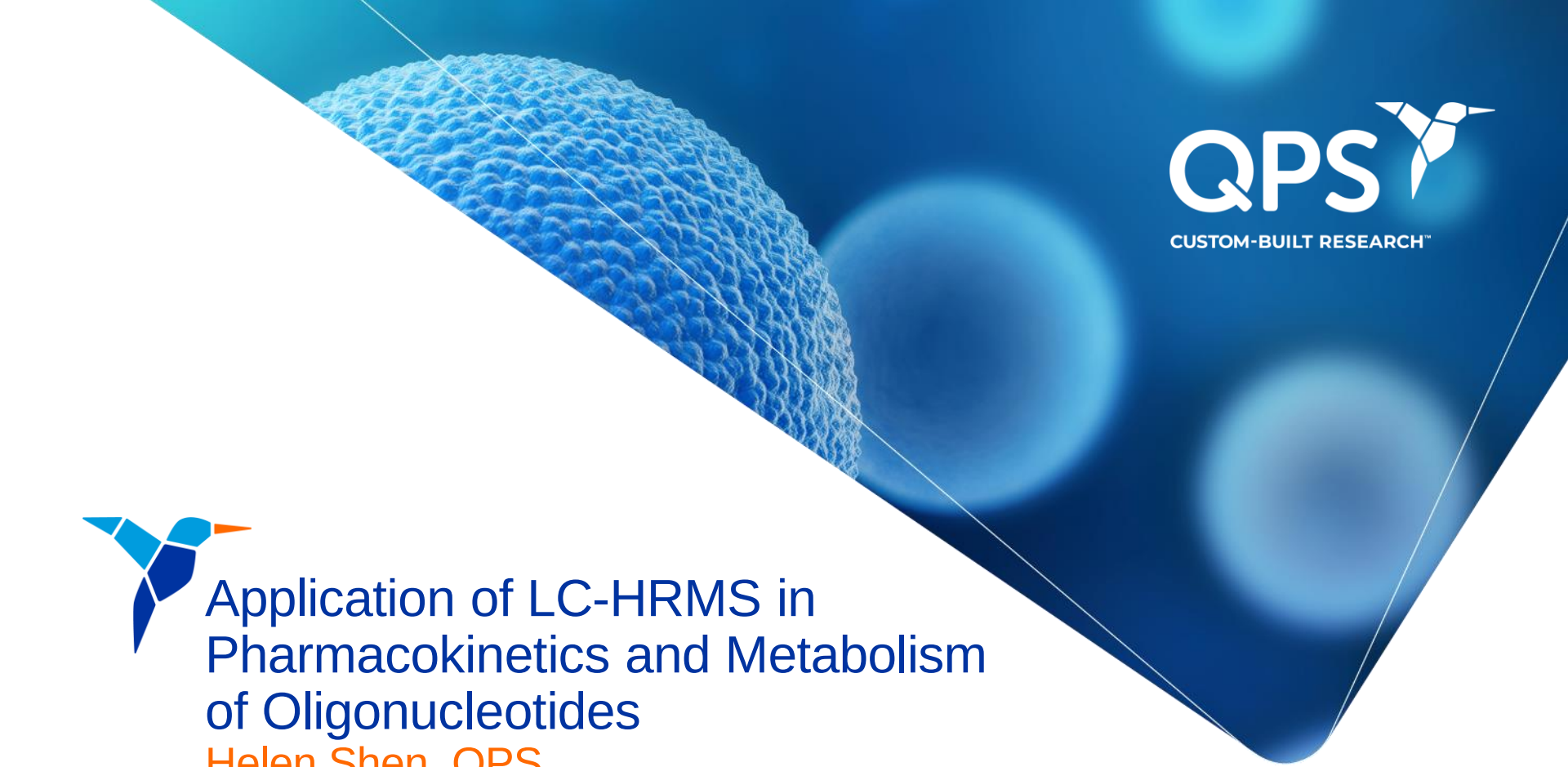


Application of LC-HRMS in Pharmacokinetics and Metabolism of Oligonucleotides

Helen Shen, QPS

JANUARY 2020

Oligonucleotides Quantitation - siRNA

LC–TOF–MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues

Lakshmi Ramanathan & Helen Shen

Bioanalysis (2019), Vol. 11, No. 21, 1983-1992

Oligonucleotides Quantitation - siRNA

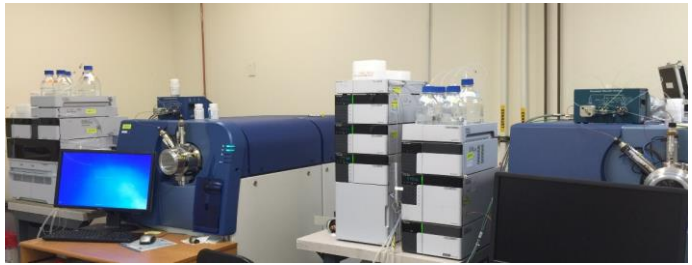
Bioanalytical approaches that have been used for the quantification of siRNA in biological matrices,

- such as S1 nuclease protection “cutting ELISA”,
- fluorescent probe hybridization HPLC,
- HPLC UV,
- LC-MS/MS
- LC-MS/HRAM

Oligonucleotides Quantitation – siRNA Using LC-HRMS

This work focused on:

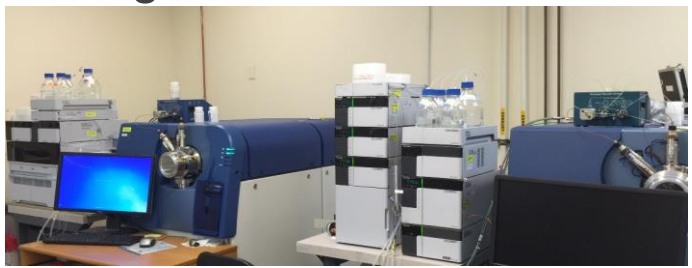
- Validated plasma assays for several oligonucleotides such as GalNAc-conjugated siRNA, using UHPLC and high resolution mass spectrometer by TOF detection
- Application to oligonucleotide quantitation in lysate of various tissues as well as urine and feces samples
- The analytes are GalNAc conjugated siRNA. The duplex of the full length oligonucleotides or N-1 metabolite



Oligonucleotides Quantitation – siRNA Using LC-HRMS

This approach provides:

- quantitative information for the target oligonucleotides
- but also qualitative information on unknown metabolites.
- One advantage of HRMS in full-scan mode is selectivity and can improve the detection limit considerably
- LLOQ generally 5-10 ng/mL for 18-22 mer oligonucleotides
- LLOQ of 1 ng/mL was achieved in specific ASO



Oligonucleotides Quantitation – siRNA Using LC-HRMS

Challenges:

- Non-specific binding of oligonucleotides to various surfaces and containers might result in low recoveries or inconsistent recovery through the concentration range.
- Formation of sodium adducts can severely reduce the signal of the ion of interest and decrease the sensitivity of the assay.
- Degradation of oligonucleotides by nucleases is another source that might affect the recovery and decrease the method precision.

Oligonucleotides Quantitation – siRNA Using LC-HRMS

Many therapeutic oligonucleotides have chemical modifications to improve their stability.

- Most therapeutic oligonucleotides contain phosphorothioate in the backbone
- 2’-modification on the ribose ring.
- 2’-Modification tends to improve stability
- Basic Structure of siRNA:

Oligonucleotides Quantitation – siRNA Using LC-HRMS

SPE Extraction method:

- Extraction was performed using a semi-automated TomTec Quadra 4 SPE 96-well format liquid handler.
- The SPE cartridges were conditioned using methanol and equilibration buffer sequentially

  The equilibration buffer consisted of ammonium acetate, NaN3 and K2EDTA in water and the pH was adjusted with acetic acid to 5.5
- Internal standard – an analog of siRNA
- Plasma (100 µL) was added IS and Clarity OTX buffer and loaded onto the column.

Oligonucleotides Quantitation – siRNA Using LC-HRMS

- SPE Extraction method (continued):
- The cartridges were washed using washing buffer 1 and 2, and the analytes were eluted using elution buffer into a plate containing RNA recovery solution.

  The washing buffer 1 consisted of ammonium acetate in 50:50 water:acetonitrile and pH was adjusted with acetic acid to 5.5.

  The washing buffer 2 consisted of ammonium acetate in 10:90 Water:Acetonitrile and the pH was adjusted to 5.5 with acetic acid.

  The elution buffer contained ammonium bicarbonate, pH adjusted to 8.8 with ammonium hydroxide, acetonitrile and THF.
- The collected solutions were evaporated to dryness and reconstituted with reconstitution solution.

  The reconstitution solvent contained water, methanol, diisopropylamine (DIPA), hexafluoroisopropanol (HFIP), EDTA in water, acetonitrile, and ammonium hydroxide.
- Ten microliters of the reconstituted solution was injected into the uHPLC-TOF system for analysis.

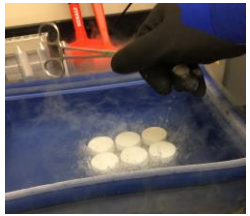
Oligonucleotides Quantitation – siRNA Using LC-HRMS

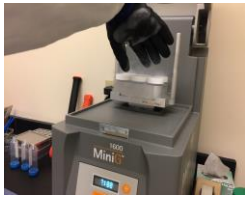
Sample preparation method for tissues

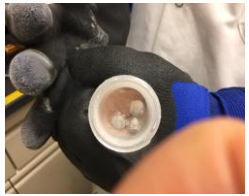
- Tissue samples underwent disruption to produce a frozen powder using a SPEX Geno/Grinder.
- Tissue powder was homogenized by adding Clarity OTX Lysis-Loading buffer to produce a 100 mg/mL mixture of tissue homogenate.
- After mixing for three hours at ambient temperature the tissue homogenate was centrifuged.
- Same extraction method as for plasma
- The calibration standards ranged from

  10-10,000 ng/mL in tissue lysate

  100-100,000 ng/g in terms of tissue concentration.







Oligonucleotides Quantitation – siRNA Using LC-HRMS

Instrumentation LC-TOF-MS Conditions

- Samples were analyzed using reversed-phase UHPLC with Electrospray Ionization (ESI) TOF-MS detection using high resolution Triple TOF™ system (AB Sciex LLC, MA, USA).
- In TOF-MS quantitation, each peak in the isotopic envelope of the $[M-4]^{4-}$ and $[M-3]^{3-}$ charged states was extracted using a 50-75 mDa extraction window.
- Accurate mass of ten most intense ions (m/z) for each strand of the analyte, antisense and sense, and each strand of IS, antisense and sense, were monitored in the negative ion mode.
- The peak area for the analyte or IS was the sum of the response from the respective ten ions.
- Waters Acquity BEH C18 column (2.1 X 50 mm, 1.7 µm, Waters Corporation, MA, USA) was used with a flow rate of 0.85 mL/min and the following mobile phases:

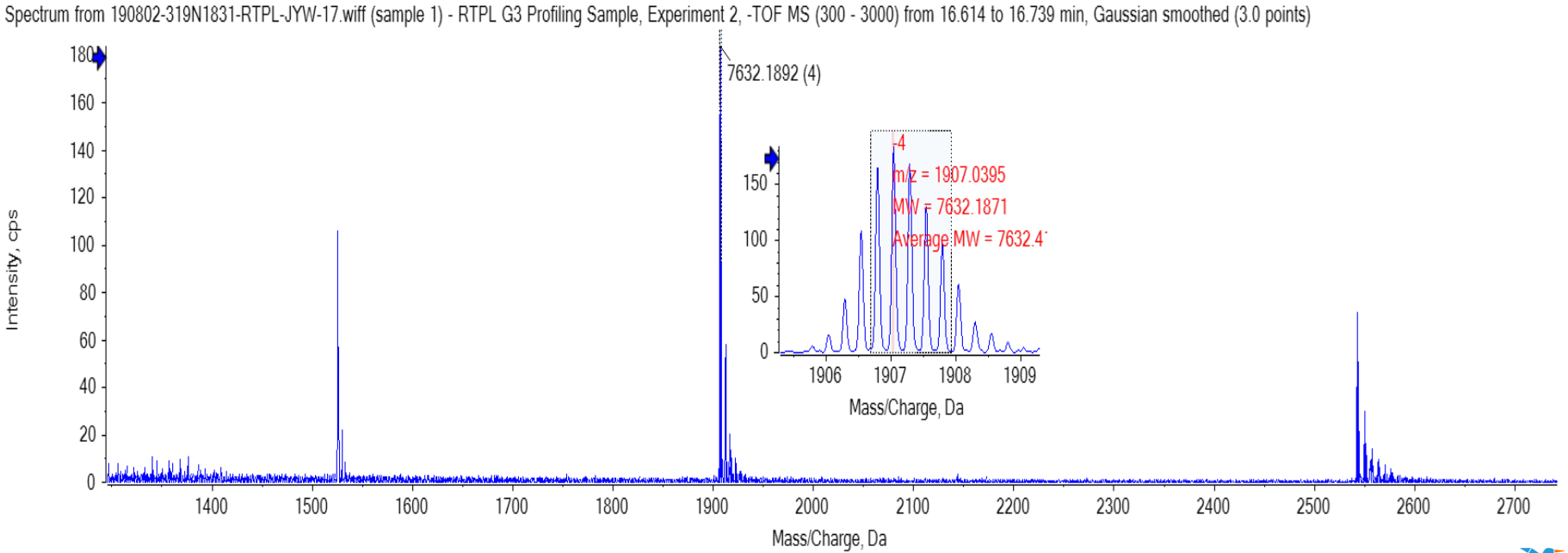
  Mobile phase A is H2O:DIPA:HFIP (100:0.15:0.264 v/v/v)

  mobile phase B is H2O:MeOH: DIPA:HFIP (50:50:0.15:0.264, v/v/v/v).

  The gradient was from 30%B increased to 50%B in 1.6 minutes, then to 100%B in 0.1 minute and maintained for 0.5minutes, followed by re-equilibration with 30%B for 0.6 minutes.

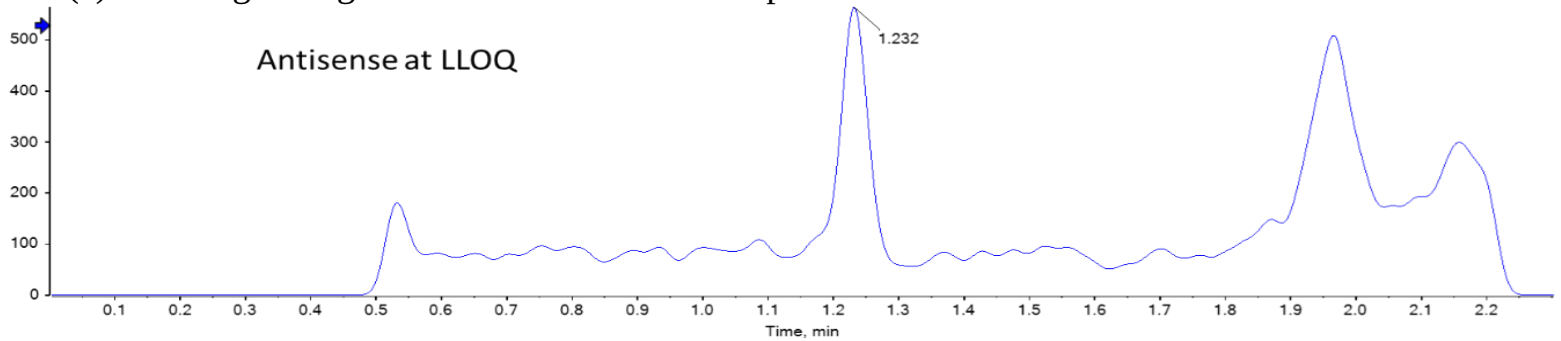
  The column temperature was maintained at 70°C.

Example Mass Spectrum from a Pooled Rat Plasma

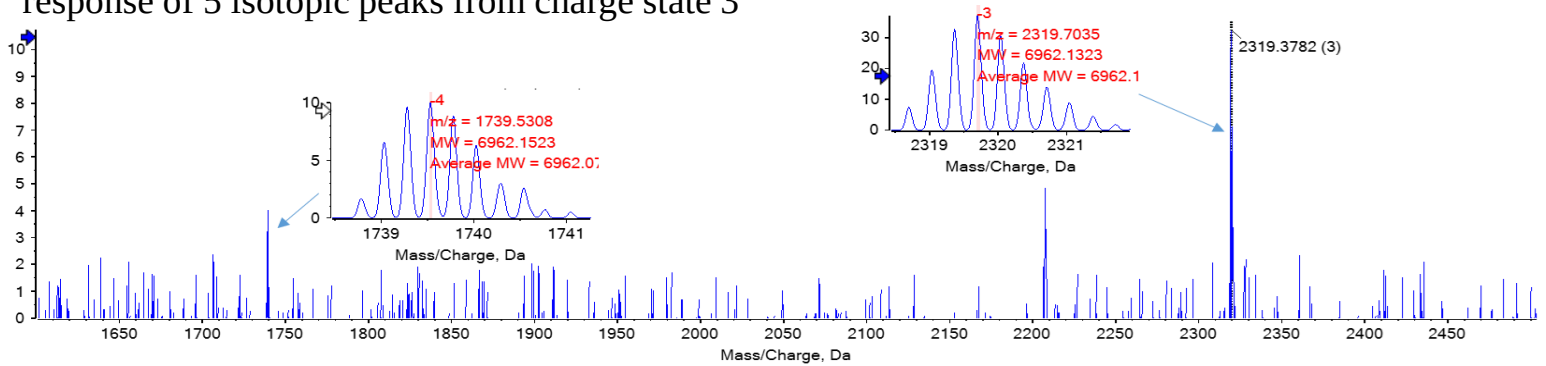


Typical chromatogram and full scan mass spectrum of siRNA

(a) Full length Oligonucleotide extracted from plasma

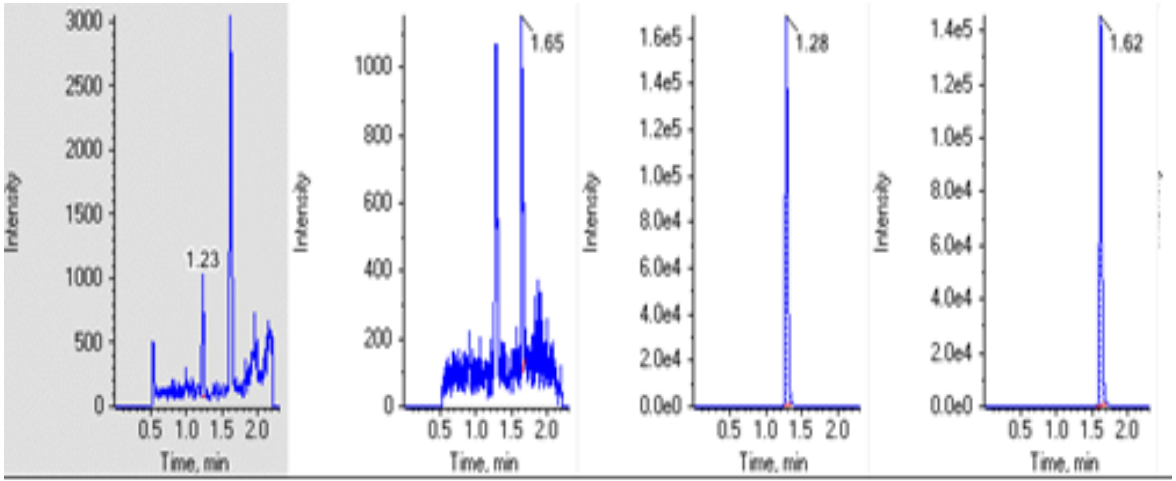


(b) showing different charge state and isotopic peaks for each charge state Mass spectrometer response of 5 isotopic peaks from charge state 3

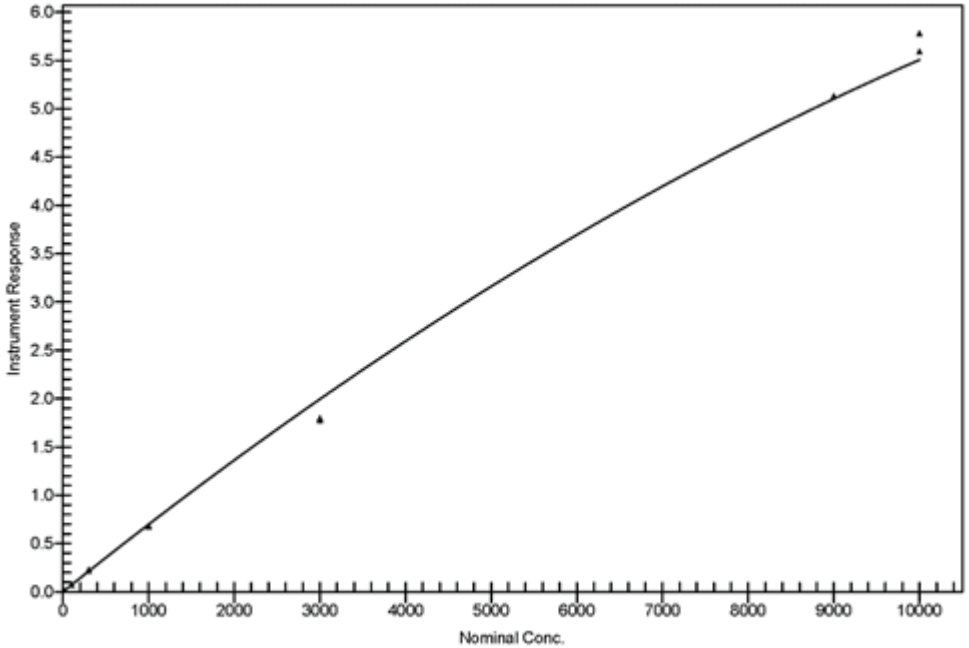


Chromatograms of analytes and IS

There are four columns for each chromatogram, the first two columns are for the full length siRNA monitored for the antisense and sense strand; third and fourth columns are for the internal standard (by antisense and by sense)



Example Calibration Curve (Antisense)



Intraday and interday accuracy and precision of QC samples in rat plasma by measuring antisense strand

QC Concentration of the duplex (ng/mL)	Intra-run (n = 6)			Inter-run (n = 18)		
	Mean Concentration	%RE	%CV	Mean Concentration	%RE	%CV
10	10.1±0.9	0.7	9.4	10.0±1.7	0.3	17.4
30	31.7±2.4	5.8	7.6	31.9±2.3	6.4	7.2
400	431±10	7.8	2.3	430±15	7.5	3.4
4000	3623±94	-9.4	2.6	3774±256	-5.7	6.8
8000	8049±360	0.6	4.5	8091±387	1.1	4.8

Example Extraction Recovery from Rat Plasma (Antisense)

QC Concentration (ng/mL)	% Recovery	
	Oligonucleotide by AS	Internal Standard
30	100.4	96.9
400	105.1	94.0
8000	78.9	83.3
Mean Recovery	94.8	91.4
Precision (%)	14.8	7.9

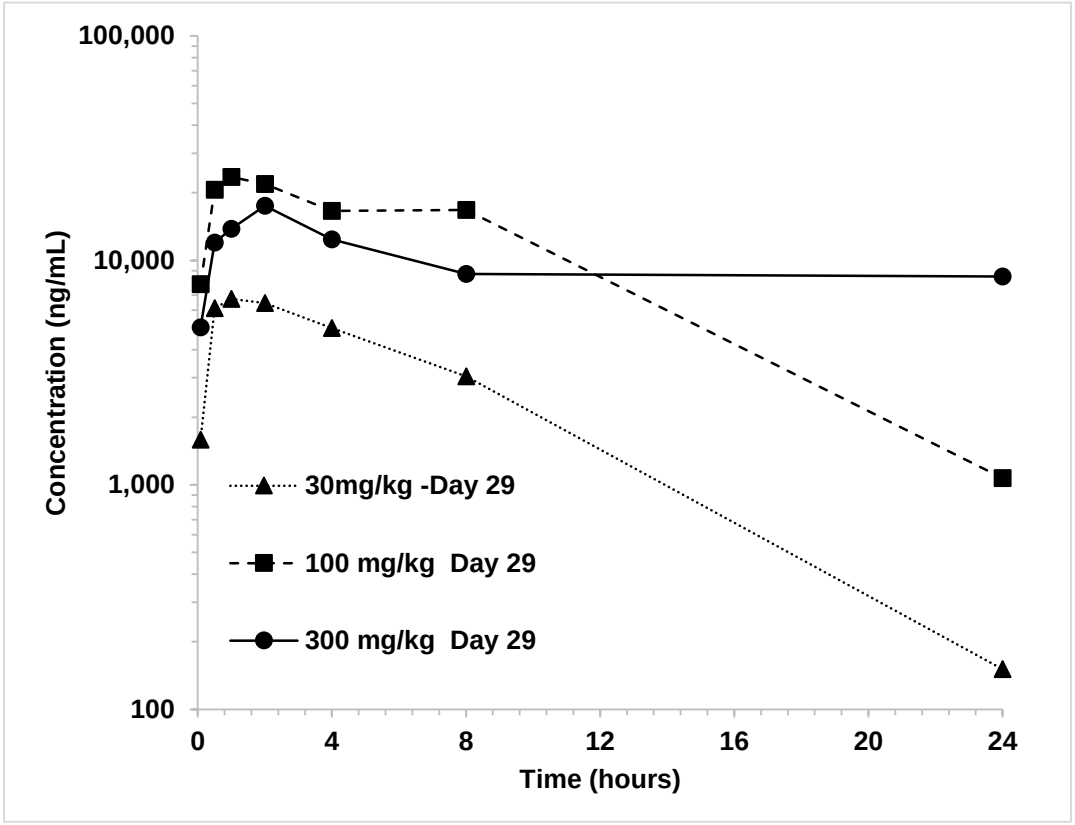
Stability of oligonucleotide (AS) in rat plasma under four different conditions (n = 5)

Storage Condition	Low QC (30 ng/mL, n = 5)			High QC (8000 ng/mL, n = 5)		
	Mean Concentration	%RE	%CV	Mean Concentration	%RE	%CV
Room Temperature (24 h)	35.6	8.6	6.0	7702	-3.7	2.7
Freeze Thaw (-20 °C, 5 cycles)	30.6	2.1	5.0	8821	10.3	4.8
Freeze Thaw (-70 °C, 5 cycles)	29.8	0.6	2.9	8407	5.1	2.3
Processed Samples (112 hours at RT)	32.9	9.7	3.7	8622	7.8	4.9
Long Term Stability (370 days at -20 °C)	26.4	-12.1	12.0	7617	-4.8	3.9
Long Term Stability (370 days at -70 °C)	27.0	-9.9	12.3	7720	-3.5	2.3

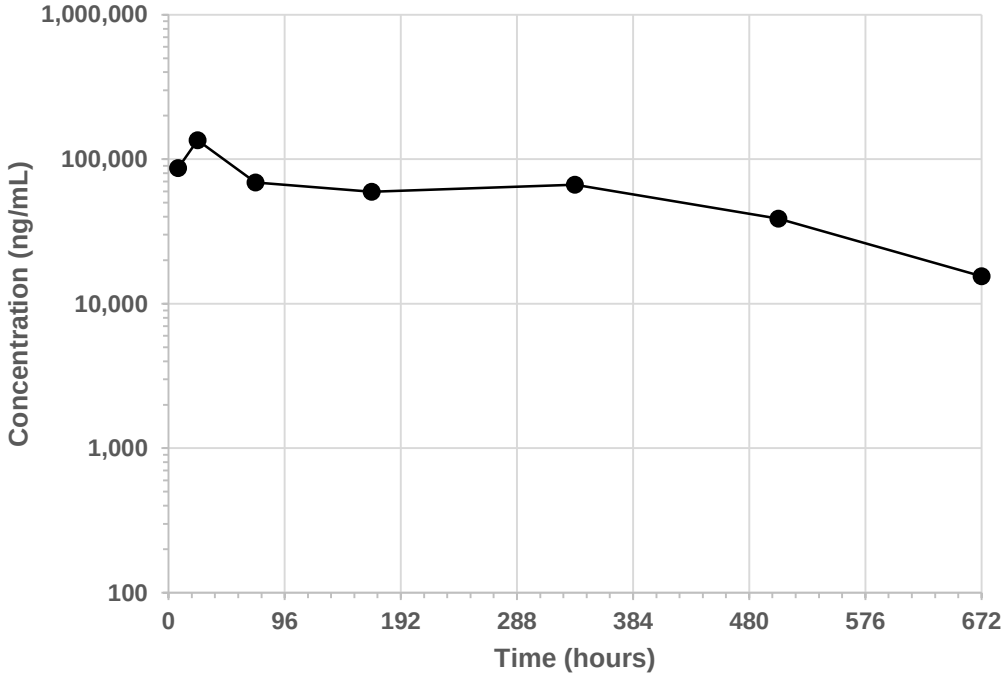
Intraday accuracy and precision of QC samples in rabbit liver and kidney by measuring antisense strand

QC Concentration of the duplex (ng/mL)	Intra-run (n = 6)-Rabbit Liver			Intra-run (n = 6)-Rabbit Kidney		
	Mean Concentration	%RE	%CV	Mean Concentration	%RE	%CV
20	18.0±1.2	-9.8	6.7	21.9±2.2	9.6	9.8
60	51.7±3.7	-13.9	7.1	61.1±4.4	1.9	7.2
400	436±10	9.0	2.2	465±20	16.2	4.4
4000	4064±160	1.6	3.9	4413±244	10.3	5.5
8000	8552±611	6.9	7.1	8749±268	9.4	3.1

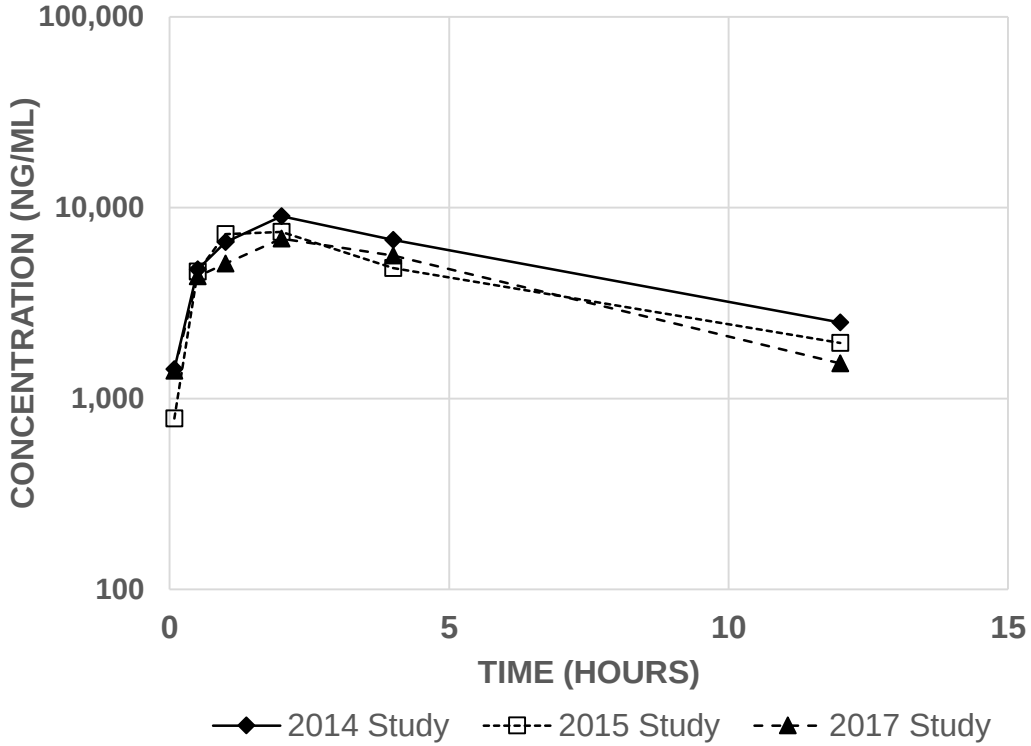
Plasma concentration of full length oligonucleotide in monkeys following SC administration of 30, 100, and 300 mg/kg dose



Mean liver concentration of full length oligonucleotide in monkeys following SC administration of 10 mg/kg dose



Mean plasma concentration of full length oligonucleotide in monkeys following SC administration of 30 mg/kg dose, showing reproducibility over a period of 3 years

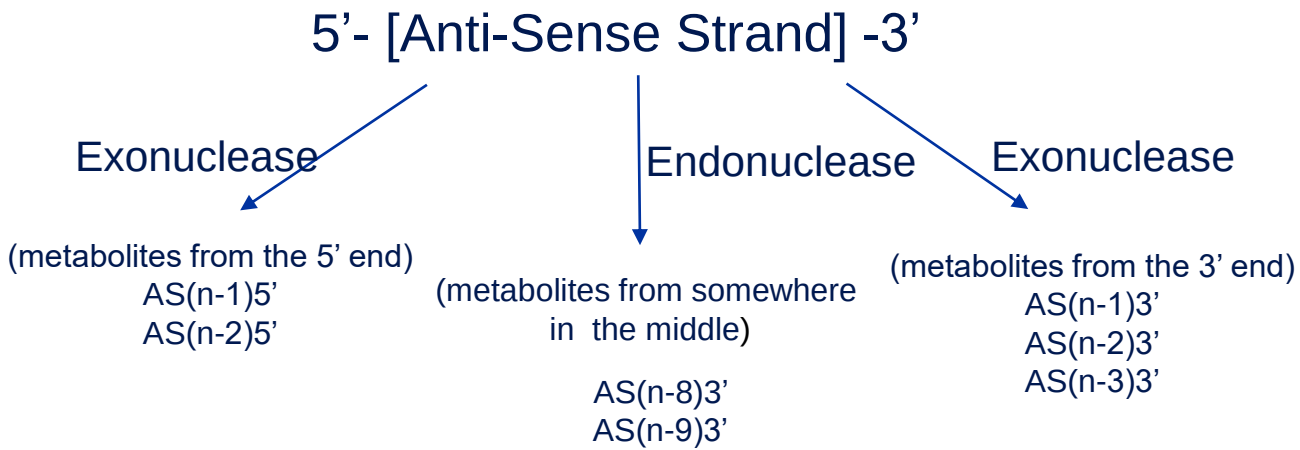


Oligonucleotides Quantitation – siRNA Using LC-HRMS

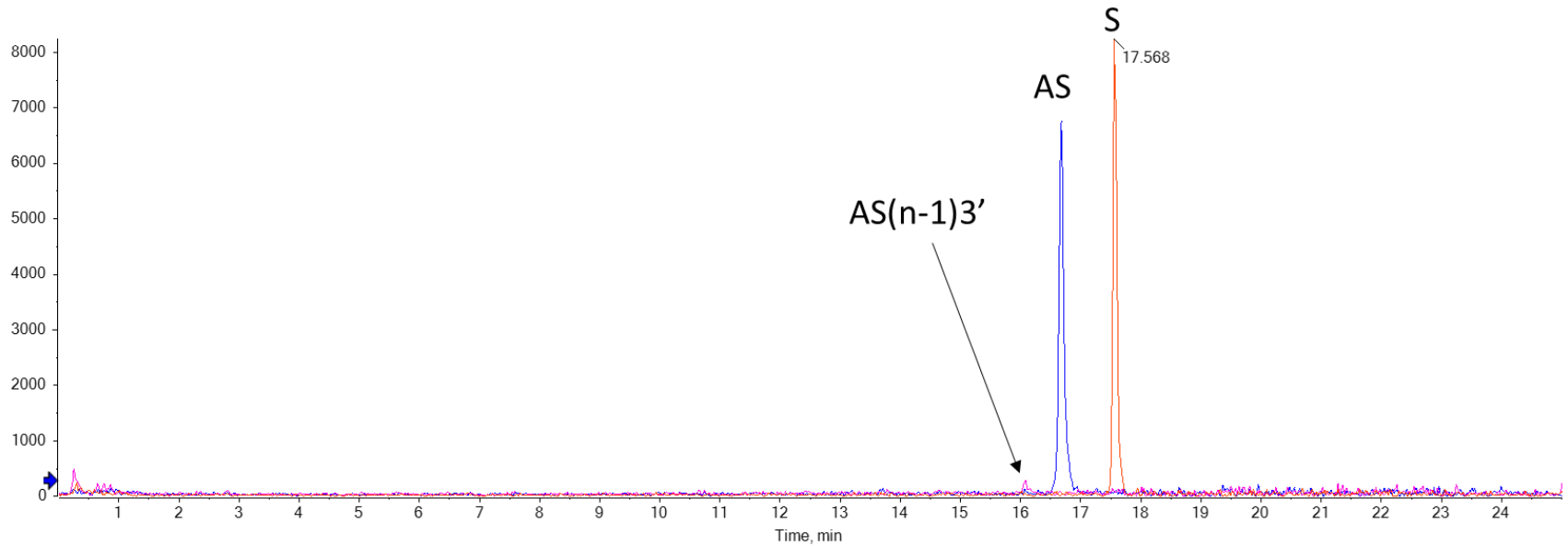
The method was successfully validated in plasma

with respect to linearity, sensitivity, accuracy, precision, dilution linearity, selectivity, hemolysis effect, recovery, matrix effect, and carryover. Validated method was used for regulated preclinical studies, using a 100 μL sample volume, the dynamic range was 10 to 10,000 ng/mL.

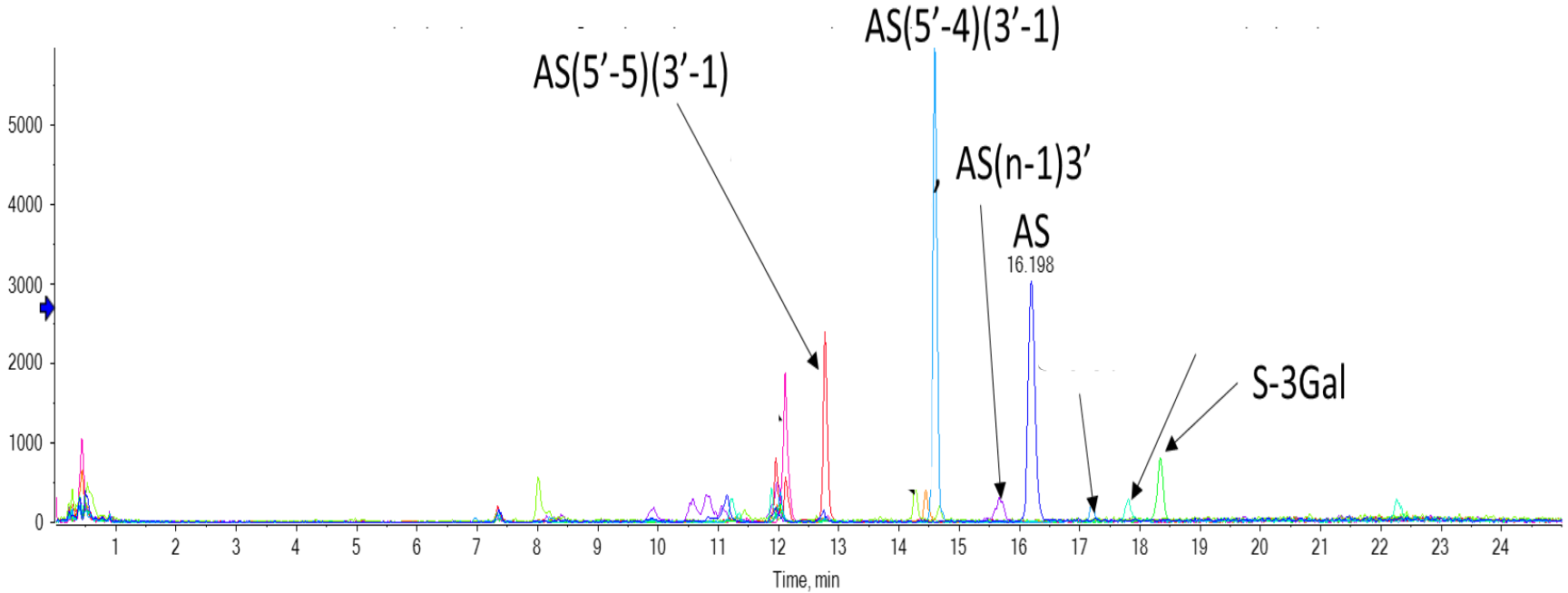
Example metabolic pathway



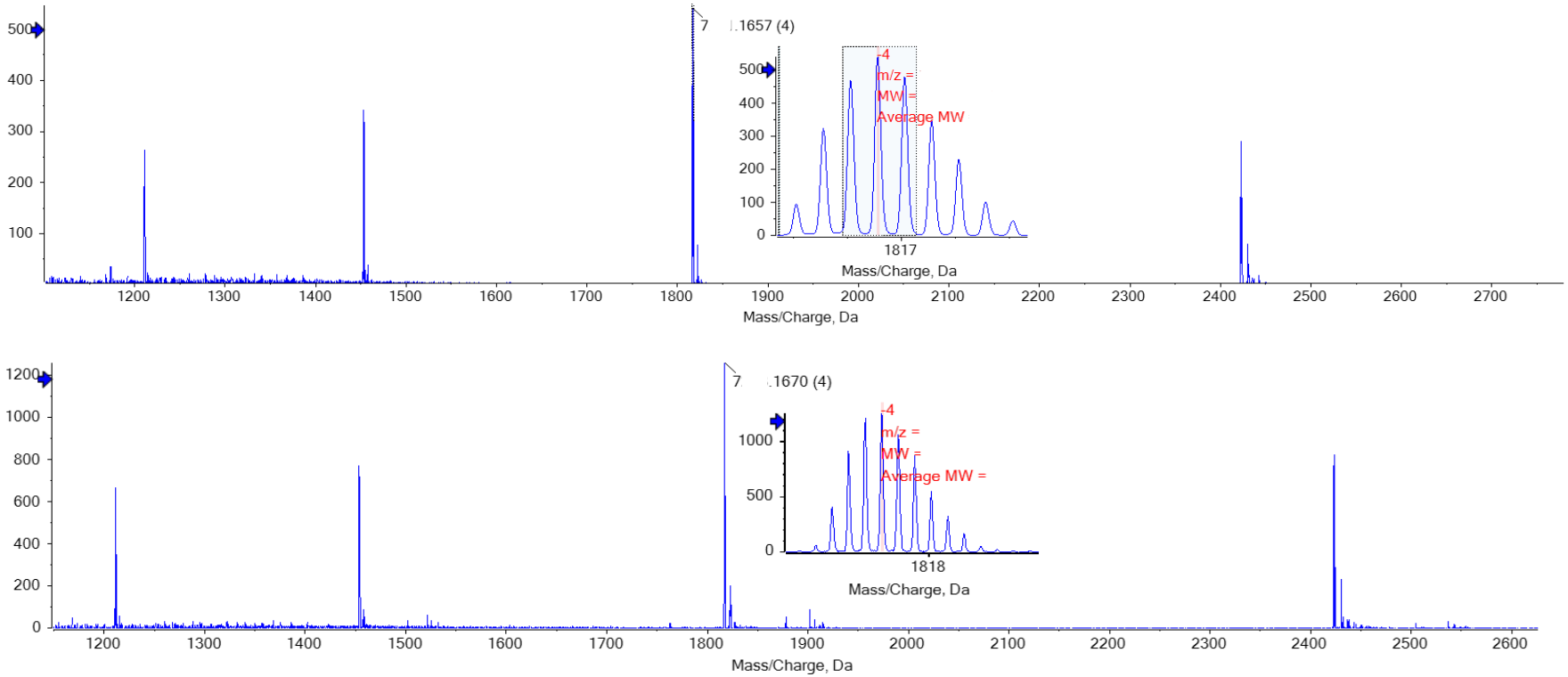
Extracted Ion Chromatogram for the Profiling Plasma Samples (0-6 h)



Extracted Ion Chromatogram from pooled Kidney Sample



Negative Ion Mass Spectrum of AS(N-1)3’ Standard (top) and from rat sample



Acknowledgement

Lakshmi Ramanathan, MS

Timothy Snow, Ph.D.

Jiyi Wang, Ph.D.

Zhihua Yang, Ph. D.

Amelie Chen

Bill Montgomery

Karen Wang

Tapan Majumda, Ph.D.