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LCMS BIOANALYSIS OF OLIGONUCLEOTIDES: OPTIMIZATION OF ALKYLAMINES AND FLUORINATED ALCOHOLS AS SOLVENT MODIFIERS

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INTRODUCTION

Conventional reversed-phase LC separation of oligonucleotides typically uses triethylammonium acetate (TEAA), where alkylamine enhances retention and carboxylic acid lowers solvent pH. However, TEAA is incompatible with electrospray mass spectrometry due to ion suppression. Hexafluoroisopropanol (HFIP), introduced by Apffel et al. in 1997 as an acidic modifier, has become popular for LC-MS analysis of oligonucleotides. The triethylamine (TEA)-HFIP method significantly improves LC-MS analysis by maintaining a low pH for better peak resolution. HFIP's higher volatility allows it to deplete from electrospray droplets, raising pH and facilitating the release of negatively charged oligonucleotides into the gas phase. Various strategies have emerged in oligonucleotide bioanalysis, focusing on alkylamine and fluorinated alcohol choice, their concentrations, optimal solvent pH, and elution strategies.Here we summarize the key features of oligonucleotide bioanalysis, through optimization of alkylamines and fluorinated alcohols as solvent modifiers, based on publications and our own experiences in WuXi AppTec.

METHODOLOGY

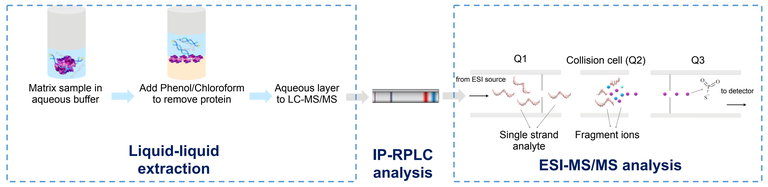

Figure 1: General workflow of plasma sample liquid-liquid extraction and ion pairing reversed-phase LC-ESI-MS/MS analysis of siRNA analytes.

Sample Preparation

Plasma, urine, and liver homogenate samples (30 µL) from rats and monkeys were mixed with 30 µL of an internal standard (an analogue siRNA) and 300 µL of ammonium hydroxide solution. Following this, 50 µL of phenol/chloroform solution was added for liquid-liquid extraction. The aqueous layer was collected after centrifugation, evaporated under nitrogen, and reconstituted with an aqueous solvent.

LC-MS/MS Analysis

Chromatographic separation utilized a binary solvent management system. N,N-diisopropylethylamine (DIEA), N,N-diisopropylamine (DIPA), and Di-N-butylamine (DBA) were dissolved in water with 5% methanol and 0.5% Hexafluoroisopropanol (HFIP) as aqueous solvents, while 95% methanol with the same alkylamines and HFIP served as organic solvents for ion pairing separation. A triple-quadrupole mass spectrometer with an electrospray ionization (ESI) source was used for quantitative analysis of oligonucleotide analytes in biological matrices, analyzing multiple charged molecular ions and their collision-induced dissociation (CID) ions in selected reaction monitoring (SRM) mode.

RESULTS AND DISCUSSIONS

Choice of alkylamine and fluorinated alcohols

When selecting alkylamine reagents, key properties include boiling point, LogP, and gas-phase basicity. Higher volatility alkylamines enhance ion emission during electrospray desorption, reducing charge state distribution in oligonucleotide ionization, while low volatility can cause signal suppression. Longer alkyl chains increase hydrophobicity, making these reagents preferable for separating longer oligonucleotides (e.g., 40 to 100 mer), though they require higher organic solvent percentages, which may broaden peaks. Using a fluorinated alcohol like 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP), can improve hydrophobic alkylamine solubility in aqueous solutions, while gas-phase basicity aids in oligonucleotide dissociation from ion pairs.

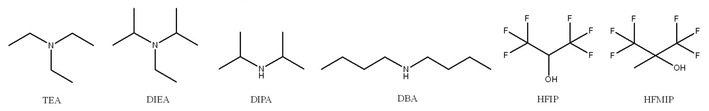

Figure 2: Chemical structures of alkylamines and fluorinated alcohols: Triethylamine (TEA), N,N-diisopropylethylamine (DIEA), N,N-diisopropylamine (DIPA), Di-N-butylamine (DBA), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP).

RESULTS AND DISCUSSIONS

Concentrations of alkylamine and fluorinated alcohol and the influence of solvent pH

To study the effect of alkylamine concentration on oligonucleotide retention in reversed-phase chromatography, researchers from Bartlett's group employed two approaches using 50 mM HFIP and varying concentrations of N,N-diisopropylethylamine (DIEA). In the first approach, without pH adjustment (7.8 to 10.7), maximum retention (> 25 min) occurred at lower DIEA concentrations (2-20 mM), followed by a sharp decrease at 50-100 mM. In the second approach, with a constant pH of 9.1, retention increased from 2 min to 15 min as DIEA concentrations rose from 2 mM to 20 mM, maintaining retention at higher concentrations. It was suggested that at elevated concentrations and pH, excess alkylamines remain in the mobile phase, forming positively charged micelles that reduce oligonucleotide retention. Thus, optimizing alkylamine concentration and solvent pH is crucial for enhancing LC retention and MS response in oligonucleotide analysis.

LC elution and separation considerations

At WuXi AppTec, LC-quadrupole MS/MS methods were developed for the bioanalysis of a triantennary N-acetyl galactosamine (GalNAc) conjugated siRNA therapeutic in plasma, urine, and liver tissue from rats and monkeys. The method aimed to chromatographically separate the antisense strand (AS) and its truncated metabolites (AS 3'n-1 and AS 3'n-2) to minimize crosstalk interferences. Reversed-phase ion pairing separation utilized 0.2% DIEA, 0.2% N,N-diisopropylamine (DIPA), and 0.2% Di-N-butylamine (DBA) with 0.5% HFIP to evaluate peak shape and retention in various solvent modifiers.

Column selection

Three columns were tested: Phenomenex bioZen Oligo, Shimadzu Shim-pack Scepter Claris C4-300, and Waters Xbridge Premier Oligo BEH C18. Results indicated that the Shim-pack column produced the most symmetric and narrow peaks for the analytes.

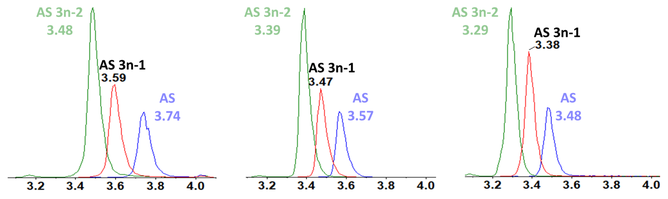

Figure 3: Chromatographic separation of the antisense strand (AS) and its truncated metabolites (AS 3'n-1 and AS 3'n-2) by different analytical columns: (A) Phenomenex bioZen Oligo, 2.6 µm, 2.1x50 mm, (B) Shimadzu Shim-pack scepter claris C4-300, 1.9 µm, 2.1x100 mm, (C) Waters Xbridge premier Oligo BEH C18, 2.5 µm, 2.1x100 mm.

Alkylamine reagent selection

The Shim-pack column exhibited varying retention and separation of analytes across solvent systems. While DIPA and DBA provided excellent peaks, DIPA lacked retention, affecting resolution. DIEA and DBA showed good retention, but DIEA produced broader peaks. Ultimately, 0.2% DBA and 0.5% HFIP were chosen for optimization.

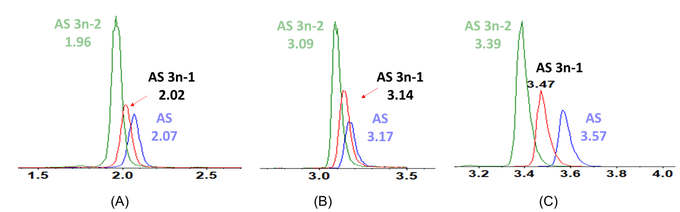

Figure 4: Chromatographic separation of the antisense strand (AS) and its truncated metabolites (AS 3'n-1 and AS 3'n-2) by Shim-pack column with different selections of modifying reagents: (A) 0.2% DIPA and 0.5% HFIP, (B) 0.2% DIEA and 0.5% HFIP, (C) 0.2% DBA and 0.5% HFIP.

RESULTS AND DISCUSSIONS

Flow rate tests

Then the flow rate and column temperature were optimized for peak resolution and mass spectrometric response. The 0.30, 0.35, 0.40 and 0.45 mL/min flow rates were studied in parallel and the 0.30 mL/min flow rate showed the best peak shape, response and separation.

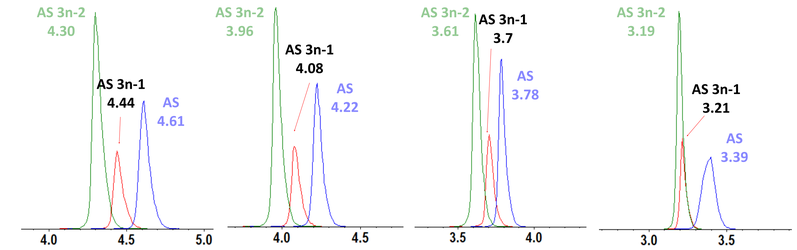

Figure 5: Chromatographic separation of the antisense strand (AS) and its truncated metabolites (AS 3'n-1 and AS 3'n-2) by Shim-pack column (with 0.2% DBA and 0.5% HFIP as modifying reagents) at different flow rates: (A) 0.30 mL/min, (B) 0.35 mL/min, (C) 0.40 mL/min, (D) 0.45 mL/min.

The column temperature was compared at 55, 60 and 65°C and results showed that the peak resolution and mass spectrometric response were not influenced by the variations. The methods finally established can achieve a 10 ng/mL lower limit of quantitation (LLOQ) for each analyte in plasma, urine and liver homogenate samples from rats and monkeys. The retention times were 4.31, 4.43, 4.62 and 4.80 min for AS 3'n-2, AS 3'n-1, AS and the internal standard respectively, with a minimum peak overlap of the two truncated metabolites and except for that, all peaks could be base-line separated.

CONCLUSION

This poster reviewed the arts of selection and concentration optimization of ion paring alkylamines and fluorinated alcohols in the bioanalysis of oligonucleotide therapeutics. The experience obtained from WuXi AppTec's projects was also examined regarding reagent selection and optimization, column selection, optimization of flow rate and column temperature, etc. We believe that future studies are needed for more thoroughly understanding of ion-pair chromatographic and mass spectrometric behaviors of oligonucleotides, which may then greatly facilitate the analysis in different biological matrices.

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