T1130-01-03

Sensitive LC-MS/MS Method for Cationic Ligand-Conjugated siRNA Therapeutics

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PURPOSE

Compared to traditional drugs, siRNA demonstrates revolutionary potential for genetic diseases and rare diseases. The conjugation of siRNA with ligands can significantly enhance the therapeutic potential of siRNA by improving its stability, specificity, and efficiency in targeting and entering desired cells. These ligands include small molecules, lipids, peptides, aptamers, antibodies, proteins, carbohydrates, and non-coding RNA [1], etc. The emergence of these conjugated siRNAs has also brought challenges to bioanalysis, and effective extraction methods are particularly crucial for detection. The extraction methods of siRNA typically include solid-phase extraction (SPE) and liquid-liquid extraction (LLE). Compared to other siRNA, ligand-conjugated siRNA often presents challenges such as low extraction recovery and low mass spectrometry response. Taking cationic ligand-conjugated siRNA as an example, low extraction recovery is encountered when using liquid-liquid extraction for sample preparation in plasma. In this study, we optimized the pre-extraction processing prior to LLE to improve extraction recovery, and developed a highly sensitive LC-MS/MS method for the quantification of cationic ligand-conjugated siRNA in rat plasma.

METHOD(S)

An aliquot 100 µL of plasma was mixed with 100 µL of 50% $NH_3 \cdot H_2O$ solution. Subsequently, 200 µL of PCI extraction reagent and 300 µL of dichloromethane were added, followed by vigorous mixing. The mixture was then centrifuged at 12,000 x g for 30 minutes. Following centrifugation, 100 µL of the upper aqueous supernatant was carefully transferred, mixed with 100 µL of water with 2% formic acid, and the resulting solution was injected to LC-MS/MS analysis. Detailed testing information is shown in Table 1.

Table 1. LC-MS/MS Method Information

LC Instrument	Waters ACQUITY UPLC I- Class PLUS system
Mass Spectrometer	Sciex Triple Quad ™ 6500+
Column	ACQUITY UPLC Protein BEH C4 Column 300 Å, 1.7 µm, 2.1×50 mm
Column Temperture	60℃
Mobile Phase A	0.3% dibutylamine and 0.2% hexafluoropropanol in methanol/ water (v:v=1:9)
Mobile Phase B	0.3% dibutylamine and 0.2% hexafluoropropanol in methanol/acetonitrile (v:v=2:8)

RESULT(S)

In the early stage of method development, we optimized the mass spectrometric parameters, MRM ion pairs, and mobile phase composition, which achieved optimal analyte MS response in neat solution. However, these measures could not resolve the issue of low response in rat plasma. The analyte peak was nearly undetectable when the LLE method was performed using the commonly employed extraction solvent mixture of phenol: chloroform: isoamyl alcohol (25:24:1) and ammonium acetate buffer (pH = 10). Although the SPE method can solve this problem the material cost is expensive and the process is complex. After optimizing the LLE pretreatment method, it was found that when $NH_3 \cdot H_2O$ of different concentrations was used as buffer, the different response of ligand-conjugated siRNA was found (Figure 1). When the concentration of $NH_3 \cdot H_2O$ is 50%, the sensitivity increased by 200-fold compared to the pre-optimized method, and LLOQ decreased from 2000 ng/mL to 10.0 ng/mL (Figure 2 & Figure 3). The introduction of $NH_3 \cdot H_2O$ leads to the deprotonation of amine groups on the ligand ($NH^{2+} \rightarrow$ -NH), reducing their positive charge and weakening their interaction with the matrix, thus preventing co-precipitation with the proteins in matrix. At the same time, the increase of OH^- results in the deprotonation of the siRNA phosphate backbone ($-PO_4H^- \rightarrow -PO_4^{2-}$), increasing its negative charge and enhancing hydrophilicity, which favors retention in the aqueous phase and improves extraction efficiency.

In the final method, the sample was prepared using LLE extraction, and 50% $NH_3 \cdot H_2O$ was added before sample extraction to get better recovery. The linearity range was 10.0 to 1000 ng/mL in rat plasma, with good linearity (r = 0.9952, Figure 4) and excellent precision and accuracy (Table 2).

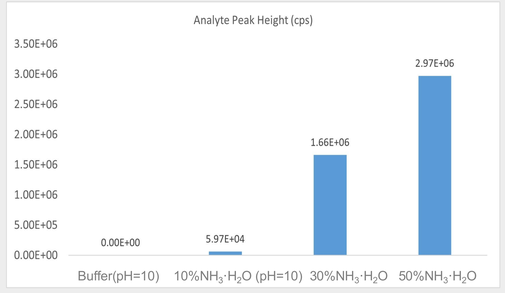

Figure 1. Responses of the analyte with different concentrations of $NH_3 \cdot H_2O$

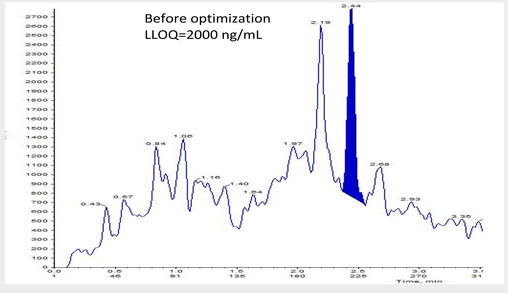

Figure 2. LLOQ in rat plasma (extracted by 10% $NH_3 \cdot H_2O$)

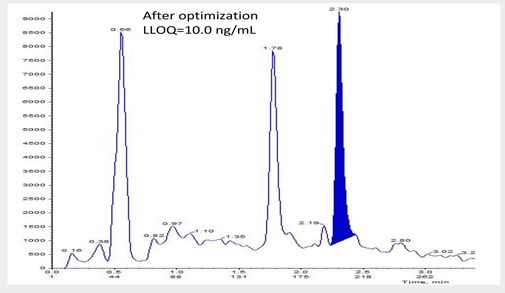

Figure 3. LLOQ in rat plasma (extracted by 50% $NH_3 \cdot H_2O$)

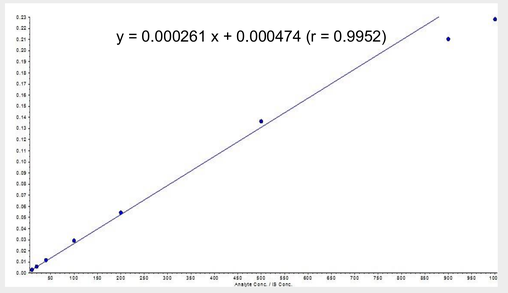

Figure 4. Chromatograms of calibration curves

Table 2. Accuracy and Precision Samples Spiked in Rat Plasma (n = 6)

Matrix	LLOQ 10.0(ng/mL)		LQC 30.0(ng/mL)		MQC 300(ng/mL)		HQC 800(ng/mL)	
	Calculated Conc.	Bias%	Calculated Conc.	Bias%	Calculated Conc.	Bias%	Calculated Conc.	Bias%
Rat plasma	10.3	3.0	33.1	10.3	322	19.1	831	3.9
	8.03	-19.7	35.2	17.3	325	1.6	833	4.1
	9.14	-8.6	34.6	15.3	341	6.6	824	3.0
	7.75	21.4	33.9	13.0	335	4.7	855	6.9
	10.6	6.0	33.5	11.7	332	3.8	776	-3.0
	8.79	-12.1	35.8	19.3	318	-0.6	759	-5.1
Within-run Mean Conc.	9.10		34.4		329		813	
Within-run SD	1.16		1.04		8.66		37.1	
Within-run %CV	12.7		3.0		2.6		4.6	
Within-run %Bias	-9.0		14.7		2.8		1.6	

CONCLUSION(S)

- A simple and sensitive LC-MS/MS method was developed for quantifying cationic ligand-conjugated siRNA in rat plasma.
- Using 50% $NH_3 \cdot H_2O$ as the extraction buffer, the method attained an LLOQ of 10.0 ng/mL in rat plasma samples. Validation results confirmed:
  - Excellent linearity (r = 0.9952) across the calibration range
  - Full compliance with accuracy and precision requirements
  - Cost-effective sample preparation versus SPE methods
- This method can serve as a reference for the analysis of other modified siRNA in plasma samples.

REFERENCE

[1] Bo Hu, Liping Zhong. *et al.* Therapeutic siRNA: state of the art. Signal Transduction and Targeted Therapy (2020) 5:101.

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