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Quantitative Analysis of GalNAc-siRNA Conjugates in Tissue Homogenate Using Two-Dimensional Liquid Chromatography Coupled with Tandem Mass Spectrometry

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PURPOSE

Metabolism of GalNAc-siRNA conjugates is mainly catalyzed by exonucleases and endonucleases, resulting in various sizes of shortened oligonucleotides, which could interfere the quantitation of GalNAc-siRNA conjugates. Therefore, bioanalytical scientists have to use a long injection time to separate analyte from metabolites when using LC-MS/MS for quantitation. Ion-pair chromatography is the recommended method for determining oligonucleotides at reverse chromatography, but its disadvantage is the difficulty to rebalance (return column to initial condition) the column system within short time before next injection. Meanwhile, as the GalNAc-siRNA conjugates contain large numbers of phosphate groups which make the molecules negatively charged. This characterization lets the analyte easily adsorb into material surfaces such as polypropylene vials, pipette tips and the column solid phase. Therefore, the significant challenges of oligonucleotide bioanalysis are a bad peak shape, low recovery at lower concentration, bad linearity relationship, and enormous carryover when using LC-MS/MS for determination in complex bio samples. How to determine oligonucleotides, or separate them within a short time is another problem to solve.

To overcome those challenges, we found an additive and applied a Two-Dimensional (2D) LC system coupled with tandem mass spectrometry to quantitatively analyze GalNAc-siRNA conjugates in tissue homogenate. The adsorption effect can be minimized, injection time can be shortened from 5.5 to 3.5 mins, and the chromatographic separation, accuracy and precision would not be affected.

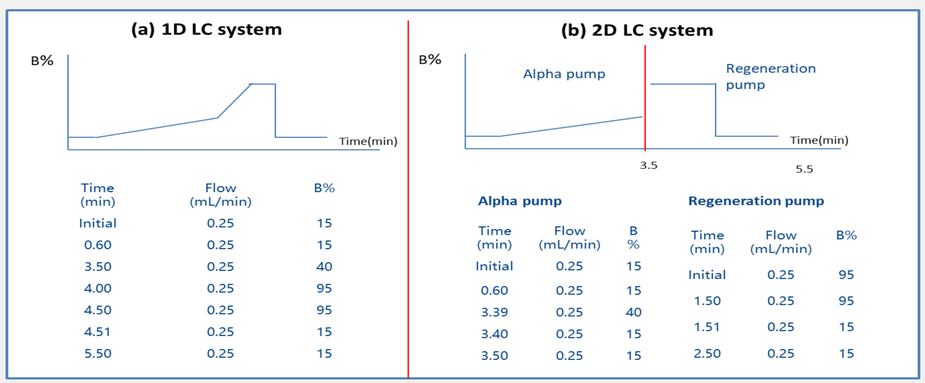
METHOD(S)

Ion-pair reagents (dibutylamine (DBA) and hexafluoroisopropanol (HFIP)) coupled with Waters ACQUITY™ Premier Peptide BEH C18 column were used as chromatography conditions. Tissue homogenate samples were added with an additive to improve peak tailing and increase recovery before purified and concentrated by SPE processing. The extracts were analyzed by liquid chromatograph (Waters ACQUITY UPLC system and Waters ACQUITY UPLC Premier 2D system) coupled with triple quadrupole mass spectrometry (SCIEX TripleQuad™6500+ and Waters Xevo TQ Absolute).

RESULT(S)

The 2D LC system has two binary solvent managers (BSM) and a column manager with two six-way 2-position valves, so sample analysis alternates between two identical columns with the same flow path. When samples are injected, separated, and regenerated on one column, the other column is re-balanced to allow more samples to be analyzed in a given time. By using the 2D LC system instead of the traditional UHPLC, the injection time of a GalNAc-siRNA conjugate could be shortened from 5.5 to 3.5 mins without affecting the separation efficiency, which can highly increase the yield of the instrument. The within-run accuracy was 0.8% to 4.9% and precision was 3.5% to 8.9%.

The commonly used method such as increasing the column temperature (this will shorten column life) or adding more EDTA in mobile phase (this can inhibit analyte ionization) cannot improve peak shape and minimize carryover for quantitation of GalNAc-siRNA conjugates using LC-MS/MS. We found an additive, which is extracted from animals' excrement, could effectively improve peak shape and minimize carryover. By adding the additive into tissue homogenate before SPE, the peak shape was significantly improved (as shown in Fig.2). In addition, the linearity relationship got well (the linearity correlation factor "r" is improved from 0.9851 to 0.9968, as shown in Fig.3.) and carryover was reduced from 3.1% to 0.5% of ULOQ peak area. This additive had been used to 20 tissues homogenate for several GalNAc-siRNA conjugates and they had similar efficiency. Maybe there are some small peptides and lipids which can be adsorbed into the solid phase of the column and protect the GalNAc-siRNA conjugates from being adsorbed severely.



Time (min)	Flow (mL/min)	B%
Initial	0.25	15
0.60	0.25	15
3.50	0.25	40
4.00	0.25	95
4.50	0.25	95
4.51	0.25	15
5.50	0.25	15

Alpha pump

Time (min)	Flow (mL/min)	B %
Initial	0.25	15
0.60	0.25	15
3.39	0.25	40
3.40	0.25	15
3.50	0.25	15

Regeneration pump

Time (min)	Flow (mL/min)	B%
Initial	0.25	95
1.50	0.25	95
1.51	0.25	15
2.50	0.25	15

Figure 1. *The gradient method of the two UPLC systems.*
The retention time of Analyte-01 is about 2.8 mins. The two UPLC systems had similar gradient before 3.5 mins. But the difference was that the 2D LC system has two pumps and it needs two columns to work together. These two columns were used alternatively by a switching valve. One was used to separate analytes by the Alpha pump while the other was rebalanced by the Regeneration pump at the same time, allowing the run time to be cut to 3.5 mins.

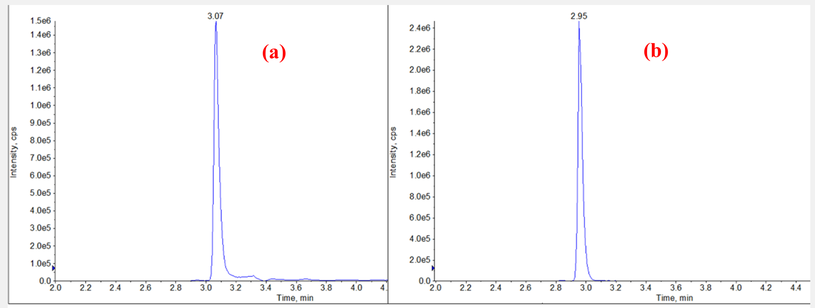


Figure 2. *The chromatogram of Analyte-01 for liver homogenate after SPE*
(a) Without additive. (b) With additive.

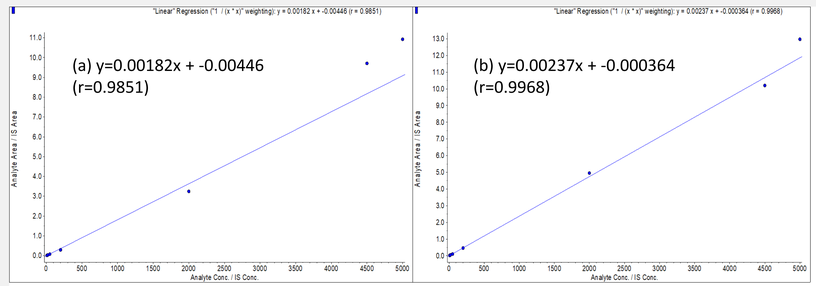


Figure 3. *The linearity of Analyte-01 for liver homogenate after SPE*
(a) Without additive. (b) With additive.

CONCLUSION(S)

The injection time was shortened from 5.5 to 3.5 mins by using the 2D LC system. The non-specific binding can be eliminated by adding the additive into tissue homogenate. A high-throughput and robust method was developed and used to accurately quantify multiple GalNAc-siRNA conjugates in bio matrices.

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