Optimization of Solid-Phase Extraction Conditions for LC-MS/MS Quantification of Oligonucleotide Therapeutics in Serum

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Introduction

Oligonucleotide therapeutics are attracting increasing attention as next-generation pharmaceuticals due to their high target specificity and the rapid development enabled by chemical synthesis. Therapeutic oligonucleotides, typically around 20 nucleotides (20mer) in length with molecular weights of approximately 5,000 - 7,000 Da, require highly sensitive bioanalytical methods based on HPLC mass spectrometry (LC-MS/MS) for pharmacokinetic studies. However, bioanalysis of serum and other biological matrices remains challenging because of the low recovery and poor reproducibility of oligonucleotides during sample preparation, as well as significant matrix effects caused by endogenous components. In this study, we focused on solid-phase extraction (SPE) and targeted to establish an optimized extraction procedure and analytical workflow to overcome these challenges.

Optimization of the SPE Method to Reduce Matrix Effects

To improve the analytical performance of LC-MS/MS, the following two approaches were evaluated. The LC-MS/MS conditions are shown in Fig. 1, and the sample preparation workflow is illustrated in Fig. 2.

1. Wash Buffer
2. Reconstitution Buffer

【Standard】
18mer All-PO, 18mer All-PS, used in LC-MS/MS analysis
5'-catgacgttcctgatgct-3'
5'-c^a^t^g^a^c^g^t^t^c^c^t^g^a^t^g^c^t-3'

20mer All-PO, 20mer All-PS, used in LC-MS/MS analysis
5'-tccatgacgttcctgatgct-3'
5'-t^c^c^a^t^g^a^c^g^t^t^c^c^t^g^a^t^g^c^t-3'

^ = phosphorothioate

Analysis conditions:

System:	QTRAP 6500+ LC-MS/MS System (Sciex)
Column:	InertSustain AQ-C18 (1.9 µm, 2.1 mm I.D. x 50 mm)
Mobile phase:	A) 100 mM HFIP + 5 mM TEA + 0.05% Acetylacetone in 10% CH_3OH aq. B) 0.05% Acetylacetone in 90% CH_3OH aq. A/B = 98/2 (0.5 min) - 3.5min - 60/40 - 0.01min – 2/98 (2 min) - 0.01 min - 98/2 (4 min)
Flow Rate:	0.3 mL/min
Col. Temp.:	50 °C
Detection:	MS/MS (Low Mass, ESI, Negative, MRM)

Fig. 1. LC-MS/MS Method

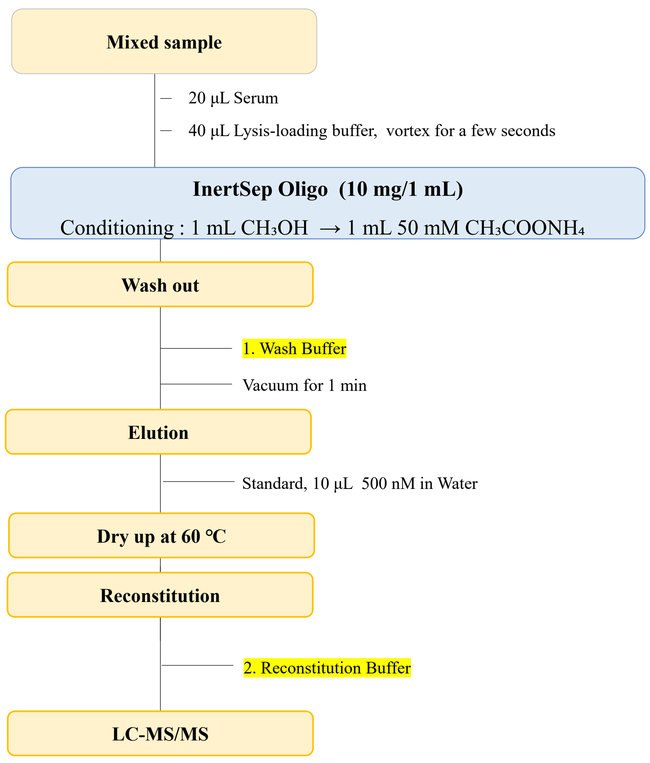

Fig. 2. SPE Method

1. Wash Buffer

The trueness obtained using three different wash buffers is summarized in Table 1, and representative chromatograms of the standard and the post-spike sample (Wash Buffer : 500 mM CH_3COONH_4 / IPA = 1 / 1) are shown in Fig. 3. Among the wash buffers evaluated, 500 mM CH_3COONH_4 / IPA (1:1) provided the best trueness. These results suggest that the use of isopropanol (IPA) as the organic solvent can reduce ion suppression caused by hydrophobic matrix components, thereby improving the sensitivity.

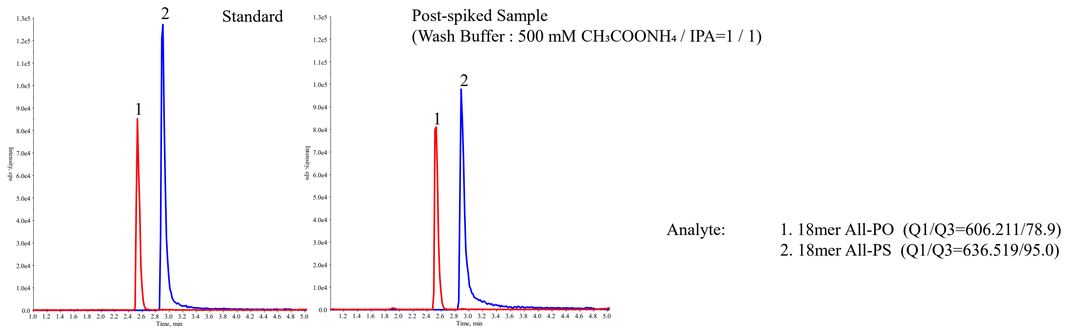

Fig. 3. Standard and Post-spiked Sample (Wash Buffer : 500 mM CH_3COONH_4 / IPA = 1 / 1)

Table 1. Comparison of Trueness for Different Wash Buffers (Standard Peak Area = 100%)

Compounds	Wash Buffer		
	500 mM CH_3COONH_4 / CH_3CN = 1 / 1	500 mM CH_3COONH_4 / CH_3OH = 1 / 1	500 mM CH_3COONH_4 / IPA = 1 / 1
	Trueness (%), n=3	Trueness (%), n=3	Trueness (%), n=3
18mer All-PO	72.2	30.3	**96.3**
18mer All-PS	68.5	73.8	**80.3**

2. Reconstitution Buffer

The trueness obtained with different reconstitution buffers is summarized in Fig. 5, and representative chromatograms of the standard and the post-spike sample (Reconstitution Buffer : 5 mM TEA in 10% MeOH aqueous solution) are shown in Fig. 4. The organic solvent composition significantly affected the trueness. Among the reconstitution buffers evaluated, 5 mM TEA in 10% MeOH aqueous solution resulted in the highest trueness.

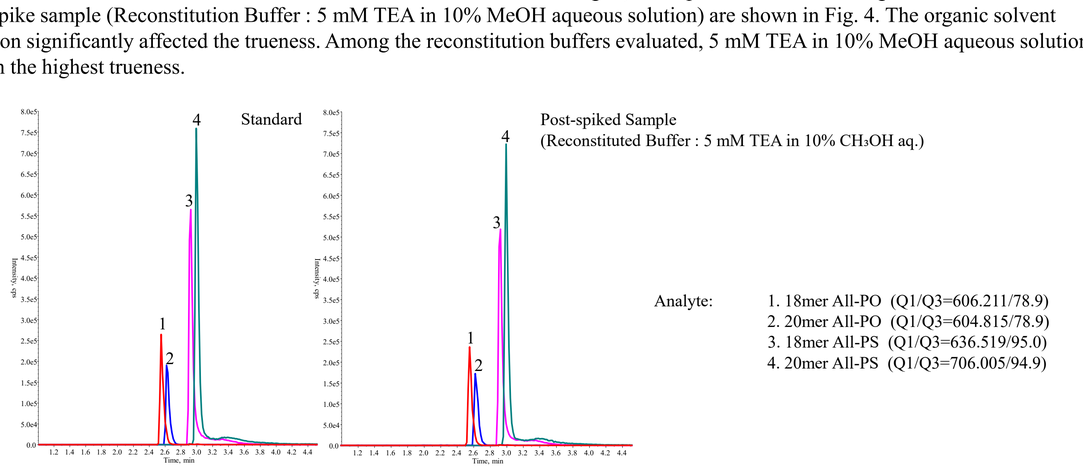

Fig. 4. Standard and Post-spiked Sample (Reconstituted Buffer : 5 mM TEA in 10% CH_3OH aq.)

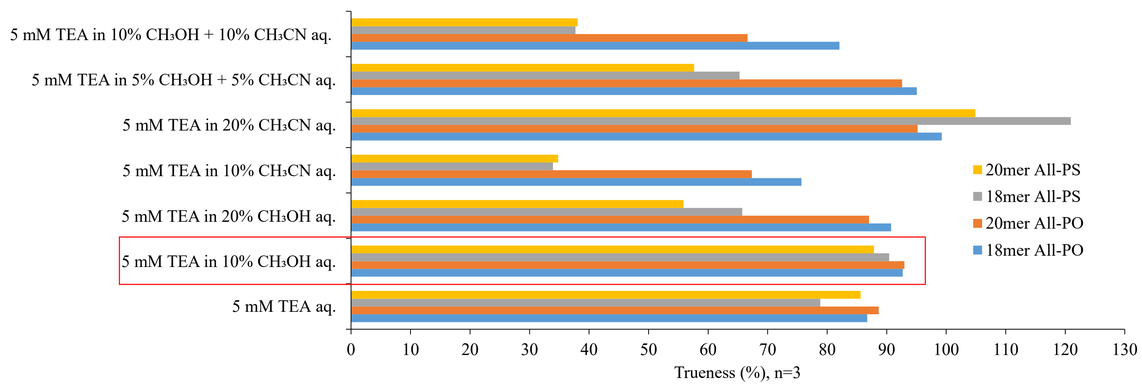

Fig. 5. Comparison of Trueness Obtained with Different Reconstitution Buffers (Standard Peak Area = 100%)

Optimization of the SPE Method to Improve Recovery and Reproducibility

To improve the recovery and reproducibility, the following investigations were conducted using LC-UV. The LC-UV conditions are shown in Fig. 6, and the sample preparation workflow is shown in Fig. 7.

3. Water Dilution Factor during Sample Preparation
4. Elution Buffer and Method

【Standard】
19mer All-PO, 19mer All-PS, used in LC-UV analysis
5'-ccatgacgttcctgatgct-3'
5'-c^c^a^t^g^a^c^g^t^t^c^c^t^g^a^t^g^c^t-3'

^ = phosphorothioate

Analysis conditions:

System:	Nexera i HPLC System (Shimadzu)
Column:	InertSustain AQ-C18 (1.9 µm, 2.1 mm I.D. x 100 mm)
Mobile phase:	A) 100 mM HFIP + 5 mM TEA + 0.05% Acetylacetone in H_2O B) 0.05% Acetylacetone in (CH_3OH/CH_3CN=1/1) A/B = 98/2 (0.5 min) - 6.5 min - 60/40 - 0.01min – 2/98 (2 min) - 0.01 min - 98/2 (6 min)
Flow Rate:	0.2 mL/min
Col. Temp.:	50 °C
Detection:	UV 260 nm

Fig. 6. LC-UV Method

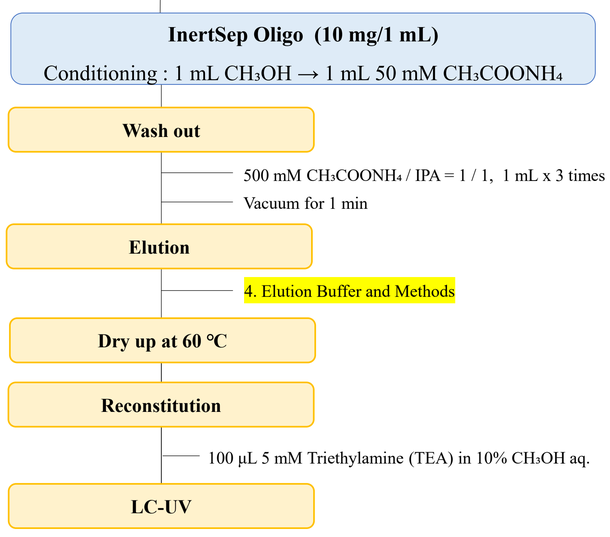

Fig. 7. SPE Method

3. Water Dilution Factor during Sample Preparation

The recovery and reproducibility obtained with serum diluted 2- to 8-fold during sample preparation are summarized in Fig. 9, and chromatograms of the standard and serum-spiked samples (4- and 8-fold dilution) are shown in Fig. 8. The highest recovery was observed with an 8-fold dilution for the PO oligonucleotide and a 4-fold dilution for the PS oligonucleotide. These results shows that dilution of the serum with water improved the retention of oligonucleotides on the SPE sorbent, thereby enhancing both recovery and reproducibility.

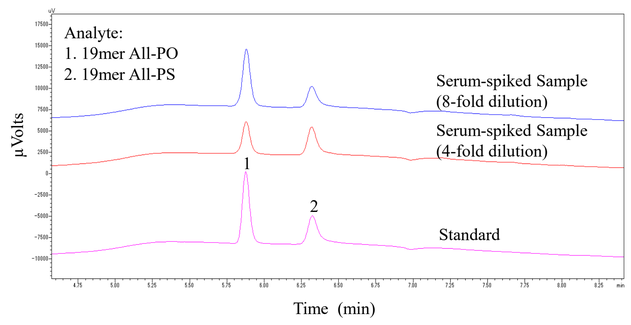

Fig. 8. Standard and Serum-spiked Sample (4- and 8-fold dilution)

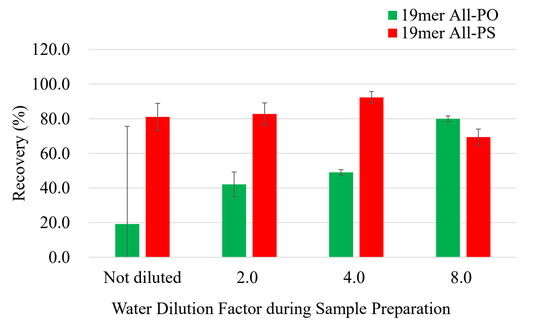

Fig. 9. Recovery and Reproducibility at Different Water Dilution Factors during Sample Preparation (n = 3)

4. Elution Buffer and Methods

The recovery and reproducibility obtained using different elution buffers and elution protocols are summarized in Fig. 11, and chromatograms of the standard and the serum-spiked sample (eluted with 100 mM TEA in 40% IPA aqueous solution, 250 µL x 4) are shown in Fig. 10. Among the conditions evaluated, elution with 100 mM TEA in 40% IPA aqueous solution using four sequential aliquots of 250 µL provided the highest recovery and the best reproducibility.

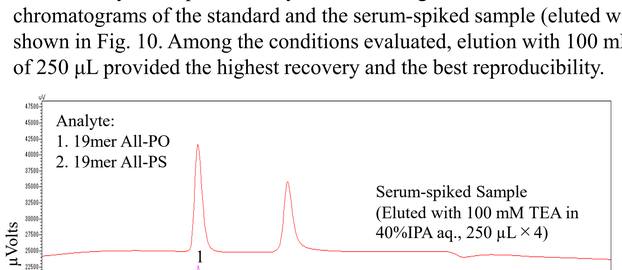

Fig. 10. Standard and Serum-spiked Sample (Eluted with 100 mM TEA in 40% IPA aq., 250 µL x 4)

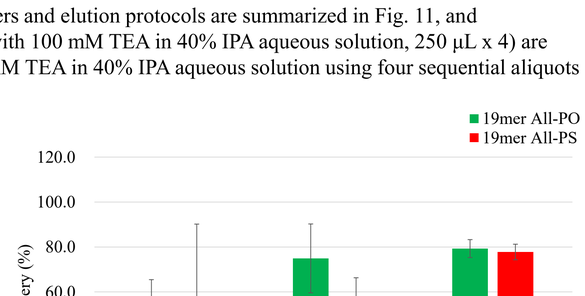

Fig. 11. Recovery and Reproducibility with Different Elution Buffers and Methods (n = 5)

Evaluation of the Inter-Day Reproducibility of Recovery

Spike and recovery tests were performed using the optimized method, and the inter-day reproducibility of recovery was evaluated by LC-MS/MS. The results are summarized in Table 2, and the chromatograms of serum samples are shown in Figs. 12 and 13. The optimized method provided consistent recovery of both phosphodiester (PO) and phosphorothioate (PS) oligonucleotides from serum, with recoveries of 70.6 - 93.1% and 77.7 - 93.7%.

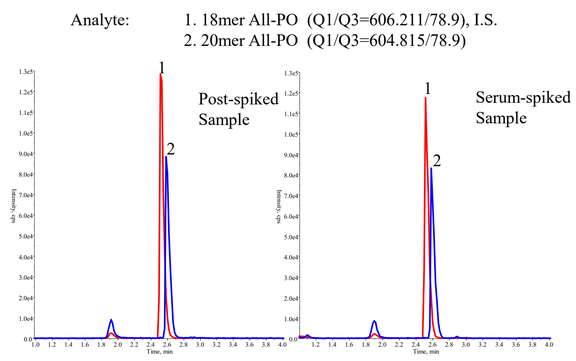

Fig. 12. Results for 20mer All-PO Oligonucleotide in Serum

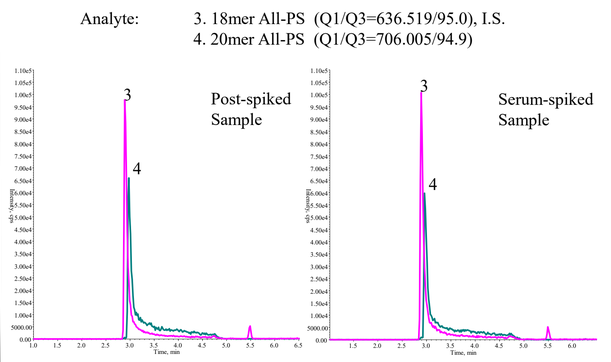

Fig. 13. Results for 20mer All-PS Oligonucleotide in Serum

Table 2. Inter-Day Reproducibility of Recovery (Final Concentration: 50 nM, n = 3 x 3 days)

Compounds	Recovery (%)									Ave.(%)	RSD(%)
	Day 1			Day 2			Day 3				
	n=1	n=2	n=3	n=1	n=2	n=3	n=1	n=2	n=3		
20mer All-PO	75.5	70.6	82.2	78.8	77.6	81.0	85.2	88.3	93.1	**81.4**	**8.4**
20mer All-PS	77.7	78.0	80.1	83.3	90.0	91.0	93.3	93.7	91.4	**86.5**	**7.7**

Conclusions

SPE conditions were optimized from the perspectives of sample preparation, washing, elution, and reconstitution to achieve stable recovery of oligonucleotides from serum. Using the optimized method, the inter-day reproducibility of recovery was evaluated. Recoveries of 70.6-93.1% (RSD: 8.4%) for 20mer phosphodiester (PO) oligonucleotides and 77.7-93.7% (RSD: 7.7%) for 20mer phosphorothioate (PS) oligonucleotides were achieved. These results demonstrate that the proposed method is a useful and reliable approach for the quantitative bioanalysis of oligonucleotide therapeutics in pharmacokinetic studies.

We have no financial relationships to disclose for this presentation.