Liquid Chromatography-tandem Mass Spectrometry Based Bioanalytical Strategies for Lipid-oligonucleotide Conjugates

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Introduction

On a global scale, the market has seen the commercialization of 20 distinct oligonucleotide drugs, including 12 ASOs, 7 siRNAs, and one aptamer. Of these, 15 employ specialized delivery systems; N-acetylgalactosamine (GalNAc) has emerged as the predominant approach and is an effective strategy for liver-targeted delivery [1]. Delivering oligonucleotides to non-hepatic organs remain a greater challenge. Conjugation with small-molecule ligands has been used to enhance cellular uptake, improve pharmacokinetics, and prolong therapeutic duration. Compared with hydrophilic delivery modalities such as GalNAc-siRNA, lipid–oligonucleotide conjugates—characterized by a lipophilic tail attached to a hydrophilic phosphodiester backbone — are intrinsically amphiphilic and strongly associate with plasma proteins, complicating dissociation and quantitative recovery in toxicokinetic (TK) bioanalysis.

Herein, we report the development and optimization of LC-MS/MS assays for lipid–siRNA conjugates, with a particular focus on sample-preparation methods. In addition to evaluating conventional liquid–liquid extraction (LLE) and solid-phase extraction (SPE), we implemented a novel hybridization-based workflow that incorporates a post-hybridization magnetic-bead affinity capture step. This approach preserves biotin–streptavidin interactions that are susceptible to disruption at elevated hybridization temperatures and more effectively liberates lipid–siRNA from protein-bound complexes, resulting in substantially improved recovery and reduced matrix effects. Finally, we present a head-to-head comparison of LLE, SPE and hybridization capture, and provide practical recommendations for selecting sample-preparation strategies for lipid–siRNA conjugates.

Methodology

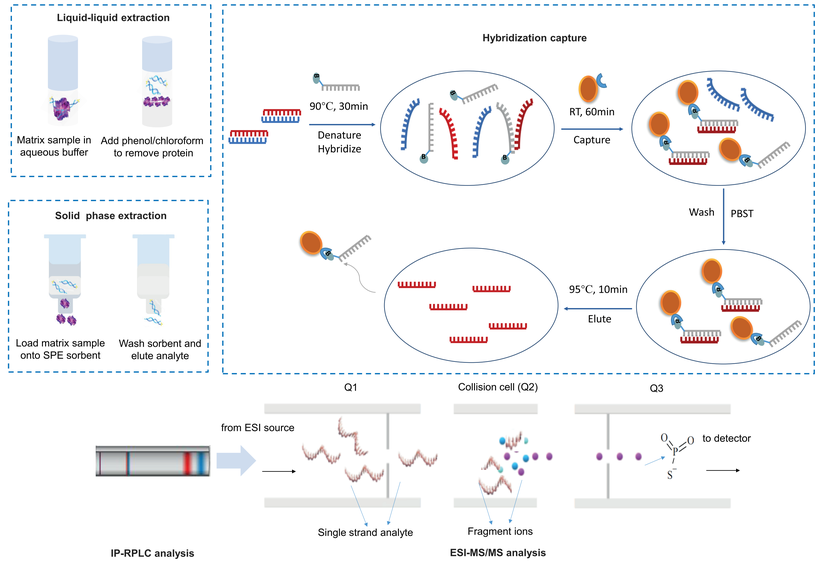

Figure 1. *Sample treatment workflow of LLE, SPE, and hybridization-capture based LC–MS/MS methods for lipid-siRNA conjugates*

Methodology

Sample Preparation

Liquid-liquid extraction

Plasma samples were extracted with an ammonia solution and an RNA extraction reagent. After centrifugation, the supernatant was evaporated to dryness under a stream of nitrogen and reconstituted prior to injection.

Solid phase extraction

Plasma samples were treated with a lysis/loading buffer and then loaded onto a Clarity OTX SPE plate. Following repeated washing procedure, the lipid–siRNA conjugates were eluted and evaporated to dryness under a stream of nitrogen, and finally reconstituted for subsequent injection analysis.

Hybridization capture

Plasma samples were hybridized with biotin-labeled PNA probe at 90°C for 30 min to unwind double strands, allowing AS-probe hybridization during annealing. After adding streptavidin-coated magnetic beads, the mixture was shaken at room temperature for 1 hr. Following washing procedure, reconstitution solution was added. The complexes were heated at 95°C for 10 min to release captured AS. The supernatant was collected and injected.

LC-MS/MS Analysis

Chromatographic separation was performed at 0.5 mL/min on a Shimadzu LC-30AD system equipped with a Thermo DNAPac RP column (4 μm, 2.1 × 100 mm) using a mobile phase of hexylamine/hexafluoroisopropanol (HA/HFIP). Chromatograms were acquired in multiple reaction monitoring (MRM) mode on an AB Sciex TQ6500+ system.

Results and Discussions

Magnetic Bead Capture Optimization: Pre-hybridization vs. Post-hybridization Workflow

Post-hybridization capture using streptavidin-coated beads markedly enhanced lipid–siRNA recovery. Double-stranded lipid–siRNA requires thermal denaturation to release the antisense (AS) strand for probe hybridization; elevated temperatures can induce thermally driven dissociation of the biotin–streptavidin interaction, thereby reducing capture efficiency. By introducing the streptavidin beads after the high-temperature denaturation/hybridization step, the biotin–streptavidin complex is spared thermal exposure, preventing binding disruption and improving overall recovery.

Parametric Optimization: Magnetic Bead and Probe Amounts

Probe and magnetic-bead inputs were optimized in ULOQ-level plasma sample (5,000 ng/mL). Recovery exceeded 90% when a 10-fold molar excess of probe relative to the ULOQ-level sample and 30 μL of 10% (v/v) bead slurry were employed. Further increases in probe excess or bead volume yielded no additional recovery improvement, indicating saturation of the capture reaction under these conditions.

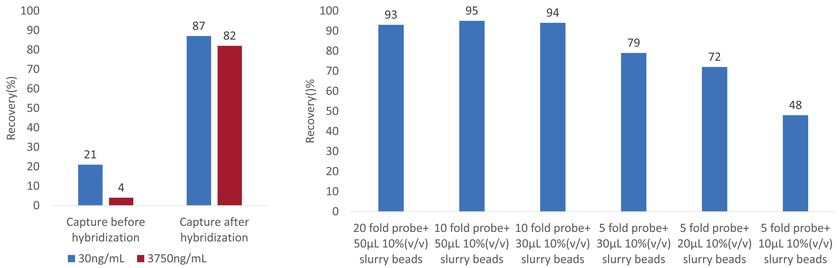

Figure 2. *Recovery optimization via adjust capture procedure and amount of PNA probe and magnetic beads*

Results and Discussions

Methodology Comparison: LLE vs. SPE vs. Hybridization Capture based LC-MS/MS

Three sample preparation methods (LLE, SPE, and hybridization-capture) have been successfully developed for the quantitative analysis of lipid-siRNA conjugates by LC-MS/MS, with achieved lower limits of quantification of 20.0 ng/mL, 10.0 ng/mL, and 5.00 ng/mL, respectively. The hybridization method demonstrated superior recovery (>90%) and complete matrix effect elimination, outperforming SPE (50% recovery, full elimination) and LLE (60% recovery, partial elimination).

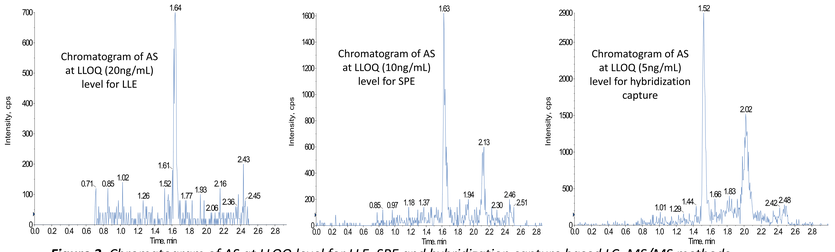

Figure 3. *Chromatogram of AS at LLOQ level for LLE, SPE and hybridization capture based LC–MS/MS methods*

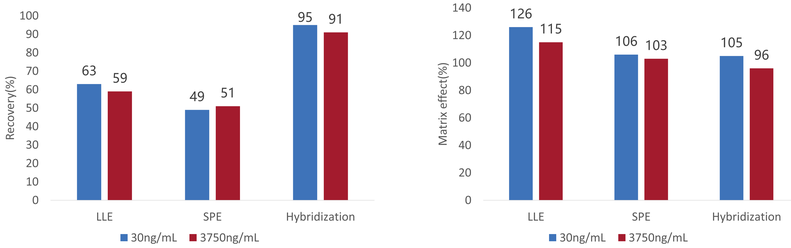

Figure 4. *Recovery and matrix effect data for LLE, SPE, and hybridization-capture sample treatment procedure*

Conclusion

Robust and GLP-compliant bioanalytical LC-MS/MS methods have been established towards the toxicokinetic profiling of lipid-oligonucleotide conjugates. A hybridization-capture workflow with post-hybridization magnetic-bead affinity preserves biotin–streptavidin interactions, more effectively liberates lipid-siRNA from protein-bound complexes, and markedly improves recovery while reducing matrix effects. Furthermore, the analytical performances of LLE, SPE, and hybridization-capture were systematically compared, aiming to provide a practical guidance for selecting the optimal sample preparation approach for method development.

Reference

[1] Debacker A. J., Voutila J., Catley M., Blakey D. and Habib N. Delivery of oligonucleotides to the liver with GalNAc: from research to registered therapeutic drug. Mol. Ther., 2020, 28, 1759–1771.