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Strategies for Bioanalysis of Oligonucleotides Using LC-MS/MS- Assay development for oligonucleotide compounds in urine and skin

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Abstract

Quantitation of in vivo oligonucleotide levels is necessary to evaluate pharmacokinetics /pharmacodynamics (PK/PD) and a drug's exposure-toxicity relationship. Therefore, a fast and reliable bioanalytical method is desired for both preclinical and clinical development. Intact oligonucleotide macromolecule analysis using LC-MS/MS presents challenges due to multiple charging, cation adduction, high protein binding, and nonspecific binding of the analyte during extraction. This analytical method development aims to provide a simpler, faster LC-MS/MS assay for an oligonucleotide in a clinical PK/PD study. Sample preparation consisted of a liquid-liquid extraction (LLE) using a mixed solvent of phenol/chloroform, followed by a wash with dichloromethane under basic conditions. The oligonucleotide analyte was separated on a Waters XBridge OST C18 column at 60°C, using a step-gradient elution. The mobile phases consisted of water and methanol containing 15 mM triethylamine (TEA) and 400 mM hexafluoroisopropanol (HFIP). Analyte-1 was detected using an AB Sciex 4000 mass spectrometer in negative electrospray ionization (ESI) mode, operating in multiple reaction monitoring (MRM) mode with a transition of m/z 778.1 → 94.9. A linear dynamic range of Analyte-1 from 10–10,000 ng/mL in both urine and skin homogenate was successfully qualified with good accuracy and precision. A two-step LLE LC-MS/MS bioanalytical method was thus successfully developed and qualified for the quantification of intact Analyte-1 in human urine and skin tissue to support a clinical PK study.

Keywords: Oligonucleotide bioanalysis, LC-MS/MS assay development, Liquid-liquid extraction, Urine and skin matrix, Negative electrospray ionization.

Introduction

With recent advances in molecular biology and genetics, oligonucleotides (ON) have been studied extensively as potential therapeutic agents for viral infection, inflammation, cancers, and genetic disorders. Oligonucleotide drugs are usually sequences of 16-30 bases of single-stranded DNA that hybridize to specific mRNAs by Watson-Crick base pairing. Phosphorothioate backbone oligonucleotides, with one oxygen element replaced by sulfur at a non-bridging position of the phosphodiester linkage, represent one of the most commonly used oligodeoxynucleotides. These macro-compounds can efficiently inhibit expression of a gene by reducing the translation of the target mRNA to its corresponding protein.

Figure 1 (A) illustrates the partial structures of oligodeoxynucleotide molecules; and Figure 1 (B) shows a schematic diagram of the mechanism of oligonucleotide therapeutics. Many oligonucleotide compounds possess favorable pharmacological characteristics, such as increased nuclease resistance and the ability to recruit and activate RNase H (enzyme-dependent antisense drugs), which make them suitable for in vivo administration.

An oligonucleotide drug, Fomivirsen (Vitravene), was first approved by the US FDA

in 1998 for the treatment of cytomegalovirus retinitis-AIDS. At present, there are approximately 80 ON drug candidates registered at the stage of clinical trials in the biopharmaceutical industry in the US. Quantitation of in vivo oligonucleotide levels is necessary to evaluate their PK/PD and a drug's exposure-toxicity relationship. Therefore, a fast and reliable bioanalytical method is desired for preclinical and clinical development in the biopharmaceutical research industry.

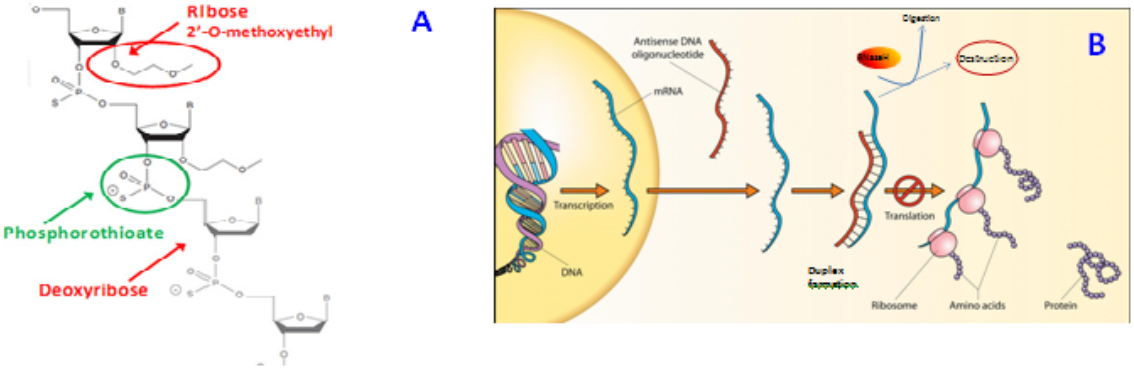

Figure 1: A) Schematic structure of an oligonucleotide molecule, B) Schematic mechanism of oligonucleotide therapeutics

Early traditional methods for quantification of oligonucleotides in biological matrices relied on ultraviolet (UV) detection methods such as HPLC-UV and CGE-UV methods [93–95]. However, UV detection usually does not have sufficient sensitivity to measure oligonucleotides at low ng/mL concentrations required to characterize the terminal elimination phase of analytes in plasma during a pharmacokinetic (PK) study.

Hybridization enzyme-linked immunosorbent assays (HELISA) have demonstrated very high sensitivity with minimal sample cleanup. However, these assays may quantify drug-related material, including 3′ and/or 5′ chain-shortened metabolites, potentially leading to overestimation or false positives of the parent drug in vivo, thereby reducing the accuracy of PK/PD relationship development. Additionally, hybridization ligand binding assay (LBA) methods often have narrow linear dynamic ranges and reduced precision, particularly at lower ng/mL analyte concentrations in biological matrices [1-2].

Similar to peptide and protein applications, electrospray ionization mass spectrometry (ESI-MS) has been a useful method for sequencing, identifying, and characterizing oligonucleotides over the past decade. However, intact oligonucleotide analysis using LC-MS/MS presents challenges due to limited ionization efficiency, multiple charging of analytes, extensive cation adduction, high protein binding, and nonspecific binding during extraction. These issues result in reduced assay sensitivity and low analyte recovery from biological matrices

Several preliminary methods for oligonucleotide quantitation in biological matrices using LC-MS/MS have been reported, but the results indicate that low recovery and high matrix interference remain major challenges in this field.

This paper describes several unique bioanalytical strategies that were used to address these analytical challenges. These approaches have proven feasible for the development of oligonucleotide LC-MS/MS bioanalytical methods with good selectivity, sensitivity, accuracy, and precision.[3]

Materials and Methods

The test oligonucleotide compounds and the analog internal standards were synthesized from internal company sources. Organic solvents including hexafluoroisopropanol (HFIP), phenol, chloroform, and dichloroethane were obtained from Sigma-Aldrich Inc. (St. Louis, MO). HPLC-grade water, acetonitrile, methanol, triethylamine (TEA), Triton-X100,

and ammonium hydroxide were purchased from J.T. Baker Inc. (Philipsburg, NJ).

Biological control matrices including human plasma, urine, and skin used for the preparation of standards and control samples were obtained either from Bioreclamation Inc. (Hicksville, NY) or Biochemed (Winchester, VA). Polypropylene sample processing vials (0.5, 1.5, or 2.0 mL with caps), LoBind™ vials, and LoBind™ 96-deep-well (DW) plates were purchased from Eppendorf NA (Hauppauge, NY).

A Tomtec Quadra 3 automatic liquid-handling station was obtained from Tomtec, Inc. (Hamden, CT, USA). The Quadra 3 was efficiently used for transferring the organic layer to a clean plate during the LLE process. The TurboVap 96 evaporator was obtained from Zymark Corp. (Hopkinton, MA, USA).

Centrifuge equipment, models 5415C (with centrifuge tube rotor) and 5810R (with racks holding 96-well plates), were obtained from Eppendorf (Brinkmann Instruments Inc., Westbury, NY). A Sorvall Legend XTR centrifuge from Thermo Fisher Scientific (Osterode, Germany) was also used for plate centrifuging. A Hamilton liquid handler, model Microlab Star, was from Hamilton Robotics Inc. (Reno, NV). This was used for standard and QC sample preparation in different matrices.

Liquid Chromatographic Conditions [4]

The HPLC system used to perform reversed-phase liquid chromatography (RP-LC) for analyte separation consisted of two Shimadzu LC-20AD HPLC pumps (Figure 5.2-C), a CTO-20AC column oven, and a SIL-20AC HT autosampler (Shimadzu Inc., Columbia, MD). A Waters X-Bridge OST C18 column, 2.1 x 50 mm, 2.5 µm (Waters Corp., Milford, MA), was used. The column temperature was maintained at 60ºC during the chromatographic separation. The autosampler tray was maintained at 4ºC.

Initial Mobile Phases:

Mobile Phase A (MP-A): 100 mM hexafluoroisopropanol, 8 mM triethylamine, with 2% methanol in water

Mobile Phase B (MP-B): 100 mM hexafluoroisopropanol, 8 mM triethylamine, with 2% water in methanol

Concentrations of the ion-pair reagents were further optimized and varied to improve separation efficiency during LC-MS/MS method development. The HPLC gradient with time profile is shown in Table 1.

Table 1 HPLC mobile phase gradient-time program in LC-MS/MS assay

Time (min)	FlowRate (µL/min)	MP-A (%)	MP-B (%)
0	200	100	10
1	200	100	10
1.1	200	75	25
6	200	69	31
6.1	200	5	95
8	200	5	95
8.1	200	5	10
10	200	100	10

Mass Spectrometric Conditions [5]

The API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada), equipped with a TurboIonspray™ source, was employed for the detection of oligonucleotide analytes.

The MS system was operated in multiple-reaction monitoring (MRM) mode under negative ionization, with dwell times of 100 ms for each analyte channel and 50 ms for the internal standard (IS).

All possible high-response MRMs of the analytes were used individually and summed when necessary to improve signal intensity.

Optimized Instrument Parameters:

- Turbo probe source temperature (TEM): 500°C
- Nebulizer gas: 50

- Desolvation gas: 55
- Curtain gas (CUR): 20
- TurboIonspray voltage: –5500 V
- Entrance potential (EP): –10 V

Mass spectrometric parameters for Analyte-1 and the Internal Standard (IS) were optimized for:

- Q1 selection
- Q2 fragmentation
- Q3 selection

using the Declustering Potential (DP) and Collision Energy (CE).

Table 2 – Optimized MRM Transitions and MS Parameters for Oligonucleotides

AnalyteID	MS/MS (m/z)	DwellTime (ms)	DP(V)	CE(V)	CXP(V)
Analyte-1	778>95	100	-80	-110	-8
Analyte-2	658>95	100	-60	-94	-8
IS	772>95	50	-85	-115	-9

Sample Preparation and Extraction

The stock solutions of oligonucleotide analytes and the internal standard (IS) were prepared in HPLC-grade water at concentrations of 1.0 mg/mL and 2.0 mg/mL, respectively. These stock solutions were stored at 4°C in a refrigerator prior to use.

Calibration Standards and Quality Control (QC) Samples [6]

- Calibration Standards (STDs) at concentrations of 10, 20, 50, 200, 1000, 5000, 8000, and 10,000 ng/mL were prepared by serial dilution in control urine or water. Automation was performed using a Hamilton liquid handler (Hamilton Company, Reno, NV, USA).
- Quality Control (QC) samples were prepared at three concentration levels: 30, 500, and 7500 ng/mL, using a similar procedure.

Internal Standard (IS) Working Solution

A 100 µg/mL IS working solution was prepared in water from the 2.0 mg/mL IS stock solution (SS).

Automated Workflow

A schematic flowchart of the oligonucleotide bioanalysis process with automated sample preparation is provided (not shown here). This workflow helps streamline and ensure consistency in:

- Standard and QC preparation
- Sample extraction and cleanup
- Transfer and injection for LC-MS/MS analysis

Sample Preparation for Urine Matrix [7]

Sample extraction of urine was carried out using the following procedure:

1. Add sample and buffer:
   Combine 200 µL of urine with 100 µL of Triton-X solution (0.05% Triton-X100 in 2% Ammonium Hydroxide in water) in a 1.5 mL LoBind® 96-deep-well plate.
   This yields a final sample pH of approximately 9.
2. Add internal standard (IS):
   Add 10 µL of internal standard to each well.
   Lightly vortex the plate to mix.
3. Add extraction solvent:
   Add 40 µL of extraction solvent (Phenol:Chloroform, 2:1, v/v) to each well.
4. Vortex and centrifuge:
   - Vortex the plate for 10 minutes at 2000 rpm.
   - Centrifuge for 10 minutes at 4000 rpm to allow phase separation.
5. Transfer aqueous layer:
   Using a Tomtec liquid handler, transfer approximately 250 µL of the aqueous layer into a new 96-deep-well plate for further analysis.

6. 20 µL of dichloroethane was added to each well containing the transferred aqueous layer.
7. The plate was vortexed for 5 min at 2000 rpm and then centrifuged for 10min at 4000 rpm to allow separation of the layers.
8. Approximately 200 µL of the aqueous layer was transferred again to a new 96-deep well plate using a Tomtec liquid handler.
9. The plate was centrifuged for 5 min at 4000 rpm to allow any remain in ghomogenate particles or organic to settle to the bottom of the well.
10. 20µL of the supernatant sample was injected into an LC-MS/MS system.

Sample Preparation for Skin Tissue Matrix

1. Homogenization of Skin Tissue:

- Based on the recorded tissue weight, a 10-fold dilution (w/v) with water was performed using MPbio Lysing Matrix D tubes (containing ~20 zirconia-silica beads).
- The sample mixture was homogenized using the FastPrep® instrument (MPbio, Inc.) for 3 × 45 seconds at a speed setting of 6.
- Tubes were placed on ice for 5 minutes between runs to cool the samples.
- After homogenization, samples were centrifuged at 14,000 × g for 5 minutes to pellet debris.
- The supernatant was collected and used as the skin homogenate sample.

2. Sample Extraction:

- Combine the following in a 1.5 mL LoBind® 96-deep-well plate:
  - 100 µL of skin tissue homogenate
  - 100 µL of standard spiking solution
  - 100 µL of Triton-X solution (0.05% Triton-X100 in 2% Ammonium Hydroxide in water)

3. LLE Extraction:

- The liquid-liquid extraction (LLE) procedure then follows Steps 2 to 10 of the urine LLE extraction protocol outlined

Method Qualification

For exploratory PK studies, **method qualifications** are typically performed prior to the analysis of unknown samples.

- Calibration curves for urine and skin tissue homogenate matrices were constructed using the peak area ratios of the analyte to those of the internal standard (IS), and applying a weighted ($1/x^2$) least-squares linear regression analysis.
- Assay precision, defined as percent relative standard deviation (%RSD), and assay accuracy, defined as percent relative error (%RE), were calculated for the three QC sample levels.
- Duplicates of each QC level were analyzed to determine intraday accuracy and precision.
- The acceptance criteria for both accuracy and precision were within ±20%, in accordance with laboratory regulations.

Results and Discussion

Method Development of Oligonucleotide Using Mass Spectrometry

For LC-MS/MS analysis of oligonucleotides, the first step always involves the acquisition of accurate full-scan ESI-MS spectra of the analytes' charge-state distribution.

During the infusion experiment, cation adduction of the analyte due to the high-affinity binding of Na^+ and K^+ to the polyanionic backbone of the oligonucleotide was observed. Therefore, in order to reduce the formation of adducts, the selection of an appropriate infusion solution that contains ion-pairing reagents is important for obtaining correct precursor and product ion spectra.

The solution of 10 µg/mL analyte, in a buffer composed of 100 mM HFIP and 80 mM TEA in water/methanol (50:50, v/v), was delivered by an infusion pump (Harvard Apparatus, Holliston, MA, USA) through a "T" connector into a conventional HPLC flow at a rate of 0.2 mL/min using:

- Mobile phase A: 60 mM HFIP and 10 mM TEA in water

- Mobile phase B: 60 mM HFIP and 10 mM TEA in methanol

With a gradient of 15–50% MP-B over a 10-minute run, a multiple charge-state MS spectrum was generated.

The second approach involved a direct infusion of a 5 µg/mL analyte neat solution using the same infusion pump.

The overlay scan function (MAC) on the API-4000 MS instrument was used to enhance acquisition of high-quality MS spectra.

The API 4000 MS full scan spectra of Analyte-2 under negative ESI-MS conditions. This oligonucleotide analyte contained 19 nucleotide subunits, with a molecular weight (MW) of 4608 Da.

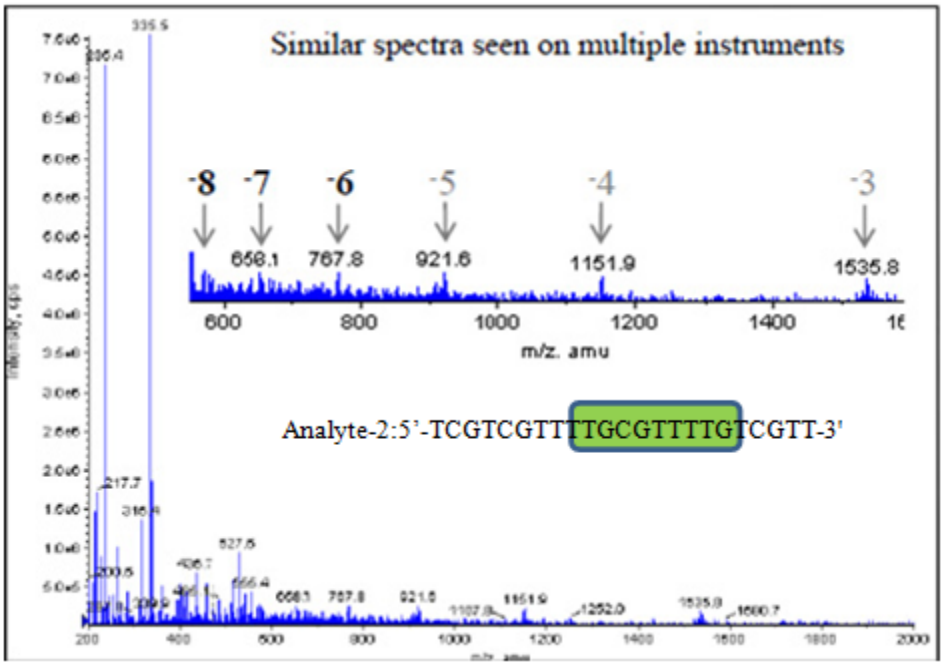

Figure 2: AB Sciex API 4000 MS full scan spectrum of Analyte-2 (MW = 4608 Da) at negative ESI conditions

Relatively low ion intensities for most of multiply charged states were observed, suggesting that a low ionization efficiency of Analyte-2withcurrent solvent. This was mainly due to the multiple charge states of the parent ions and the high surface tension and conductivity of droplets in the gas phase when high aqueous and low organic mobile phase had to be used for chromatography. To this end, the ratio of solvents HFIP: TEA in the sample and mobile phase is likely to have critical impact on the signal responses of oligonucleotide analyte.

HPLC method development

In order to achieve the highest sensitivity and optimal selectivity for the oligonucleotides LC-MS/MS assay, the concentration and chemical properties of the ion-pairing agents used in the mobile phase were found to be important.

It is suggested that triethylamine (TEA) forms ion pairs with the negatively charged phosphate groups of the oligonucleotides, which promotes retention and ensures a good peak shape of ON analytes.

The volatility of the organic solvent hexafluoroisopropanol (HFIP) likely aids in the desolvation of the solvent droplet during the MS electrospray ionization (ESI) process.

Additionally, during the MS desolvation process, the pH of the solvent droplet is reported to increase from 7.5 to 10 due to the evaporation of the more volatile HFIP, which leaves the basic TEA–oligonucleotide complex in the droplet.

This process reduces the presence of other cation adducts during ionization, thereby increasing the intensity of the unadducted

oligonucleotide analyte peaks monitored in the LC-MS/MS assay.

After the experiments, the Waters X-Bridge C18 column was demonstrated among the column options to provide the best sensitivity, capacity, and reproducibility for the oligo analytes. Using a low flow rate of 0.2 mL/min and preconditioning of any new columns were found to be necessary. This approach significantly enhanced analyte sensitivity and improved the ruggedness performance of the assay.

Preconditioning was carried out by 15-20 repeated injections of an oligo analyte (at a concentration of ~1 µg/mL in matrix). This process could be beneficial in improving chromatographic separation and assay reproducibility.

Oligonucleotide chromatography was found to be very sensitive to minor adjustments/changes in mobile phase composition and column equilibration. Therefore, to ensure good quality chromatography, sample equilibration times should be given before the start of the run and built into the gradient to allow reproducible peak shape and retention time.

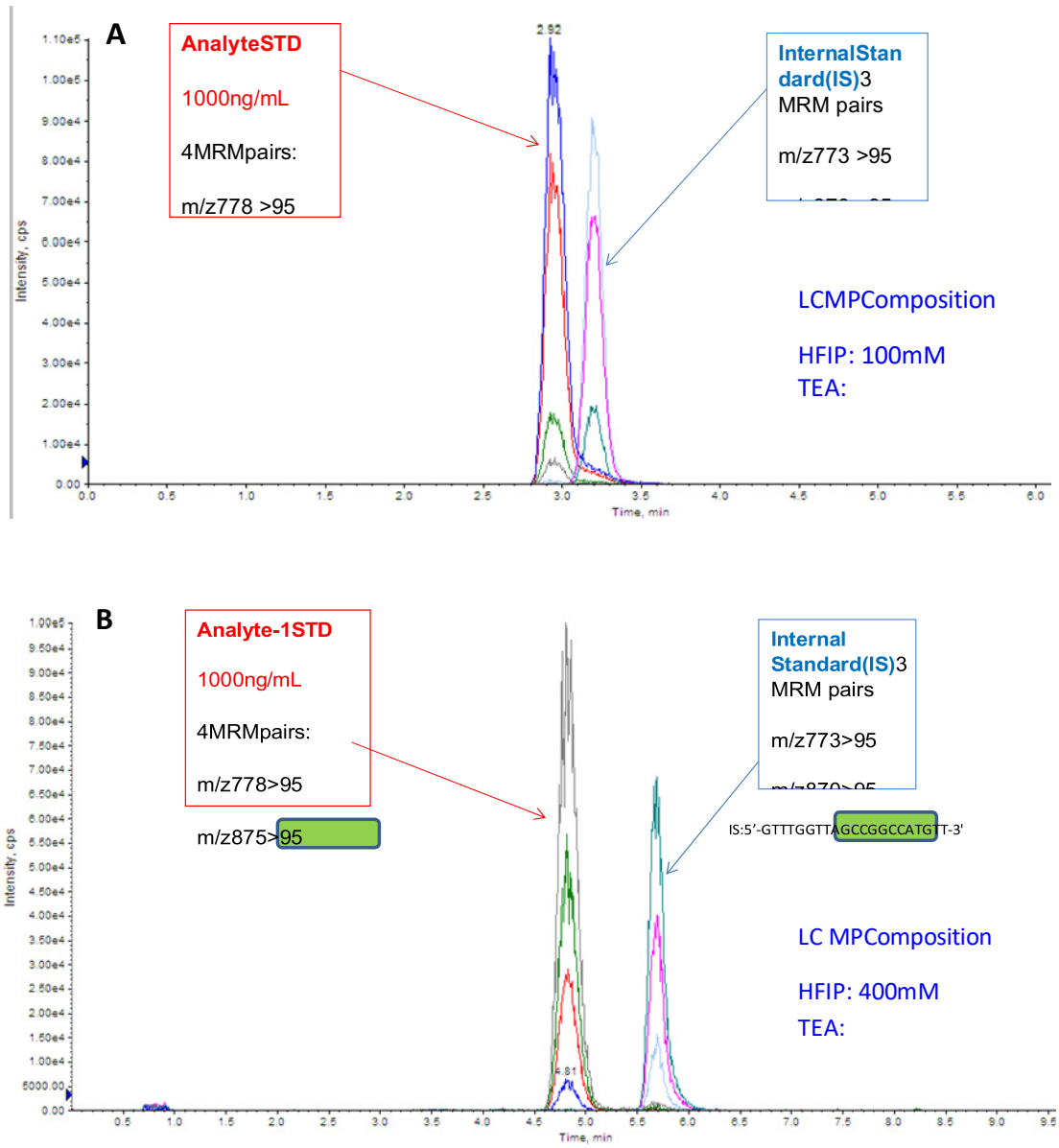

Figure 3: Different mobile phase compositions affected on LC separation efficiency between the analyte and the IS.

A) Low concentration of HFIP and TEA with a lower HFIP/TEA ratio resulted in a poor LC separation efficiency;

B Higher concentration of HFIP and TEA with a higher HFIP/TEA ratio increased the separation resolution of the analyte and the IS on a reverse phase C18 column

Conclusion:

There are many analytical challenges encountered during the investigation of oligonucleotide bioanalysis with LC-MS/MS. The key issues that have been discussed include:

- Multiply charged ions
- Extensive cation adduction of the analyte in the MS source
- Mobile phase ion-pair strength
- Compounds co-eluting in LC separation
- Nonspecific binding in sample preparation

These factors are unique to this class of macromolecules, and failure to adequately control and address them can have adverse consequences on analytical methods.

For example, it is important to carefully evaluate both the type of ion-pair agent and its concentration early in method development, since ion-pair chromatography provides excellent chromatographic separation and mass spectrometric signal intensity. In addition, it is crucial to be aware of the limited solubility of common mobile phase additives in acetonitrile, which is often used in LC analysis. For this reason, most LC-MS methods for oligonucleotides use methanol as the organic solvent.

Bioanalytical strategies have been demonstrated and discussed in this chapter, which can overcome these analytical problems in oligonucleotide assay development. These approaches have been proven to be feasible for quantification of oligonucleotides with LC-MS/MS assays.

A rapid and sensitive bioanalytical method using LLE with LC-MS/MS has been developed and qualified for the analysis of oligonucleotides in urine and skin tissue matrices. Both assays have demonstrated good precision, accuracy, and sensitivity, with linear dynamic ranges of 10-10,000 ng/mL in the corresponding matrices. The matrix control samples showed no significant interference response in either the analyte or the internal standard MRM channels, indicating that the assay is highly selective.

This bioanalysis should be useful in the quantitative determination of potential oligonucleotide drug candidates in preclinical and clinical PK/PD studies, which will aid lead optimization and late-phase drug development.

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