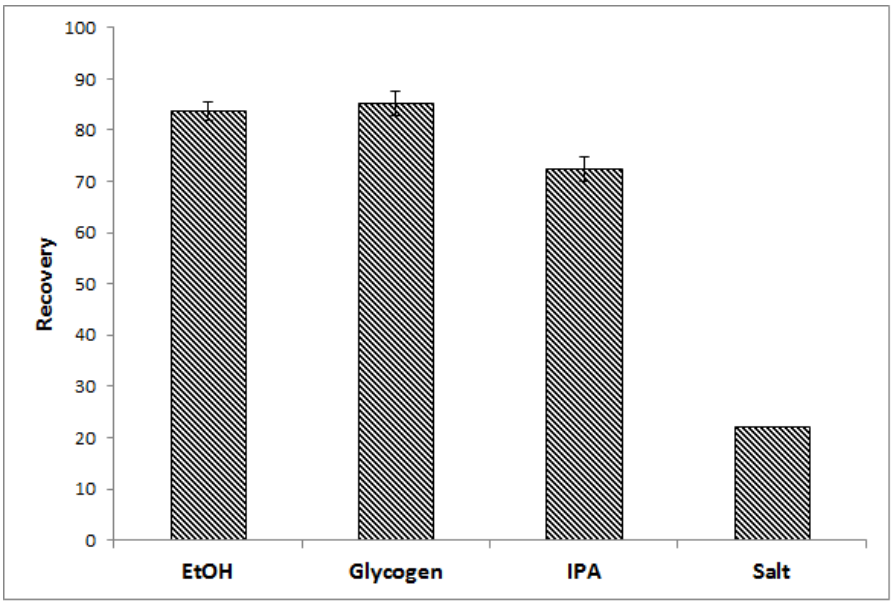


Figure 5: Impact of ethanol precipitation condition on recovery of the 24mer. Detailed conditions are described in the text

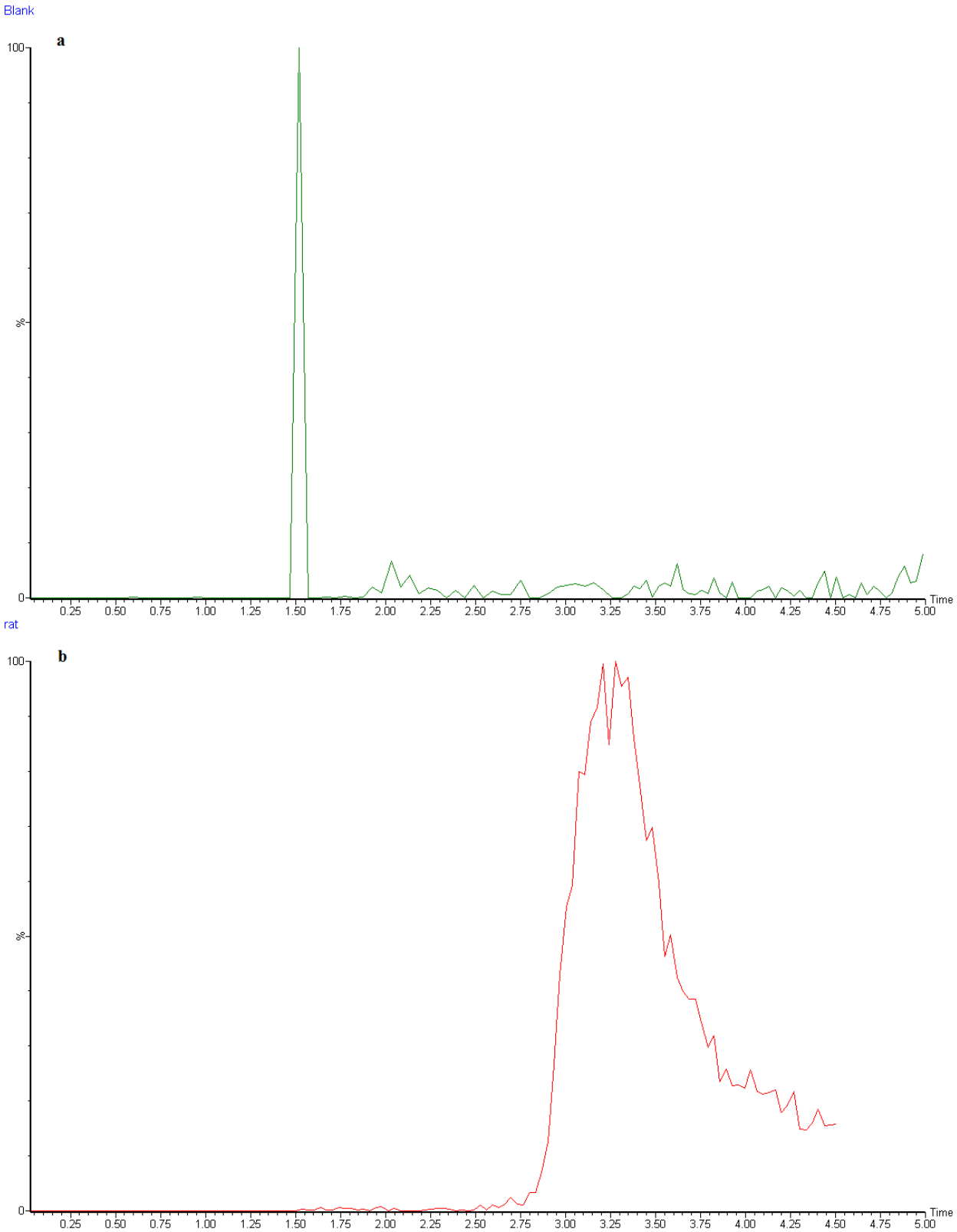


Figure 6: Artifacts caused by anticoagulant. **a**: EDTA Na_2; **b**: Heparin Na

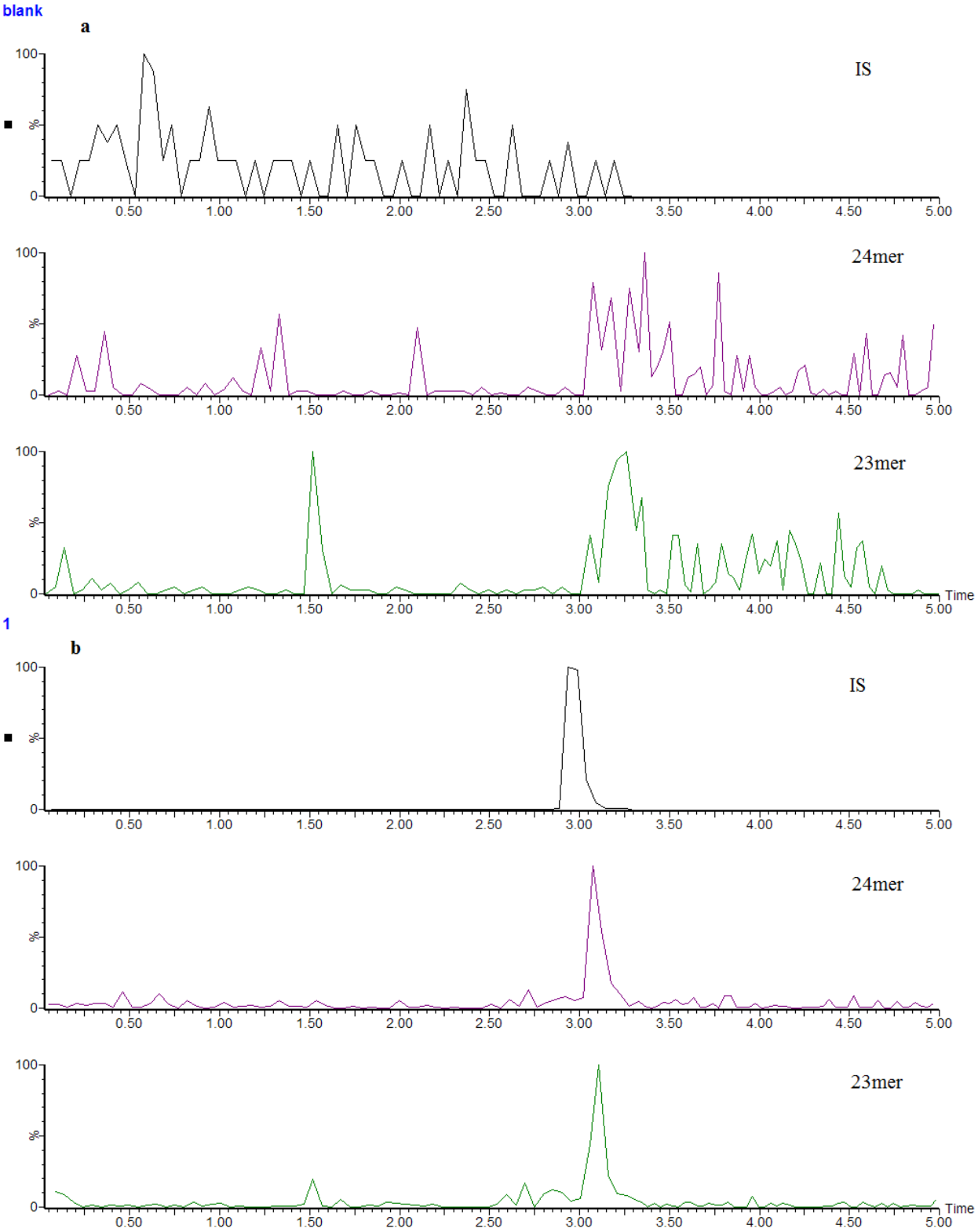


Figure 7: Chromatograph of **a**: extracted blank plasma; **b**: plasma spiked with 2.5ng/ml 23mer and 24mer and 2µg/ml IS (LOQ)

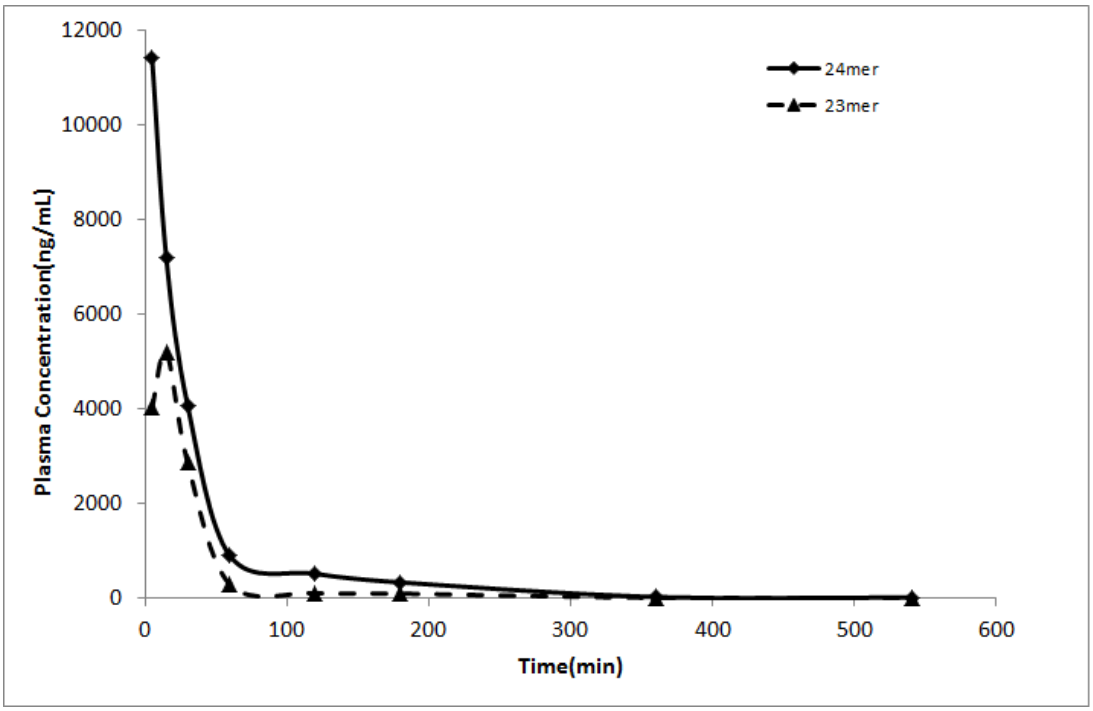


Figure 8: Plasma concentration of 23mer and 24mer in a male Sprague Dawley rat after a 1mg/kg i.v. bolus injection of 24mer

Chapter 6

Conclusions

Bioanalysis of oligonucleotide therapeutics using LC-MS based assays is challenging in various aspects. Enhancing the sensitivity and simplifying sample preparation procedures are two major challenges. This study systematically evaluated the chemical composition of the mobile phase in respect to their impact on chromatographic separation and electrospray desorption efficiency for oligonucleotides. The Henry's Law Constant of an ion-pairing agent was found to play an imperative role in the charge state distribution and ionization efficiency of oligonucleotides. This finding led to the discovery of an ion-pairing agent DIEA, which provides more ionization enhancement than TEA for oligonucleotides. The "wrong way around" ionization facilitating effect of HFIP was attributed to its high Henry's Law Constant and moderate acidity. The dielectric constant of an organic solvent affects its interactions with ion-pairing agents and HFIP significantly. Ethanol and isopropanol increased the desorption efficiency of oligonucleotides almost twice when compared to the most commonly used solvent methanol. A one-step solid phase extraction (SPE) method and a liquid-liquid extraction (LLE) followed by an ethanol precipitation procedure were developed for extracting an oligonucleotide from rat plasma. A longer more hydrophobic internal standard was used in the SPE method to prevent non-specific binding during the extraction. The concentration of the internal standard was optimized to avoid interference with analytes due to "cross-talk" between MS/MS channels. LLE with ethanol precipitation leaves minimal residual substances therefore it suits high throughput UHPLC methods best. Other issues such as optimizing LC column dimensions, choosing appropriate flow

rates and scan rates, preventing oxidation during drying the process and eliminating artifacts caused by anti-coagulants were also addressed. Both methods were fully validated according to FDA guidance and applied to proof of concept animal experiments.

Appendix

Determination of therapeutic oligonucleotides using capillary gel electrophoresis

Chen B and Bartlett MG. *Biomedical Chromatography*, 2012; **26***:409-418.* Reprinted with permission of publisher

Abstract: Oligonucleotides have developed into highly versatile and selective therapeutics over the past twenty years. More than five discrete mechanisms of action have been reported and more than ten different chemical modifications have been used to extend their in vivo half life and reduce their toxicity. Capillary gel electrophoresis (CGE) has been used extensively for the quantitative analysis of oligonucleotide therapeutics in both preclinical and clinical studies since the 1990's. The success of CGE is based on its extraordinary resolving power which allows for the simultaneous determination of the parent drug and its metabolites. More recently, capillary gel electrohporesis has seen renewed interest with the emergence of replaceable gels with single-base resolving power and new capillary electrophoresis – mass spectrometry interfaces. This review discusses the bioanalysis of therapeutic oligonucleotides showing the evolution of the field over the past two decades leading to the current new approaches. Included in this review are topics such as different gel types, sample introduction modes, sample extraction procedures, separation conditions and detection methods used in CGE along with discussions of the successes and limitations associated with each.

Introduction: In the two decades since the first antisense oligonucleotide drug Vitravene was approved for use against cytomegalovirus-induced retinitis by local injection (Crooke, 1998) over 20 oligonucleotide therapeutics have been submitted to the United States Food and Drug Administration for Investigational New Drug approval (Lee, 2008). Oligonucleotides as a new class of therapeutic agents have gained ever increasing interest because of their versatility and unparalleled selectivity. Numerous types of oligonucleotides, interfering with gene expression at

different levels, have been used for therapeutic purposes including antisense oligonucleotides, aptamers, ribozymes, DNAzymes, siRNAs and miRNAs. It has been reported that antisense inhibitors have already been developed for as many as 4000 genes (Crooke, 2004). The sequencing of the human genome and the elucidation of the many molecular pathways of important diseases has opened a plethora of previously inaccessible therapeutic targets for oligonucleotides. They have emerged as promising candidates for drug therapy against a wide range of diseases, including cancer, AIDS, Parkinson's disease, Alzheimer's disease and cardiovascular disorders (Paril, 2005). Currently, there are two therapeutic oligonucleotides approved in the United States with another fifty-eight undergoing clinical trials. Of these 58, 36 are antisense DNAs, 11 are aptamers and 11 are siRNAs. The blossoming of oligonucleotide therapeutics has truly initiated the era of gene therapy.

As versatile as they are, unmodified oligonucleotides are highly susceptible to nuclease degradation and therefore impractical for direct therapeutic use. Typically, the half-lives of phosphodiester DNAs range from 15-60 minutes in most sera and RNAs are even more unstable (Crooke, 1992). Therefore, a wide range of modifications have been used to enhance the stability of oligonucleotides (Figure 1). The first of these modified oligonucleotide analogs were the phosphorothioates, which were first synthesized in 1969 by DeClercq et al.. (DeClercq et al.. 1969). In phosphorothioates, an oxygen atom in the phosphodiester linkage is replaced with a sulfur atom resulting in a compound with significant resistance to nuclease degradation. Unlike analogs with modifications on the sugar moiety, phosphorothioates maintain DNA-like properties and are excellent substrates for RNAase H (Crooke, 2004). Phosphorothioates also have very low affinity for serum albumin and their binding is saturable at higher doses (Crooke, et al., 1996, Agrawal, et al., 1991, Iversen, 1992). The absorption and distribution of

phosphorothioates are extremely rapid following intravenous administration. The terminal elimination half-life is 40-60 hrs in animals and even longer in humans (Crooke, et al., 1994). The clearance of phosphorothioates is primarily due to metabolic degradation by exo- and endonucleases (Iversen, 1992, Cossum, et al. 1994, Cossum, et al., 1993). In addition to intravenous use, aerosol (Nyce, 1997, Templin, et al., 2000) and topical administration (Saxena, et al., 1990) of phosphorothioates have been reported with promising results.

The second generation of oligonucleotide analogs was the 2'-O-(2-methoxyethyl) (2' – MOE) chimeras. The 2'-MOE modification gives the molecule more RNA like properties and increases its affinity for target mRNA by several orders of magnitude compared to phosphorothiaotes (Crooke, 2004). However, their potency was compromised due to their lack of capacity to initiate RNAse H degradation. When made in chimeric structures, 2'MOE oligonucleotides are only five times more potent than the corresponding phosphorothioate in vivo (Stepkowski, S.M., et al.. 2000). 2'-MOE chimeras are substantially more stable than phosphorothioates with elimination half lives of 30 days in plasma and several tissues from mice, rats and monkeys (Geary, et al.. 2001a, Geary, et al.. 2001b).

Acute and transient toxicities from oligonucleotides include cardiovascular collapse and inhibition of clotting has been reported. These toxic responses are believed to be related to both peak plasma concentrations and the sequence lengths of the oligonucleotides (Henry, et al.. 1997a, Henry, et al.. 1997b, Wallace, et al.. 1996, Griffin, et al.. 1993, Nicklin, et al.. 1997). Subchronic toxicities such as immune stimulation have also been found in rodents but are far less severe in primates (Crooke, 2004).

Considering the long elimination half-lives of oligonucleotide therapeutics and their concentration related toxicities, it is necessary to develop sensitive quantification methods to

monitor the drug concentration and generate accurate and complete pharmacokinetic profiles. Given that oligonucleotides go through intensive metabolism and some chain shortened metabolites likely still have antisense activity and similar toxicities as the parent compound, quantification methods with the ability to measure and identify these active metabolites are highly desired by regulatory agencies. Furthermore with quickly emerging genetic targets, the selection of sites on a mRNA molecule for optimal antisense activity requires a robust and reproducible method with high throughput capacity. Currently, numerous quantification techniques have been reported for the bioanalysis of oligonucleotides, including quantitative PCR, ELISA and radiolabel tracer methods. A major limitation of these methods has been their inability to differentiate the metabolites from the parent drug and to identify unknown metabolites. Capillary electrophoresis has demonstrated an unprecedented ability to separate metabolites and other chain shortened products from their parent compounds. The potential of CE to be interfaced with mass spectrometry provides the ability to identify these species. However, the high molecular weight and poly-anionic nature of oligonucleotides, along with their high degree of modification has posed unprecedented challenges to the bioanalytical community. These challenges cover many areas, such as biological sample extraction, separation and sensitive detection. For the past 20 years, scientists have made enormous efforts to overcome these hurdles and significant progress has been made in all of these areas. A few recent reviews cast different technological aspects of analyzing nucleic acids using capillary electrophoresis, including CE-MS and miniaturized CE analysis (Willems, 2005; Righetti, 2002). However, the last review on the bioanalysis of oligonucleotide therapeutics was published more than 10 years ago (Chen, 1998). During this time there have been many important studies involving the new generations of oligonucleotides and substantial technological advances. In this

review, electrophoretic methods developed for quantification of oligonucleotides and their metabolites will be discussed along with the remaining challenges to this field moving forward.

Capillary Electrophoresis

Capillary Gel Electrophoresis (CGE) was widely employed in preclinical and clinical studies for quantification of oligonucleotide therapeutics in the early years of oligonucleotide drug development (Yu, et al., 2004). CGE is a technique with high resolving power for the separation of parent oligonucleotides from their chain shortened metabolites. The separation in CGE is based on the length of the oligonucleotide strand and has been described using the reptation model(deGennes, 1971, Slater, et al., 1986). In this model, the electrophoretic migration of an oligonucleotide is delayed by collisions between the oligonucleotide and polymer molecules. The mobility of oligonucleotides through the polymer solution is inversely related to the probability of collisions, which is proportional to both oligonucleotide strand length and polymer solution concentration. Besides the mechanism of separation described in the reptation model, hydrophobic interactions were also proposed in a study where separation of oligonucleotides with the same length and sequence but differing only by a modification on a single base were achieved by using poly(N-vinylpyrrolidone) (PVP) as a pseudophase (Barry et al., 1996).

Types of Gel Matrices

Matrices for CGE of oligonucleotides broadly fall into two different classes of polymers. The first type is “replaceable” gels that can be rinsed in and out of the capillary. This type of gel consists of a low-viscosity polymer network such as linear polyacrylamide, alkylcellulose (Guttman, et al., 1995) and low-melting agarose (Hjerten, et al., 1994).

Replaceable gels have long been used for double stranded (ds) DNA analysis in applications such as DNA sequencing and PCR product screening, where each gel column is considered a single use item. For example, Heiger and coworkers used linear polyacrylamide to separate dsDNA fragments ranging up to several thousand base pairs (Heiger, et al., 1990). Melted agarose gels, hydroxyethyl cellulose (HEC) solution, polyethylene oxide, and many of their mixtures or derivatives have been used for DNA sequencing. Their applications have been extensively reviewed (Guttman, 2003) and will not be repeated here. However, these types of replaceable gels historically have lacked the ability to separate single stranded (ss) oligonucleotides differing by only a single base (Guttman, et al., 1995). More recently, new types of polymers capable of achieving single base resolution have been used as replaceable gels with great success (Guttman, 2003). Chen et al. (Chen, et al., 2006) used PEG 35000 alone for phosphodiester ssDNA separations and PEG 35000 together with acetonitrile for phosphorothioate ssDNA separations respectively, in each case demonstrating single base resolution. Szekely et al.. also reported separating dsDNA using PEG and organic modifiers achieving single base resolution (Szekely, et al., 2009). Polyvinylpyrrolidone (PVP) has been used alone (Gao, et al., 1998, Barry, et al., 1996) or with other types of polymers (Song, et al., 2001) to separate both single stranded and double stranded oligonucleotides with promising resolutions. Wang et al. (Wang, et al., 2006) achieved single base resolution for both dsDNA and ssDNA using a mixture of PVP and poly-TrisA. Zhang et al. (Zhang, et al., 2005) used pluronic gels, a novel thermoresponsive copolymer consisting of hydrophobic and hydrophilic blocks, to successfully separate a RNA sequence ladder. The thermothickening properties of this polymer resulted from transient intermolecular cross-links at higher temperatures offering

advantages of easy gel replacement and handling. Nevertheless, to date there are limited applications involving bioanalysis and especially quantitative studies using replaceable gels for oligonucleotides (Shang, et al., 2004). Therefore, while the current literature shows great promise for replaceable gels, it is unclear how well this technology platform will perform in more applied experiments.

The second major type of polymers used in CGE is "fixed" gels, which are chemically anchored to the surface of the capillary column. A chemical cross linker is usually used to form the gel and to create a more viscous matrix with a smaller pore size. The higher the concentrations of the monomer and the crosslinker, the smaller the pore size of the gel. This type of gel is often used for separation of oligonucleotides and their chain shortened metabolites or impurities due to its much higher resolving power relative to replaceable gels. Guttman et al. (Guttman, et al., 1988) reported the first single base separation using a dA40-60 DNA ladder with a fixed gel and thus opened the door for CGE based nucleic acid sequencing analysis. However, in all cases gel matrices are sensitive to temperature, changes in pH, and high voltage, which usually leads to bubble formation and termination of current flow (Tanaka, 1981). The performance of fixed gel columns diminish over a relatively low number of runs. The life time of capillary gel columns has been reported to be approximately 50 injections with standard solutions and 30 or fewer injections with plasma samples. This column instability is due to gel breakdown from repetitive applications of high temperature and voltage (Reyderman, et al.. 1997). Higher running voltages and separation temperatures usually shorten analysis time and enhance separation efficiency by denaturing the oligonucleotide molecule. However, the Joule heating effects of high voltage and high temperature are still significant even with circulating cooling systems. Without sufficient

sample clean-up, protein residues from biological samples may clog the pores in the polymer matrix accelerating the deterioration of the system (Reyderman, et al.. 1997). The limited column life time creates a significant obstacle when trying to develop validated quantitative methods, as the batch-to-batch reproducibility of the CGE columns is difficult to achieve. The combination of these effects makes the analysis of large batches of samples a challenge and highlights the specific need for inter-column reproducibility for any method validation. Therefore, the development of more robust fixed gel columns or replaceable gel columns with the ability to separate full length oligonucleotides from chain shortened metabolites are critical needs for this technique to reach its full potential.

Sample introduction

There are two modes of sample introduction in capillary electrophoresis: hydrodynamic injection (pressure, vacuum) and electrokinetic injection. Electrokinetic injection introduces the sample to the capillary by the force of the electric field, rendering the introduced sample amount dependent on both the analyte mobility and composition of the sample matrix. Hydrodynamic injection, in contrast, introduces the sample as a plug into the capillary, the composition of which is exactly the same as the original sample. Typically the relative standard deviations (R.S.D.) for migration time and peak area are 0.1-0.5% and 0.5-3%, respectively for hydrodynamic injection, which are two to three times lower than for electrokinetic injections (Gordon, et al., 1996, Lee, et al., 1994). For this reason, hydrodynamic injections have been generally preferred for quantitative analysis (Guttman, A., et al.. 1995). However, more recent studies also showed that hydrodynamic injection is associated with several disadvantages compared to electrokinetic injection such as smaller sample amount injected, lower reproducibility and difficulty of detection when applied to

biological samples (Noll, et al., 2007). Most of the quantitative work of therapeutic oligonucleotides done so far utilizes “fixed” gels as the sieving matrix due to their higher resolution, leaving electrokinetic injection the only feasible sample introduction mode for most CGE applications.

Quantitation issues and solutions related to electrokinetic injection have been extensively reviewed by Krivacsy et al.. (Krivacsy et al.. 2000) and factors specifically affecting oligonucleotide quantitation by CGE have been addressed by Guttman (Guttman, et al., 1995). There are two major biases that specifically affect bioanalysis of oligonucleotides. These are termed mobility bias and matrix bias. Mobility bias is caused by the different mobilities of the substances within one sample. During electrokinetic injections, high-mobility analytes are preferentially introduced into the capillary over slow moving ones. Salts and other low-molecular weight substances in biological matrices are all favored by electrokinetic injection, resulting in a limited amount of oligonucleotides being introduced to the column. Therefore, extensive sample cleanup is usually necessary for oligonucleotide quantitation by CGE to remove these competing molecules. In addition, mobility differences between the analyte and the internal standard should also be given careful consideration. Matrix bias is caused by ionic strength differences among different samples. The relative amounts of the same analyte introduced into the capillary will depend not only on the relative concentrations but also on the relative mobility (Gordon, et al., 1996). Given that the composition of the biological matrices varies largely from sample-to-sample, an internal standard is almost always required for quantitation. Furthermore, the small sample volume typically used in CGE applications can result in ionic strength or pH changes in the sample solution due to consecutive injections.

Consequently, a decrease in the amount of sample introduced into the capillary and reduced peak areas would be observed. Guttman et al.. (Guttman, et al., 1995) used water preinjection with the same voltage and injection time as used with the sample to introduce an "ion depleted" zone at the end of the capillary and thereby increase the amount of analyte injected (Fig 2). This method was applied to another study of GC rich DNA by combining a water preinjection of 10kV×2s with sample injection of 10kV×45s (Noll, et al., 2007).

Prolonged sample injection time and elevated injection voltage are usually used to increase method sensitivity. However, the over-loading effects of these practices should always be taken into consideration. Most commercially available instruments have a ramp time for the injection voltage to increase from zero to the set value. Given the injection times are the same, the higher the injection voltage, the longer the instrument will take to achieve the set value and the shorter the time spent at the injection voltage, resulting in less reproducible injection amounts. In general, longer injection times increase the width of the sample zone and shorten the separation path, resulting in distorted peak shapes, compromised separation efficiencies and lower reproducibility. Variations caused by migration time and peak area can be addressed by use of an internal standard and correcting the integration. Internal standards are especially important when the injected sample amounts are less reproducible.

Sample extraction

Quantitation of oligonucleotides in biological matrices by CGE typically requires extensive sample clean up and careful sample handling due to endogenous interferences and the extensive binding of oligonucleotides to cations, proteins and containers. The presence of low molecular

weight anions will compromise the method sensitivity as they compete with oligonucleotides during electrokinetic injection.

Most oligonucleotides, regardless of the chemical modifications, are reported to have high protein binding ratios (Lee, 2008). Plasma sample extraction typically includes two steps of solid-phase extraction (SPE) followed by a drop dialysis process. Given the multiply-charged anionic nature of oligonucleotides, a strong anion exchange solid-phase extraction (SAX-SPE) under basic pH (around 9) has often been used as the first SPE step to remove proteins and other cations. Next, a C18 SPE extraction is typically used to isolate the oligonucleotide based on polarity. There are still anions eluted with the oligonucleotide even following this second step of SPE. Therefore, a 30 minute drop dialysis step is used to decrease the ionic strength of the extracted sample and reduce the competition of anions with higher mobility during the electrokinetic injection process. A schematic of this sample preparation strategy is shown in Figure 3. Leeds et al. (Leeds, et al., 1996) and Crooke et al. (Crooke, et al., 1996) were the first two groups to utilize this sample extraction approach. Recovery of phosphorothioate oligonucleotides were reported to be approximately 40% with the majority of the losses attributed to irreversible binding to the C18 SPE column. To date, this procedure has been successfully applied to several studies and clinical trials (Geary, et al., 1997, Yu, et al., 2001, Yu, et al., 2004, Geary, et al., 1999, Shang, et al., 2004, Geary, et al., 2003, Soucy, et al., 2006, Noll, et al., 2005, Graham, et al., 2001, Szekely, et al., 2009, Noll, et al., 2007). Tissue extraction procedures in these studies were similar, except that a proteinase K digestion and a liquid-liquid extraction using phenol/chloroform (1:1 v/v) were added prior to solid-phase extraction to remove the proteins from tissues more thoroughly (Geary, et al., 1997, Geary, et al., 1999,

Agrawal, et al., 1991). Another SPE step using a phenyl bonded column was sometimes used for tissue extraction of 2'-MOE modified oligonucleotides between the LLE and SAX-SPE steps to further remove proteins from the matrix prior to analysis (Yu, et al., 2004, Geary, et al., 2003). A similar plasma sample extraction procedure was carried out for a 33-mer hammerhead ribozyme in a 96-well plate format. The sample was incubated under denaturing conditions (90 ℃, 8M Urea) prior to extraction. A weak anion exchange (WAX) SPE plate was used as the first step instead of a SAX SPE column and a C2 SPE plate instead of C18 SPE column was used for the second step of isolation. A less laborious 96-well plate dialysis was used for desalting instead of drop dialysis using a membrane. However, this approach was more time consuming (5hrs) (Bellon, et al., 2000). These arduous sample preparation procedures highlight the significant hurdles in the bioanalysis of oligonucleotides.

Alternative strategies were attempted to simplify the sample extraction procedure in several studies. A one step liquid-liquid extraction using phenol-chloroform (1:1) followed by drop dialysis was performed to quantitate a 15mer phosphodiester DNA using laser induced fluorescence (LIF) detection by Reyderman et al.(Reyderman, et al.,1997). However, no recovery data was reported for this assay. Chen et al. (Chen, S.H., et al.. 1997) employed a single step of SAX SPE followed by desalting for extraction of a 20mer phosphorothioate antisense DNA from plasma, urine and tissues. Formamide was added to the loading and eluting buffer as a denaturant. The recovery was reported to be more than 60%. A simple sample extraction assay using one step of C18 SPE followed by drop dialysis was reported by Wu et al.(Wu, L., et al., 2009) for the quantitation of a 19mer phosphorothioate (PS) from biological fluids, tissues and feces. Biological samples were diluted with a loading buffer containing high

concentrations of NaCl (1M) and extracted using a reversed-phase C18 SPE column before a final desalting step. The absolute recovery from biomatrices was reported to range from 80-120%. The authors observed increasing recovery of the PS 19mer and internal standard (IS) with higher concentrations of NaCl. Similar effects were not observed with other typical ion-pairing agents such as TEAA and TBAS, suggesting NaCl may play a unique role in the extraction process, although the specific mechanism for this effect is not clear. Maier et al. (Maier, et al., 1998) designed a novel "solid" phase extraction procedure by encapsulating oligonucleotides from biological matrices in polystyrene nanoparticles. By using a Tris-HCl(pH=9) buffer, oligonucleotides and protein interactions were minimized without precipitating the protein-oligonucleotide complex. At pH of 9, the polyanionic oligonucleotides have high affinity to the cationic polystyrene nanoparticles due to hydrophobic and electrostatic interaction. After the oligonucleotides were isolated from plasma components into the nanoparticles, the mixture was washed twice to remove residual proteins and salts. Then oligonucleotides were released by deprotonating the cationic group of the nanoparticles in strong alkaline conditions (pH=12-13). This ingenious sample extraction procedure was linear from 10-500nM (60-3000ug/ml) for a 20mer phosphorothioate and the recovery was between 61.9 and 86.8%. However, the author conceded that other polyanionic components from the matrix could also be extracted because of the nature of the extraction mechanism. In addition, the extremely basic releasing pH might be detrimental to unmodified oligonucleotides. Although further optimization for different applications might be needed, this method is time saving (30min), economic, and automatable. It is worth further exploration since the current bioanalysis techniques allow for the detection of oligonucleotide at much lower concentrations (low ng/ml range).

Separation Conditions

More recently, two studies evaluated the separation conditions for CGE analysis of oligonucleotides with more complex secondary structures: ds DNA and GC rich DNA. Both the studies discussed the impact of column temperature on the separation of oligonucleotides. Separation temperature of CGE has similar effects on quantitative analysis as on other separation modes using capillary electrophoresis, such as decreasing migration time, increasing resolution or compromising reproducibility due to Joule heating. For oligonucleotide separations specifically, temperature is especially critical when the conformation of the analyte is more complicated. For example, Szekely and coworkers reported that elevated column temperature caused significant peak splitting, which indicated the dimeric species of dsDNA was denatured and converted into the monomeric species (Szekely et al., 2009). Noll et al.., also found that temperatures as high as 60 ℃ helped to resolve a GC-rich DNA mixture (Noll et al., 2007). However, temperatures above 30 ℃ were reported to increase fluctuations in the baseline and current. No temperature above 60 ℃ was tested in this experiment due to the thermal instability of urea used in the run buffer.

Organic modifiers, similar to elevated temperatures, are often used in CGE analysis of oligonucleotides to resolve secondary structures and improve resolution. In the same work involving dsDNA separation, Szekely evaluated the effect of acetonitrile in the buffer system on double strand annealing (Szekely, et al.. 2009). With 20% of ACN added to the buffer, dsDNAs more readily annealed, even at relatively low temperatures. Compared to the buffer without ACN, where only a portion of dsDNA was denatured at 60 ℃, almost all dsDNA was denatured in the presence of ACN at the same temperature. In order to separate the GC-rich DNA mixture,

Noll and coworkers explored various organic solvents for their resolving power in the CGE system, including ethylene chlorohydrins (EC), N-methyl-formamide (NMF), formamide (FA) and dimethylsulfoxide (DMSO) (Noll, et al.. 2007). As a result, 10% DMSO was reported to significantly improve the resolving power of the separation system due to its strong denaturing ability and higher thermal stability. Other factors such as the effect of cations in the buffer on maintaining the intact duplex structure (Szekely, et al.. 2009) and increased capillary length's effect on improving resolution (Noll, et al.. 2007) were also included in these two studies.

Detection method

The heterocyclic rings of oligonucleotides enable their detection by UV light. The maximum absorption wavelength is about 260nm for nucleic acids, 1.8 times the absorption found at 280nm, hence its more wide spread use in the quantification of oligonucleotides by CGE (Leeds, et al., 1996, Crooke, et al., 1996, Geary, et al., 1997, Yu, et al., 2001, Yu, et al., 2004, Geary, et al., 1999, Shang, et al., 2004, Geary, et al., 2003, Soucy, et al., 2006, Noll, et al., 2005, Graham, et al., 2001, Agrawal, et al., 1991, Bellon, et al., 2000, Chen, et al., 1997). UV detection is simple and straightforward but the sensitivity is still limited due to a large variety of interferences at this wavelength, such as residual phenol used in LLE for tissue extractions. The limit of detection will also be affected by the length of the oligonucleotide when measured on a mass basis because the absorption of UV light is proportional to the number of bases in the oligonucleotide molecule. The longer the oligonucleotide, the fewer molecules there are per unit of mass and the lower the signal intensity. The detection limit in molar concentration is not affected though. The lower limit of quantitaion (LLOQ) for CGE using UV detection has been approximated to be 70 ng/ml from plasma and urine and between 0.35 and 1.2 μg/g from tissues,

depending on the specific structure of the oligonucleotides (Yu, et al., 2004). Due to the extremely narrow peak widths in CGE separations, the peak areas of early migrating analytes will be observed as smaller than later migrating analytes because they spend less time at the detection window. This difference should be accounted for by applying a correction factor related to the analyte migration time.

Laser-induced fluorescence (LIF) detection is known for its high sensitivity and selectivity. Unfortunately, oligonucleotides are not inherently fluorescent compounds and derivatization is required for their detection using LIF. Two types of derivatization have been reported for LIF detection: on-column derivatization using a fluorescent dye and pre-column derivatization by ligation of a fluorescent probe to the analyte. Reyderman et al.. (Reyderman, et al., 1997, Reyderman, et al., 1995) reported an instantaneous on-column derivatization using OliGreenTM. The Oligreen solution was placed at the anode because the dye carries an overall positive charge. Oligonucleotides were introduced in to the gel-filled column from the cathode and reacted with the OliGreen during their migration toward the anode, forming an oligo-dye complex. The drawback of this method is that the fluorescence would be significantly quenched at high concentrations of urea or high temperatures, both of which are important for achieving better resolution. A run buffer containing four molar urea and a run temperature of 30 ℃ were used to gain the optimal balance of both resolution and sensitivity. Typically 7M urea and 40-50 ℃ have been used in CGE separations of oligonucleotides when using UV detection. The linear dynamic range of this method was from 0.02-1.5 μg/ml and the sensitivity was improved 3-fold when compared to UV detection. Zhang et al. (Zhang, et al.. 2005) found that mixing the gel solution with OliGreen resulted in higher S/N ratio than introducing OliGreen into the column

after the gel was formed. By using Pluronic F127, a sieving matrix with temperature dependent viscosity, they were able to separate oligos sizing markers up to a 32mer within 1.3 minutes at a high separating voltage. Gilar et al. (Gilar, et al., 1997) used a fluorescently labeled 17mer primer to ligate with the 25mer target PS-DNA. The target oligonucleotides was phorphorylated at the 5'end by T4 DNA ligase and the primer and target DNA were connected using a complementary bridging DNA template. The CGE analysis was then carried out on the ligation product resulting in a LOQ of 1 ng/ml.

CE-MS has the ability to rapidly separate and detect biological samples present in extremely small quantities (Qiugley, et al., 2004). The hyphenation of CE and MS combines the advantage of the high resolution of CE with the structure elucidation capability of MS, making it extremely tempting for the bioanalysis of oligonucleotides and their metabolites. However, the high salt concentrations and the highly aqueous buffers typically used in CE analysis pose a formidable challenge to electrospray ionization, which is the most commonly used MS ionization mode with CE. Harsch et al. (Harsch, et al., 1998) used PVP as a replaceable sieving matrix with an electrospray compatible ammonium acetate buffer for the separation of ten 3-7mer unmodified DNAs and their fluorescently labeled analogues with verification of their structures. Four 6mer isomeric DNAs were also successfully separated using this assay.

Representative applications to pharmacokinetic and metabolism studies

During the past ten years, the types of oligonucleotides under pharmaceutical development have increased exponentially. More second generation oligonucleotide analogs entered clinical trials; oligonucleotides with more complicated conformations, such as siRNA, ribozymes and CpG type oligonucleotides began to attract enormous interest in their therapeutic uses. The new

classes of oligonucleotides have posed new challenges for the bioanalytical community to develop robust methods with better sensitivity and selectivity. The following examples represent the advantages and limitations of CGE technique in coping with these challenges.

2' MOE chimeric oligonucleotides significantly enhance resistance against exonucleases. Therefore, their metabolic profile would also be distinctive when compared to phosphorothioates. Yu et al. (Yu, et al., 2004) employed CGE to characterize the metabolites of two 2'MOE and one phosphorothioate oligonucleotides in the monkey and compared them with each other. This is the first full characterization of the pharmacokinetics of 2'MOE oligonucleotides in primates. ISIS 13650 is a fully modified phosphorothioate with 2'MOE modification at both the 3' and 5' ends. ISIS 15839 is also a fully modified phosphorothiaote with 2'MOE modification only at the 3'end. ISIS 16952 is a phosphodiester with 2'MOE modification throughout its sequence. All of these drugs were administered via i.v. infusion for 2 hours. Parent drugs and their metabolites were quantitated by CGE and characterized by LC-MS. The LC-MS method only detected N-1 and N-2 metabolites of all three compounds in plasma, urine and tissues while CGE detected N-6 to N-8 metabolites in addition to N-1 and N-2 metabolites of ISIS 13650 and ISIS 15839 in tissues and urine, respectively. They were not detected by LC-MS possibly because of coelution or other interferences from the HPLC. These metabolites were considered to be the product of endonuclease degradation. Metabolites of ISIS 13650 were not found in plasma or tissues. One possibility for this may be the fast clearance of the metabolites in these matrices. The quantitation limits of the CGE method for plasma and tissue analysis are much higher than in the urine, which could also be the reason why N-6 to N-8 metabolites of ISIS 13650 were not detected in these two matrices by CGE. Because the 2'MOE modification on both ends of ISIS

13650 makes it the most stable oligonucleotide of the three compounds, its endonuclease metabolites are more likely to present in plasma and tissues at lower concentrations. This application represents the advantages of CGE analysis in metabolite profiling and quantification afforded by extremely high resolution and mobility based separations which resulted in less interference from endogenous substances. However, the quantitation limit of the CGE-UV method was a major hurdle toward discovering new metabolites from plasma and tissues.

Geary et al. (Geary, et al., 2003) conducted the first complete preclinical pharmacokinetic study for the 2'MOE chimeric oligonucleotide ISIS 104838 using CGE-UV, LC-MS and ELISA methods. CGE-UV was used to measure drug concentrations in mouse, dog, and monkey plasma, as well as mouse and monkey urine and tissues. The LOQs of CGE-UV were 0.154 ug/ml in plasma, 0.039 ug/ml in urine and 0.39 ug/g in tissue, respectively. For mouse and rat PK studies at 5 mg/kg level, CGE-UV was able to capture both the distribution and elimination phase while only the distribution phase was above the LOQ for the monkey and dog PK studies at 0.5 and 2 mg/kg level. No measureable metabolites were observed in plasma using CGE-UV. But 8-12 bases long metabolites were detected in urine and identified by LC-MS. An ultrasensitive and selective hybridization ELISA with LOQ=0.78 ng/ml was used to analyze selected monkey and human plasma samples. The ELISA method provided characterization of a slow terminal elimination phase in both monkey and human. Plasma distribution of this drug was rapid hence unlikely to influence dosing frequency strategies. Instead, dosing frequency should be determined by the slow elimination from tissue in order to minimize accumulation and to ensure continued exposure for efficacy. Because tissue sampling in clinical studies would be prohibitive, it is therefore critical to characterize a terminal plasma elimination phase

Recently, more double stranded oligonucleotides such as siRNA are under pharmaceutical development. Szekely et al. (Szekely et al., 2009) explored different conditions to maintain the stability of a dsDNA therapeutic agent and established a quantitative method of analyzing it in rat serum. In addition to the impact of temperature and organic modifiers covered in the previous section, the author also discussed the effect of sieving matrix concentration and the presence of cations on the stability of dsDNA during the separation. Theoretically lower concentration of PEG (sieving matrix) would shorten the analysis time and enhance the on-column stability of dsDNA. However, decreasing PEG concentrations from 20% to 6% did not change the separation profile significantly. The addition of sodium or magnesium ions to DNA preparation is commonly considered to enhance dsDNA or dsRNA stability. In this study, it was found that adding MgCl2 to the separation matrix resulted in increased stability of the dsDNA than adding it only to the sample solution. The optimized buffer contained 6% PEG and 1 mM MgCl2 without any organic modifiers and the separation was carried out at 20 ℃. The ssDNA and dsDNA were successfully separated but single base resolution was not achieved in this system. The linear range of the calibration curves were 0.05-0.5 mg/mL and 0.005-0.15 mg/mL and the LOQ was 5 ug/ml. This study provided valuable information on stabilizing double stranded oligonucleotide therapeutics but also presented the challenge of achieving high sensitivity and resolution simutaneously.

Conclusion and Future Perspective

In the past two decades, oligonucleotides as research tools and therapeutic agents have brought us closer than ever to unraveling the many critical functions coded for by these four nucleobases. Two oligonucleotide drugs are already on the market and more continue to advance through both

preclinical and clinical studies. Critical to advancing the development of oligonucleotides as therapeutics are the continuing improvement of analytical technologies. These include pharmacokinetic and toxicokinetic studies, pharmcology and formulation studies addressing cellular uptake efficiency, the determination of off target effects and understanding the metabolism of oligonucleotides. In addition, the determination of drug purity and quality control processes are other critical functions that would be enhanced by improved analytical technologies to support their functions.

New polymers have given us more desirable sieving matrices for capillary gel electrophoresis that have higher resolving power and easier sample injection and gel replacing procedures. The invention of CE-MS interfaces has enabled the combination of a separation technique with unrivalled resolving power with excellent characterization abilities. It remains to be seen if improved chromatographic technologies including fast high throughput purification with monolithic column to ultra-sensitive and selective separation using capillary columns will be able to challenge capillary electrophoresis moving forward. In addition, new mass spectrometry sources and analyzers have reached sub ng/ml detection limits with improved characterization accuracy for oligonucleotides.

In this article, we have reviewed the progress made with the aid of these new technologies in quantification and characterization of oligonucleotides. However, there still remain significant challenges and issues that need to be addressed such as: 1) the development of more robust and reproducible CE injection and separation methods; 2) novel fluorescence derivatization agents that afford reproducible quantification results; and 3) less complex sample extraction procedures

with high recovery. Although immunoaffinity assay is still the most prevalent method used in pre-clinical and clinical studies of oligonucleotides, the separation and identification ability of electrophoretic methods provides obvious advantages in daily use to support pharmacokinetic and metabolism studies. With the continuing emergence of new technologies, these techniques are poised to be widely applied in the discovery and development of oligonucleotide therapeutics.

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Analyte Class	Modification	Sieving Matrix	Resolution	Sample preparation	Recovery	Detection Method	LOQ(plasma)	Ref
ssAntisense DNA	PS	polyacrylamide	Single base	Plasma: SAX+C18+Drop dialysis Tissue: ProteinaseK+Phenol/Chloroform LLE	40%	UV	10nM≈60ng/ml	Leeds, 1996; Crooke, 1996; Geary, 1997, 1999; Yu, 2001; Shang, 2004; Soucy, 2006; Noll, 2005; Graham 2001.
ssDNA	unmodified	Polyacrylamide	Single base	Phenol chloroform LLE	N/A	LIF(OliGreen)	20ng/ml	Reyderman, 1997
ssDNA	PS, unmodified	PEG35000, PEG35000 with ACN	Single base	N/A	N/A	UV	N/A	Wang, 2006
ssDNA	PO + 2'MOE PS + 2'MOE	polyacrylamide	Single base	plasma: SAX+C18+Drop dialysis tissue: ProteinaseK+Phenol/Chloroform LLE+phenyl SPE	N/A	UV	70ng/ml	Yu, 2004; Geary 2003
ssDNA	Unmodified, PS	Bio-Rad Dynamic Sieving Polymer	Single base	SAX+C18+Drop dialysis	N/A	UV	N/A	Gilar, 1998
ssDNA	Unmodified	Pluronic F127	Single base	N/A	N/A	LIF(OliGreen)	N/A	Reyderman, 1996
Ribozyme	2'MOE, PS	LPA	N/A	WAX+C2+dialysis	around 50%	UV	48ng/ml	Bellon 2000
Antisense DNA	PS	polyacrylamide	Single base	SAX	more than 60%	UV	N/A	Chen 1997
dsDNA, ssDNA	unmodified	PVP	Single base/base pair, isomeric	N/A	N/A	LIF(labeled primer)	N/A	Harsch 1998, Gao 1998
ssDNA	PS	Polyacrylamide	Single base	C18+Drop dialysis	80-120%	UV	2μg/ml	Wu 2009
dsDNA	Unmodified	Polyacrylamide+PVP	N/A	N/A	N/A	LIF(EB)	N/A	Song 2001
dsDNA, ssDNA	Unmodified	PVP+polyTrisA	Single base(pair)	N/A	N/A	UV	N/A	Wang 2006

Table 1. CE applications

Figure 1. Modification of Oligonucleotide. (Adapted from Crooke, S.T. 2004)

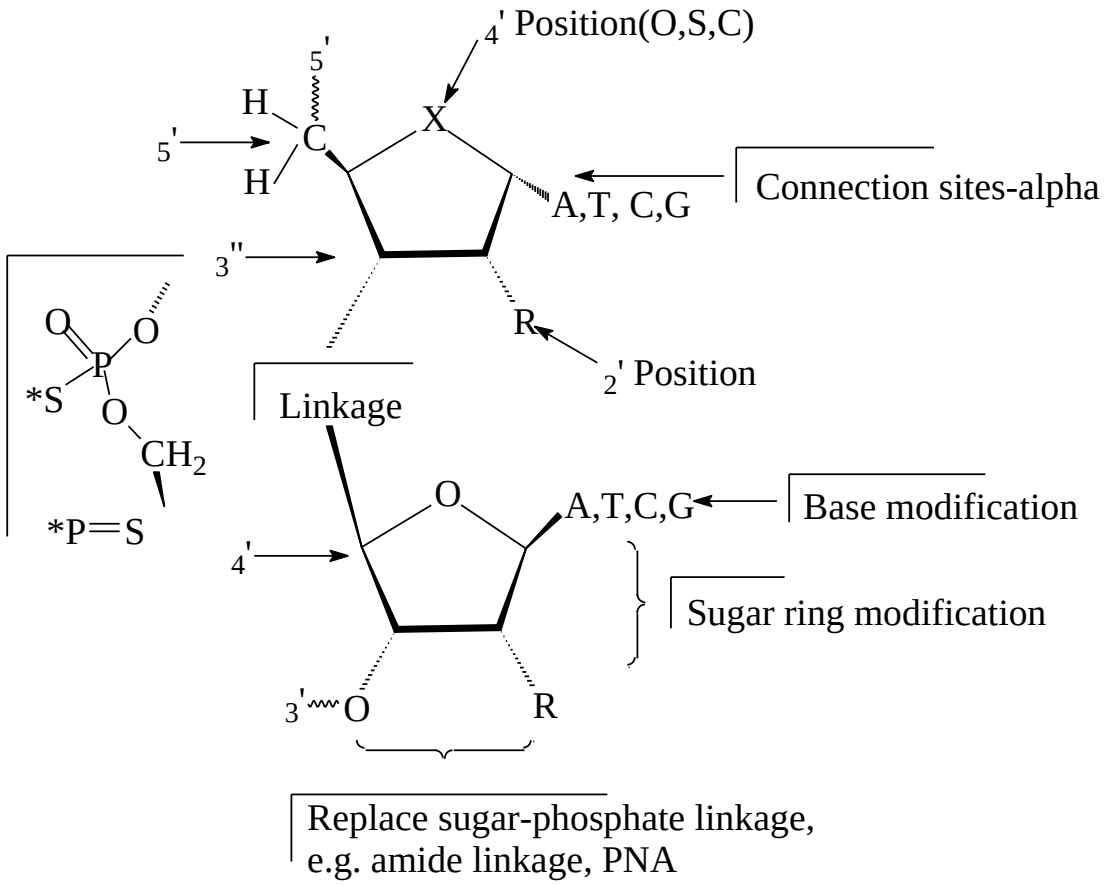

Figure 2. Peak height of the p(dA)50(absorbance units) versus numbers of consecutive injections. Inset:Capillary gel electrophoresis separation of the test mixture of p(dA)40-60; time frame, 20-27 min. Conditions:eCAP-ssDNA-100 gel filled column; E=300V/cm, t=20 ℃; sample, 7.4 μg/mL total DNA in water; (▪)injection,1.5s, 7.5kV electrokinetic injection from sample vial("regular injection"), and (•) 1.5s, 7.5kV electrokinetic injection from sample vial preceded by a 1.5s, 7.5kV electrokinetic injection from water("water preinjection")(reprinted from Guttman, A., et al.. 1995. Copyright 1995 with permission from ACS)

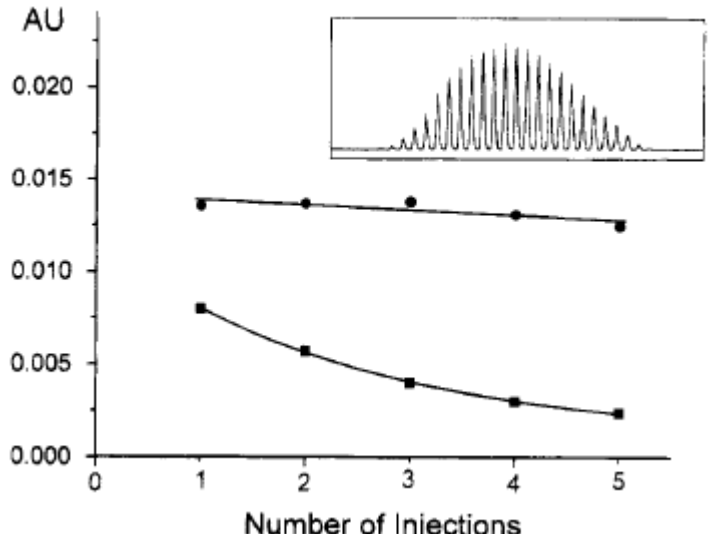

Figure 3. Typical Sample Preparation procedure of oligonucleotide: Solid-phase extraction scheme. Plasma containing phosphorothioate oligonucleotide is loaded onto the SAX-SPE cartridge after dilution into the SAX loading buffer. The phosphorothioate oligonucleotide eluted is diluted with buffer containing no acetonitrile before it is loaded on to the reverse-phase SPE cartridge. After elution and additional desalting the sample can be analyzed on a gel filled capillary. (reprinted from Leeds, J.M., et al.. 1996. Copyright 1999 with permission from ACS)