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Quantitation of a Phosphothioate Oligonucleotide in Rat Plasma Using LC/MS/MS

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Objectives

To develop and validate rugged, sensitive and high throughput methods for the quantitative determination of oligodeoxynucleotides in biological matrix.

Currently, hybridization based immunoassays and HPLC-UV methods are predominately used for the quantitative analysis of oligonucleotides in biological matrix [1]. However, the immunoassays generally have narrow calibration ranges, poor specificity and low precision. HPLC-UV methods typically have low sensitivity and very long run times. Although the increased use of UPLC has reduced the run times for GLP analysis, the sensitivity is typically still not adequate for most studies [2, 3].

To overcome these shortcomings, we have developed and validated dozens of LC-MS/MS assays to analyze DNA oligonucleotides and siRNA in biological matrices [4]. Here we present this methodology by using a model compound, an 18-mer phosphorothioate oligonucleotide (TL0901), extracted from rat plasma. The validated linear range for TL0901 in rat plasma (K2EDTA) is 10.0 - 2,500 ng/mL using a 200 µL aliquot of plasma.

Methodology

SAMPLE PREPARATION:

1. Thaw the samples in a wet ice-bath.
2. Aliquot 200 µL of samples into the corresponding labeled 2.0 mL Eppendorf centrifuge tubes.
3. Add 50.0 µL ice-cold working internal standard solution [10.0 µg/mL of TL0901 (n-6) in water].
4. Add 200 µL of pH=8.0 extraction buffer to all samples.
5. Add 200 µL of the phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) solution to all samples.
6. Vortex-mix the samples thoroughly.
7. Centrifuge the samples in the microcentrifuge at 13,000 rpm for 15 minutes.
8. Transfer the clear supernatant to corresponding wells of 96-well plate and dried down.
9. Reconstitute the samples with 200 µL mobile phase A.

CHROMATOGRAPHIC CONDITIONS

Column: Phenomenex C18, 2x50 mm. 3 µm.

Mobile Phase: A: HFIP and TEA buffered water
B: HFIP and TEA buffered MeOH

Time		2.0'	2.5'	3.5'	5.5'	
	B%	15	45	45	15	end

Injection volume: 5– 20µL

Column temperature: 55°C

Flow rate: 0.300 mL/min

AS Temperature: RT

MASS SPECTROMETER CONDITIONS

Instrument: API5000

Ionization mode: Turbo ionspray, Negative ion mode

Source Temperature: 500°C

SRM transitions:

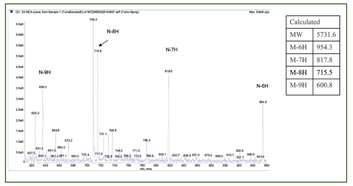
Analyte	Internal Standard	Transitions (±0.5 amu)	Typical Retention Times (min.)
TL0901		715.4 → 319.0	1.5
	TL0901(n-6)	631.7 → 319.0	1.3

Results and Discussion

METHOD DEVELOPMENT

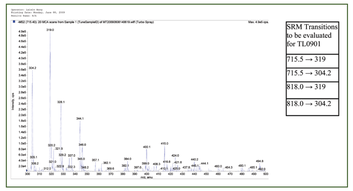
- The molecular weight of TL0901 is 5731.6. A typical multiple charged Q1 scan was obtained under optimized negative ESI conditions (Figure 1).
- The full length TL0901 can be completely separated chromatographically from its n-6 or shorter metabolites, but not the n-4 or longer metabolites under selected LC conditions (Figure 3). Fortunately, the analyte and each metabolite have multiple MRM transitions to choose to avoid interfering each other (Figure 1,2 and 4).
- SPE extractions were compared using different SPE plates, the recoveries were not as consistent as LLE for phosphorothiate oligonucleotides which are longer than 18-mer.
- The addition of EDTA to samples (EDTA as anticoagulant) can improve the sample stability, extraction recovery and column life time.

FIGURE 1. Typical Q1 scan Mass Spectrum of TL0901



Calculated	
MW	5731.6
M-6H	954.3
M-7H	817.8
M-8H	715.5
M-9H	600.8

FIGURE 2. Typical Product Ion Scan Spectrum of TL0901



SRM Transitions to be evaluated for TL0901
715.5 → 319
715.5 → 304.2
818.0 → 319
818.0 → 304.2

FIGURE 3. Liquid Chromatographic Separation of TL0901 and its Metabolites

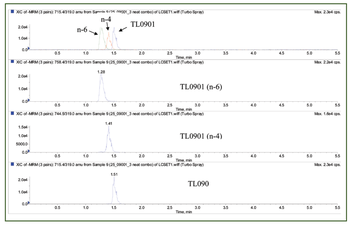


FIGURE 4. MS/MS Selectivity and LC Separation of TL0901 and its n-1 and n-2 Metabolites

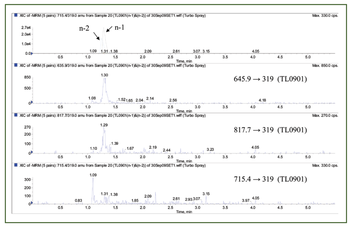


FIGURE 4. Representative Calibration Curve (10.0-2,500 ng/mL)

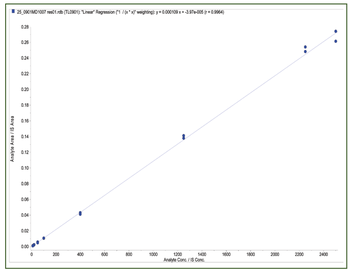


FIGURE 5. Representative Chromatogram of a Plasma Blank

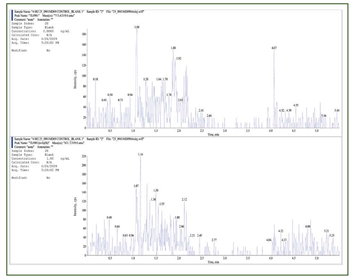


FIGURE 6. Representative Blank with Internal Standard (0-ng/mL QC)

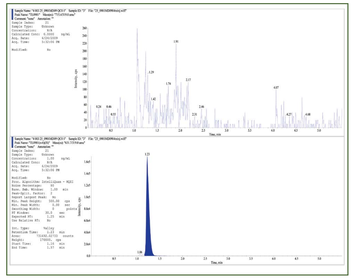


FIGURE 7. Representative Low Standard (10.0 ng/mL) for TL0901

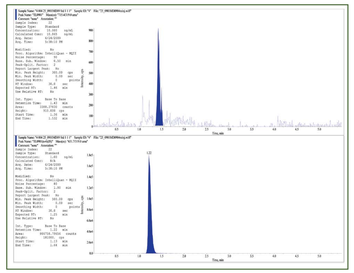
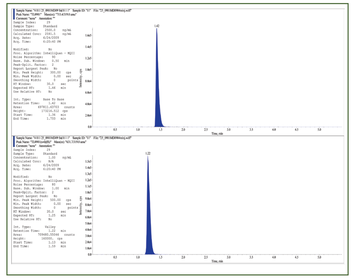


FIGURE 8. Representative High Standard (2,500 ng/mL) for TL0901



ASSAY VALIDATION

Accuracy/precision: Demonstrated at n=6 at LLOQ, Low, Medium, High concentrations over 3 validation runs. (Tables 2)

Selectivity: Demonstrated with blank and LLOQ concentrations in six sources of rat plasma. (Tables 3)

Ability to dilute: Demonstrated above ULOQ at DF=10. (Table 4)

Extraction recovery: Evaluated for analyte at Low, Medium, High concentrations (n=6). (Table 5)

Carryover: Evaluated in each run. No carryover present.

STABILITY

Stock solution: Room temperature for analytes and the I.S., 6.0 hrs
96 days at 1-8°C in water

Stability in matrix: Freezing/thawing, 4 cycles
4.0 hours at room temperature
6.0 hours on ice bath
19 days at -70°C

Extract: Batch reinjection stability, 114 hours at RT

TABLE 1. Back-Calculated Concentrations of Calibration Standards for TL0901

All concentrations are expressed as ng/mL.

Nominal Conc.	10.0	20.0	40.0	100	500	1250	2200	2500
Mean	9.98	20.5	38.6	97.7	512	1270	2220	2470
S.D.	0.959	0.631	1.72	5.20	23.1	45.2	61.8	86.5
%CV	9.6	3.1	4.5	5.3	4.5	3.6	2.8	3.5
%Bias	-0.2	2.5	-3.5	-2.3	2.4	1.6	0.9	-1.2
n	6	6	6	6	6	6	6	6

TABLE 2. Intra- and Inter-Assay Accuracy and Precision for TL0901

Accuracy and Precision Quality Control Samples from ANOVA

Nominal Conc.	LLOQ QC 10.0 ng/mL	Low QC 30.0 ng/mL	Medium QC 1000 ng/mL	High QC 2000 ng/mL
Mean Observed Conc.	9.36	26.8	989	1990
%Bias	-6.4	-10.7	-1.1	-0.5
Between Run Precision (%CV)	3.6	3.1	2.3	5.2
Within Run Precision (%CV)	5.7	4.6	3.0	2.8
Total Variation (%CV)	6.8	5.6	3.8	5.9
n	18	17	18	17
Number of Runs	3	3	3	3

TABLE 4. Selectivity in 6 Lots of Rat Plasma at the Lower Limit of Quantitation (10.0 ng/mL) for TL0901

	LLOQ 10.0 ng/mL
	10.1
	11.2
	11.2
	10.7
	8.18
	9.01
Mean	10.1
S.D.	1.24
%CV	12.3
%Theoretical	101.0
%Bias	1.0
n	6

TABLE 5. Dilution Quality Control Samples (6,000 ng/mL at DF=10) for TL0901

	Dilution QC 10000 ng/mL DF=10
	10500
	10800
	10800
	10500
	10200
	10400
Mean	10500
S.D.	234
%CV	2.2
%Theoretical	105.0
%Bias	5.0
n	6

Conclusion

A robust, sensitive and specific method was developed and validated to quantify oligonucleotides in rat plasma. Similar assays have been successfully used to analyze pre-clinical and clinical samples. All instances of Incurred Sample Reanalysis (ISR) have met the acceptance criteria with a high degree of reproducibility.

Reference

1. Guy A Tremblay and Philip R Oldfield, "Bioanalysis of siRNA and oligonucleotide therapeutics in biological fluids and tissues", *Bioanalysis (2009), 1(3), 595-609.*
2. Yanhui Zhang, Laixin Wang, Jian Chen, Yue Zhao, Min Meng and Patrick Bennett (Tandem Labs) as well as Donna Dobinson and Colin Green (Antisoma Research Limited, Welwyn Garden City, UK, "Quantitative determination of AS1411 oligonucleotide in human urine using UPLC-PDA", the 11th Annual Symposium on Chemical & Pharmaceutical Structure Analysis, October 2008, Langhorne, PA.
3. Jian Chen, Laixin Wang, Yanhui Zhang, Ming Meng, Juan Wang and Patrick Bennett (Tandem Labs) as well as Donna Dobinson and Colin Green (Antisoma Research Limited, Welwyn Garden City, UK),"Quantitative determination of AS1411 oligonucleotide in monkey plasma using UPLC-PDA", the ISSX 15th Annual Meeting, October 2008, San Diego, CA.
4. Laixin Wang, Weiwei Yuan, Yue Zhao, Jian Chen, Gregory vonArx, Min Meng and Patrick Bennett, "Quantitation of TL0901 Oligonucleotide in Human Plasma Using LC MS/MS", AAPS Annual Meeting and Exposition, November 8 - 12, 2009, Los Angeles, CA.