

Oligonucleotide bioanalytical method development - triple quadrupole and high-resolution mass spectrometric detection - the benefits and challenges of selecting the technology

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Oligonucleotides Quantitation by LC-HRMS or LC-MS/MS

IND/CTA-ENABLING STUDIES FOR OLIGONUCLEOTIDES: ALL NEED BIOANALYTICAL DATA

- ▶ The physicochemical properties of oligonucleotide therapeutics (ASO, miRNA, siRNA, nucleic acid aptamers, and antibody-oligonucleotide conjugates) make quantitation of these compounds in biological matrices very challenging.
 - ▶ Different quantitative approaches have been used, such as hELISA, hHPLC-fluorescence, HPLC-UV, hUPLC-MS/MS, UPLC-MS/MS, and UPLC-HRMS.
 - ▶ This presentation will discuss the bioanalytical differences between LC-MS/MS and LC-HRMS for this modality.
- ▶ Participant to understand
 - ◆ the chromatographic conditions to separate oligonucleotide drugs and their metabolites.
 - ◆ the differences between MS/MS format or HRMS format for oligonucleotides quantitation.
 - ◆ the 'working' mass resolution requires to quantitate oligonucleotides in HRMS.



Oligonucleotides Quantitation by LC-HRMS or LC-MS/MS

IND/CTA-ENABLING STUDIES FOR OLIGONUCLEOTIDES: ALL NEED BIOANALYTICAL DATA

▶ *In vitro*

- ◆ Metabolism
 - *Metabolic Stability / Clearance*
 - *Metabolite Profiling / Identification*
 - *Reaction Phenotyping*
- ◆ Plasma Protein Binding
- ◆ Drug-Drug Interactions
 - *Up / down regulation of drug metabolizing enzymes; Cellular Uptake/Distribution*
 - *CYP450 inhibition*
 - *CYP450 induction*
 - *Drug Transporters*

▶ *In vivo*

- ◆ PK / PD / Biodistribution Studies
 - *Rat Single and/or Multiple Dose*
 - *NHP Single and/or Multiple Dose*
- ◆ General Toxicity Studies
 - *Rat DRF & TK*
 - *Rat Definitive Toxicity & TK (4 weeks or 13 weeks)*
 - *NHP DRF & TK*
 - *NHP Definitive Toxicity & TK (4 weeks or 13 weeks)*
- ◆ Genetic Tox / Safety Pharma Studies
 - *Rat Micronucleus & TK*
 - *NHP CV & TK*

▶ Radiolabel ADME Studies

- ◆ Mass Balance / Excretion
- ◆ QWBA
- ◆ Metabolite Profiling/ID (plasma/tissue)



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IND/CTA-ENABLING STUDIES FOR OLIGONUCLEOTIDES: ALL NEED BIOANALYTICAL DATA

▶ Bioanalysis

- ◆ Modifications (thiophosphorolate, PMO, cholesterol, GalNAc, lipids, ...)
- ◆ Species
 - Mice, rat, NHP, mini-pig, rabbit, human
- ◆ Matrices
 - Hepatocytes, cellular fractions
 - Plasma, Excreta (urine, cage wash, bile, feces)
 - Tissues (liver, kidney, adrenal, thymus, thyroid, brain, CSF, lung, heart, intestine, pancreas, spleen, testes, ovaries, ...)

▶ Criteria

- ◆ GLP: plasma and 'critical' tissues
- ◆ Non-GLP: urine for excretion; tissues for biodistribution

▶ Chromatography vs. Ligand Binding

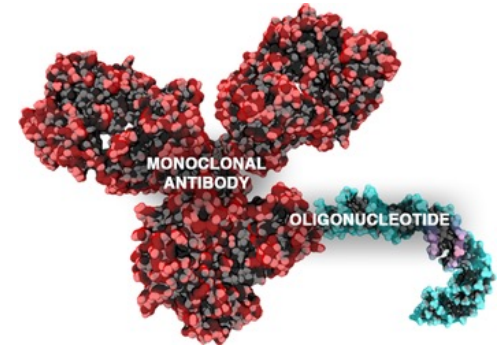
- ◆ Perception
 - *hLBA, hLC-FLD or hLC-MS more sensitive*
 - *LC-Mass Spec more specific*
- ◆ Reality
 - *Mostly based on historical data & 'comfort'*
 - *Whatever works, driven by*
 - Potential non-specific binding
 - Metabolism
 - *Nucleases (exo/endo) vs. oxidative deamination vs. glycosidases*
 - Sensitivity
 - *Tissue concentration usually high*
 - Transferability from plasma to excreta and tissues
 - LC-MS challenges: Formation of cation adducts can severely reduce the signal of the ion of interest and decrease the sensitivity of the assay



Oligonucleotides Quantitation by LC-HRMS or LC-MS/MS

ANTIBODY/PROTEIN OLIGONUCLEOTIDE CONJUGATES

- ▶ Immuno pulldown (with antibody to protein/antibody)
 - ◆ Protease digestion
 - ◆ Quantitation of *signature peptide* reflective of the 'total' Protein/Antibody
 - Typical reverse phase workflow
 - ◆ Quantitation of *oligonucleotides* reflective of the original POC
 - Typical ion-exchange / ion-pairing workflow
- ▶ Immuno pulldown (with complementary strand/antibody to oligonucleotides)
 - ◆ Protease digestion
 - ◆ Quantitation of *oligonucleotides* reflective of the original POC and 'free' oligonucleotides
 - Typical ion-exchange / ion-pairing workflow



Coat

Capture

Digest

Liquid Chromatography

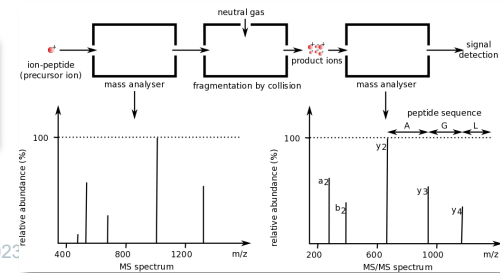
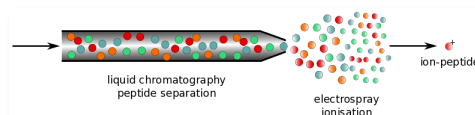
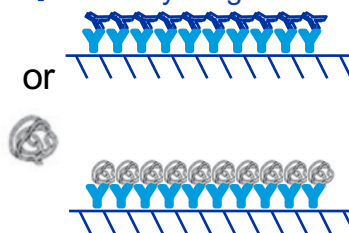
Mass Spectroscopy

Y



Magnetic beads are coated with antibody to antibody or protein target.

Y Antibody drug

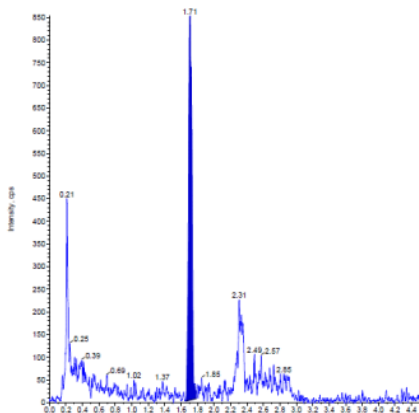




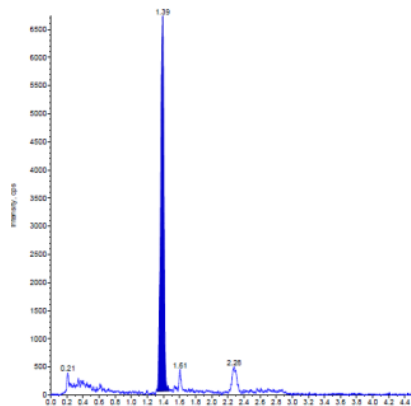
ASO (from an AOC) Quantitation by LC-MS/MS

EXAMPLE: TIC OF ANTI-SENSE STRAND & STAND CURVE IN HUMAN URINE (LLOQ @ 2 NG/ML)

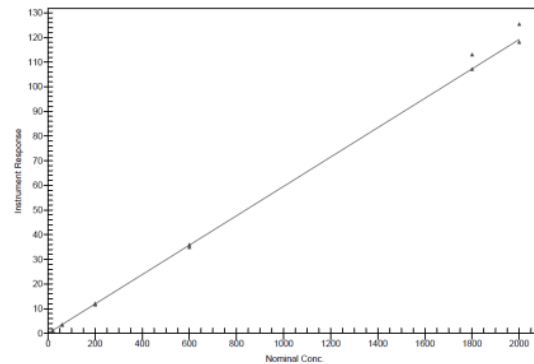
ASO



ASO IS



Run Date	Curve Number	Slope	Intercept	R-Squared
xx-xxx-xxxx	2 ^a	0.052502951	0.028351613	0.9980
xx-xxx-xxxx	3 ^a	0.054957681	0.018927791	0.9708
xx-xxx-xxxx	4	0.059605645	0.007871027	0.9988
xx-xxx-xxxx	6	0.058372671	0.004483040	0.9967
xx-xxx-xxxx	7	0.054106424	0.007662517	0.9977
xx-xxx-xxxx	8	0.050945421	0.023371753	0.9882
Mean		0.05575754	0.010847084	0.9954
S.D.		0.003980347	0.008492482	0.0048
%CV		7.1	78.3	0.5
n		4	4	4
Regression Formula(s):				
1) Resp. = Slope * Conc. + Intercept				
*Rejected run, not included in statistical calculations				





ASO (from an AOC) Quantitation by LC-MS/MS

EXAMPLE: BACK-CALCULATED CONCENTRATIONS (ng/mL) & INTRADAY PRECISIONS & ACCURACY

Run Date	Run No.	2.00	4.00	20.0	60.0	200	600	1800	2000
XX-XXX-XXXX	2 ^a	2.02	3.76	20.8	62.1	201	625	1860	2110
		2.06	3.96	19.5	54.6	198	593	1790	1940
XX-XXX-XXXX	3 ^a	2.13	3.24	23.9	53.0	235	526	1580	1800
		2.15	3.28	25.5	56.8	250	586	1640	1930
XX-XXX-XXXX	4	2.04	4.07	19.0	57.8	196	587	1800	1980
		1.93	4.11	20.3	58.5	202	603	1900	2100
XX-XXX-XXXX	6	1.86	3.79	19.6	59.9	192	588	1720	2000
		2.14	4.13	22.0	64.9	199	563	1860	2000
XX-XXX-XXXX	7	1.93	3.58	19.1	60.8	207	597	1800	1900
		2.14	4.16	20.8	59.8	200	611	1840	2010
Mean		2.01	3.97	20.1	60.3	199	592	1820	2000
S.D.		0.118	0.235	1.15	2.50	5.13	16.7	62.0	64.0
%CV		5.9	5.9	5.7	4.1	2.6	2.8	3.4	3.2
%RE		0.5	-0.8	0.5	0.5	-0.5	-1.3	1.1	0.0
n		6	6	6	6	6	6	6	6

^a Rejected run, not included in statistical calculations

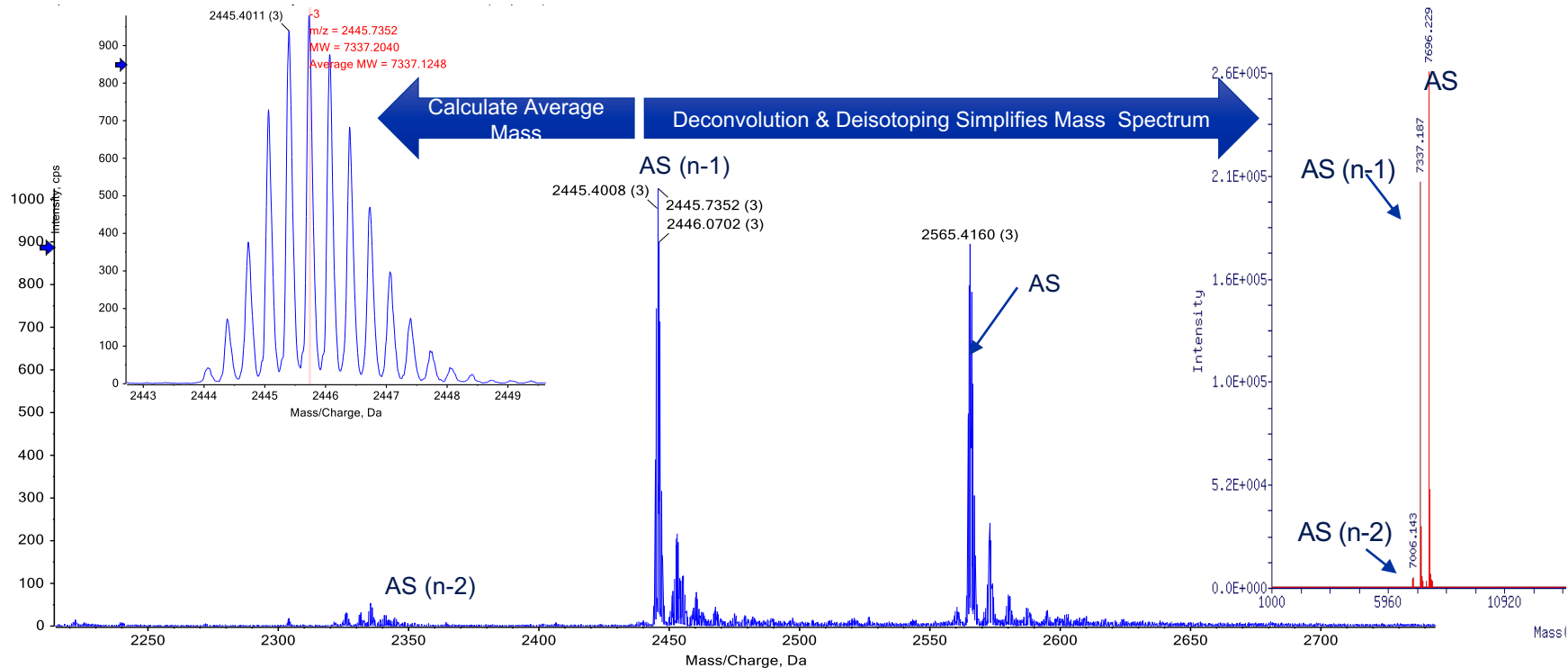
Note: No regression performed in Runs 1 and 5

Run Date	Run No.	2.00 (ng/mL)	6.00 (ng/mL)	80.0 (ng/mL)	800 (ng/mL)	1600 (ng/mL)
XX-XXX-XXXX	4	2.03	5.71	73.5	775	1530
		1.81	5.68	78.4	770	1520
		2.34	5.75	73.8	771	1490
		1.83	5.67	84.7	744	1550
		1.76	5.45	74.5	775	1560
		2.08	5.54	75.8	741	1510
Intra-run Mean		1.98	5.63	76.8	763	1530
S.D.		0.220	0.114	4.27	15.8	25.8
%CV		11.1	2.0	5.6	2.1	1.7
%RE		-1.0	-6.2	-4.0	-4.6	-4.4
n		6	6	6	6	6



siRNA Quantitation by LC-QToF-MS

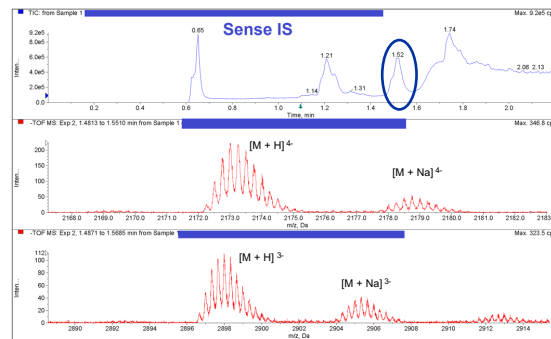
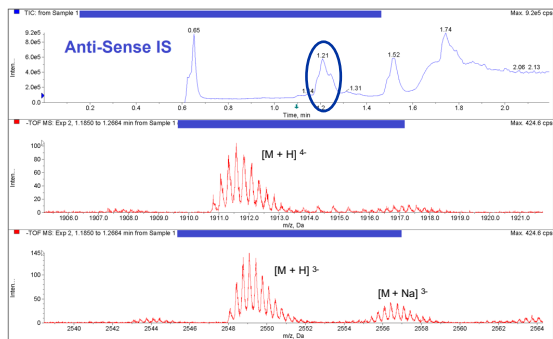
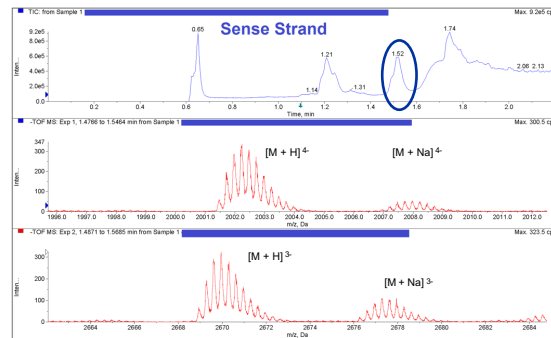
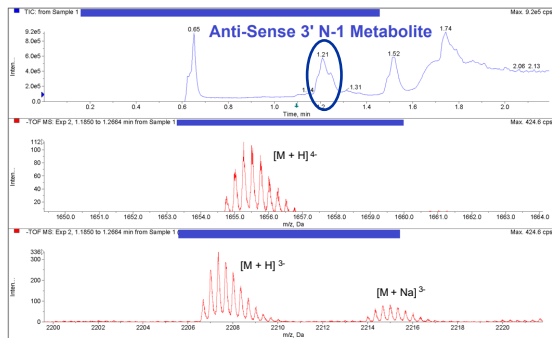
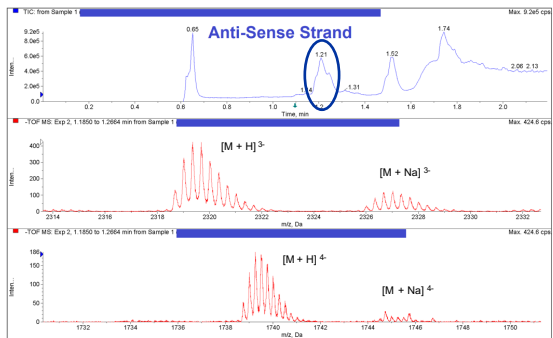
EXAMPLE: OLIGONUCLEOTIDES NEGATIVE ESI MASS SPECTRUM SHOWING AS³⁻ (PARENT, N-1, N-2)





siRNA Quantitation by LC-QToF-MS

EXAMPLE: TIC AND CORRESPONDING MASS SPECTRA OF ANTI-SENSE, AS (N-1), AND SENSE STRANDS IN HUMAN PLASMA





siRNA Quantitation by LC-QToF-MS

EXAMPLE: STANDARD CURVES OF ANTI-SENSE, AS (N-1), AS (N-2), AND SENSE STRANDS IN HUMAN PLASMA (LLOQ @ 5 ng/mL AND 1 ng/mL)

Calibration Curve Parameters for Anti-Sense Strand Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000006	0.003819670	-0.011560698	0.9951	1
Day 2	30	-0.00000012	0.004052494	-0.009053454	0.9966	1
Day 3	34	-0.00000014	0.003581962	-0.002954172	0.9969	1
Day 4	37	-0.00000010	0.004465860	0.000321158	0.9924	1
Day 5	38	-0.00000016	0.003670701	-0.004392901	0.9926	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

Calibration Curve Parameters for Anti-Sense 3' N-1 Metabolite Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000104	0.010317655	-0.005277962	0.9922	1
Day 2	30	-0.00000044	0.009893031	-0.002362463	0.9963	1
Day 3	34	-0.00000208	0.010450719	-0.002749375	0.9956	1
Day 4	37	-0.00000165	0.007991487	0.004228573	0.9935	1
Day 5	38	-0.00000313	0.012478697	-0.002909850	0.9922	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

Calibration Curve Parameters for Anti-Sense 3' N-2 Metabolite Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000157	0.012459182	-0.007114476	0.9933	1
Day 2	30	-0.00000157	0.011743405	-0.004888467	0.9912	1
Day 3	34	-0.00000395	0.012705437	-0.002720330	0.9959	1
Day 4	37	-0.00000329	0.011105167	-0.002518838	0.9942	1
Day 5	38	-0.00000415	0.014417882	-0.002621136	0.9948	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

1) Resp. = A * (Conc.**2) + B * Conc. + C

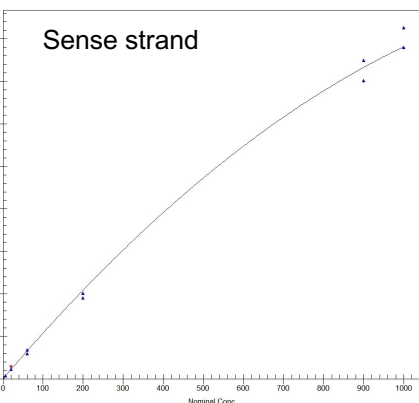
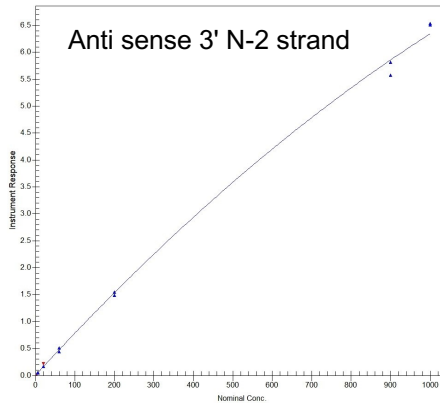
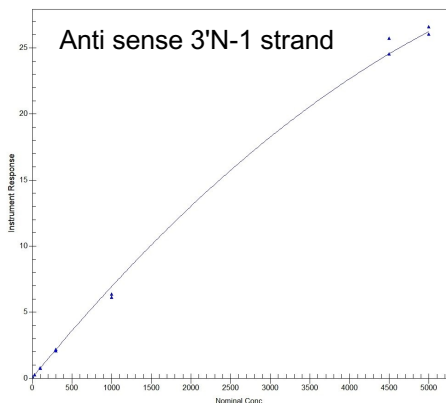
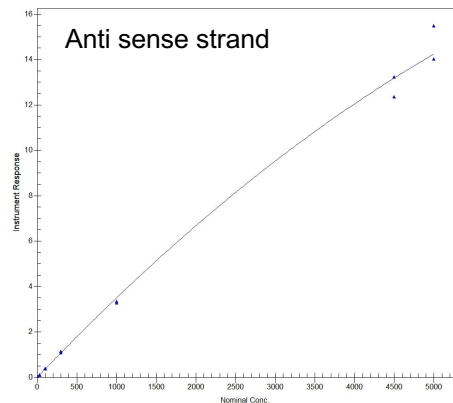
Calibration Curve Parameters for Sense Strand Calibration Standards in Human Plasma by LC-TOF-MS

Run Date	Run ID	A	B	C	R-Squared	Regression Footnote(s)
Day 1	36	-0.00000014	0.004089592	-0.007317355	0.9919	1
Day 2	30	-0.00000025	0.004474451	-0.003289805	0.9950	1
Day 3	34	-0.00000025	0.003762272	-0.003002574	0.9934	1
Day 4	37	-0.00000042	0.007331004	0.001793958	0.9923	1
Day 5	38	-0.00000159	0.004356572	-0.004784018	0.9964	1

Quadratic Regression with $1/x^2$ Weighting

Regression Footnote(s):

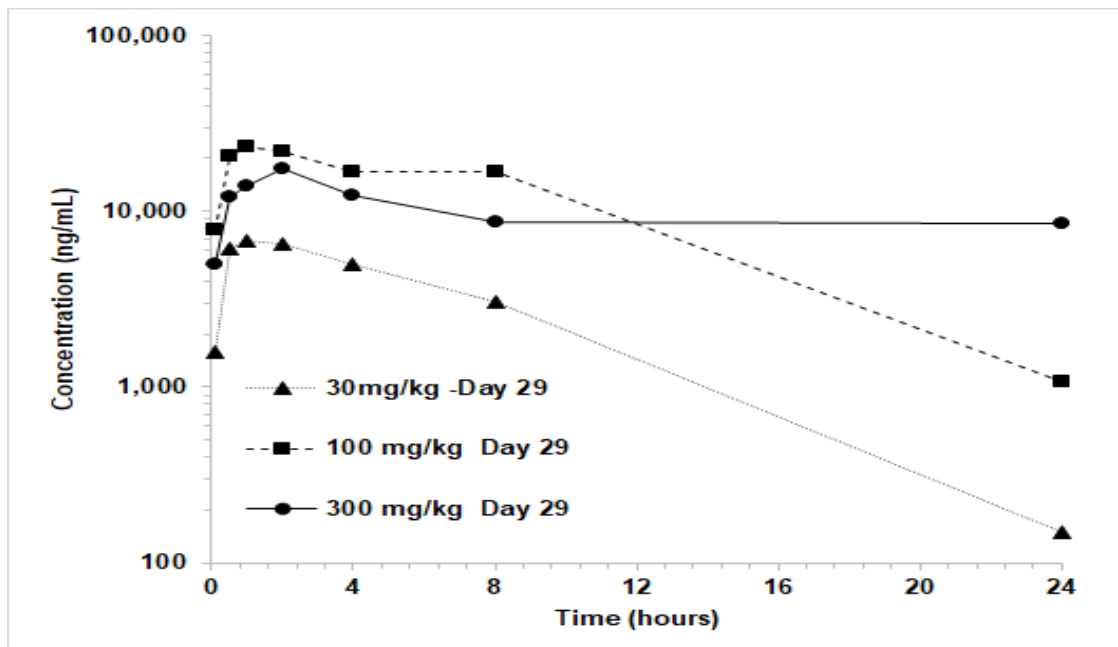
1) Resp. = A * (Conc.**2) + B * Conc. + C





Oligonucleotides Quantitation by LC-HRMS

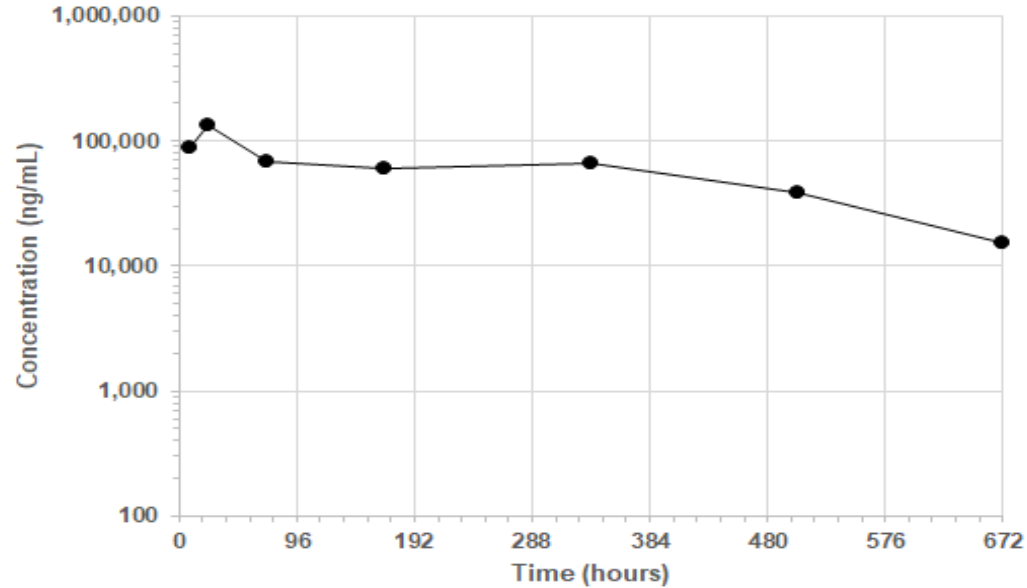
PLASMA CONCENTRATION IN NHP FOLLOWING SC ADMINISTRATION OF 30, 100, AND 300 MG/KG DOSE





Oligonucleotides Quantitation by LC-HRMS

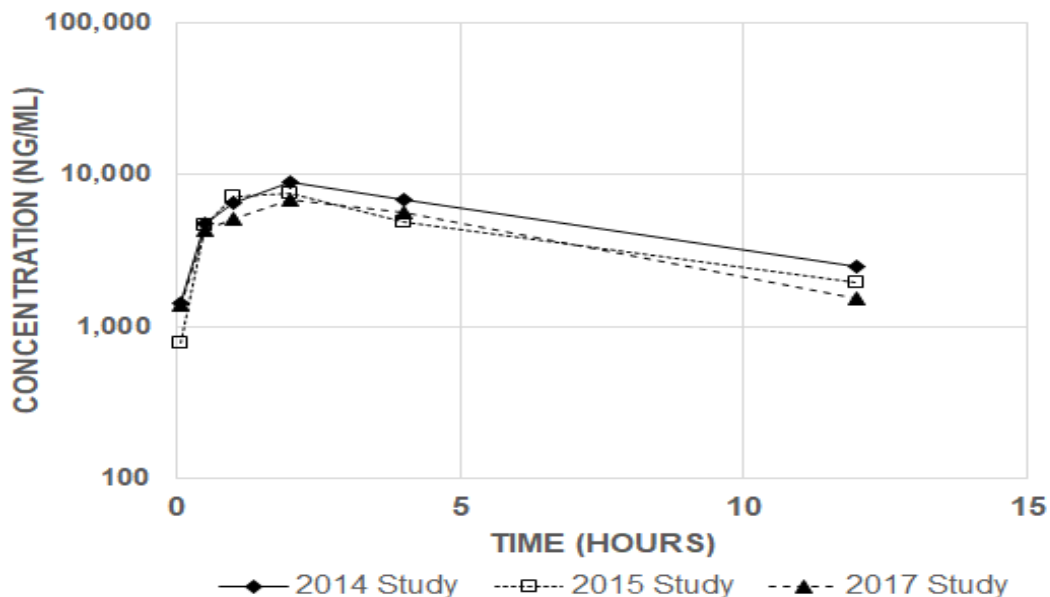
MEAN LIVER CONCENTRATION IN NHP FOLLOWING SC ADMINISTRATION OF 10 MG/KG DOSE





Oligonucleotides Quantitation by LC-HRMS

MEAN PLASMA CONCENTRATION IN NHP SHOWING REPRODUCIBILITY OVER A PERIOD OF 3 YEARS





Oligonucleotides Quantitation by LC-HRMS vs. LC-MS/MS

PROCEDURE: LC-MS (HRMS OR MS/MS) METHOD

▶ Calibration Curve

- ◆ Standalone curve vs. Standalone curve: depends on the purpose and matrix availability
- ◆ Part of method development process; whatever matrix that provide the best surrogate curve

▶ Sample Preparation

- ◆ Feces and tissues need homogenization followed by lysis
- ◆ Extraction: Recovery: $\geq 70\% \leftrightarrow \leq 95\%$

▶ Chromatography

- ◆ Run-time usually ≤ 5 min injection-to-injection
- ◆ Column
 - C18 2.1 mm x 50 mm 1.7 μm 130Å fully porous
 - Column life: 300 – 400 injections
 - 300Å for specific applications, e.g., AOC / POC

▶ Mass Spectrometry

- ◆ IS: Analogue or Stable Isotopically Labeled
 - *Analyte AS & S & analogue AS & S*
 - *Cation adducts:*
 - $\text{H}_n\text{Na}_0\text{K}_0 \rightarrow \text{H}_{n-1}\text{Na}_1\text{K}_0$; $\text{H}_{n-1}\text{Na}_0\text{K}_1 \rightarrow \text{H}_{n-2}\text{Na}_2\text{K}_0$; $\text{H}_{n-2}\text{Na}_1\text{K}_1$; $\text{H}_{n-2}\text{Na}_0\text{K}_2 \rightarrow \dots$
 - *Analogue IS: Potential overlapping isotopic mass*
 - *SIL-IS: expensive, control isotopic overlap?*
- ◆ Mass Resolution (Theoretic vs. Operating)
 - *Triple Quad (unit resolution)*
 - *QTOF (uniform resolution; $\sim 40\text{K}$) vs. Ion trap (mass dependent; $\geq 120\text{K}$ @ 200 amu)*
 - *UPLC peak width @ 3-6 seconds; with 12+ data-point operating resolution @ $\sim 35\text{K}$*



Oligonucleotides Quantitation by LC-HRMS or LC-MS/MS

PROCEDURE: LC-MS (HRMS OR MS/MS) METHOD

▶ Tripe Quadrupoles vs. QTOF

- ◆ -ve ion mode
- ◆ Assay range: 3 order
- ◆ LC-MS/MS (similar to peptides MS/MS)
 - Q1 (@ higher charge envelope, e.g., M^9) → Q3 (@ lower charge envelope, mostly <400 m/z)
- ◆ Full scan LC-HRMS
 - *Quan and Qual*
 - *Sum multiple isotopic mass @ lower charge state, e.g., M^4 and M^3*
- ◆ Data Intensity: two 96-well plates: 2MB vs. 3 GB
- ◆ LLOQ:
 - ≤ 2 ng/mL (QqQ) vs. ≤ 5 ng/mL (HRMS)
 - Stoichiometry: nM double strand ~ single strand
 - Stoichiometry: ng/mL double strand ≠ single strand
 - 100 μ L sample volume, 100 μ L recon, 3 μ L – 5 μ L injection
 - Sub-1 ng/mL (if higher sample volume, lower recon, higher volume injection)

▶ Why choose Tripe Quadrupoles

- ◆ Well known/established metabolites (per MIST guidance)
- ◆ Analogue or SIL IS has little or no to cross-talk
- ◆ Desired LLOQ @ sub-1 ng/mL

▶ Why choose QTOF

- ◆ No metabolism data available
- ◆ Analogue or SIL IS potential cross-talk
- ◆ Desired LLOQ @ 1 ng/mL

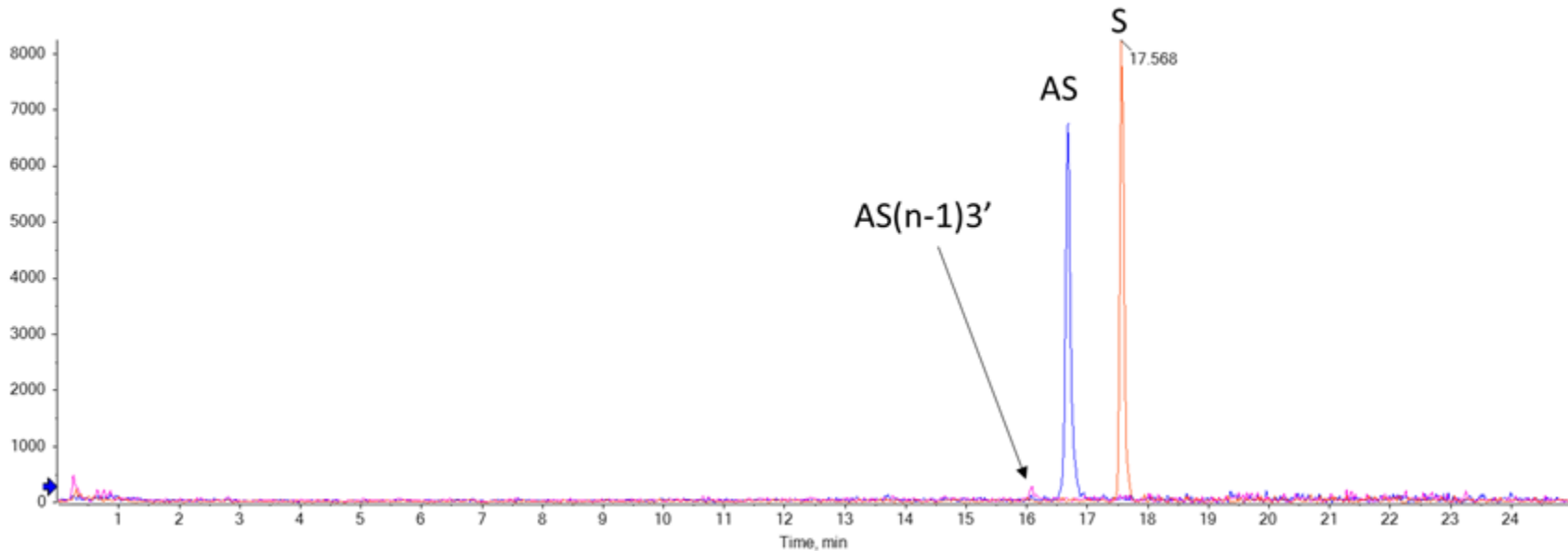
▶ Preliminary experiment

- ◆ Well-established historical metabolism data
- ◆ *In vitro* metabolism (simple 'well-stirred' hepatic model vs. more complexed long-term co-culture model)
- ◆ Confirm *in vivo* rat/NHP vs. *in vitro* metabolism data



siRNA Quantitation by LC-QToF-MS: Power of LC-HRMS

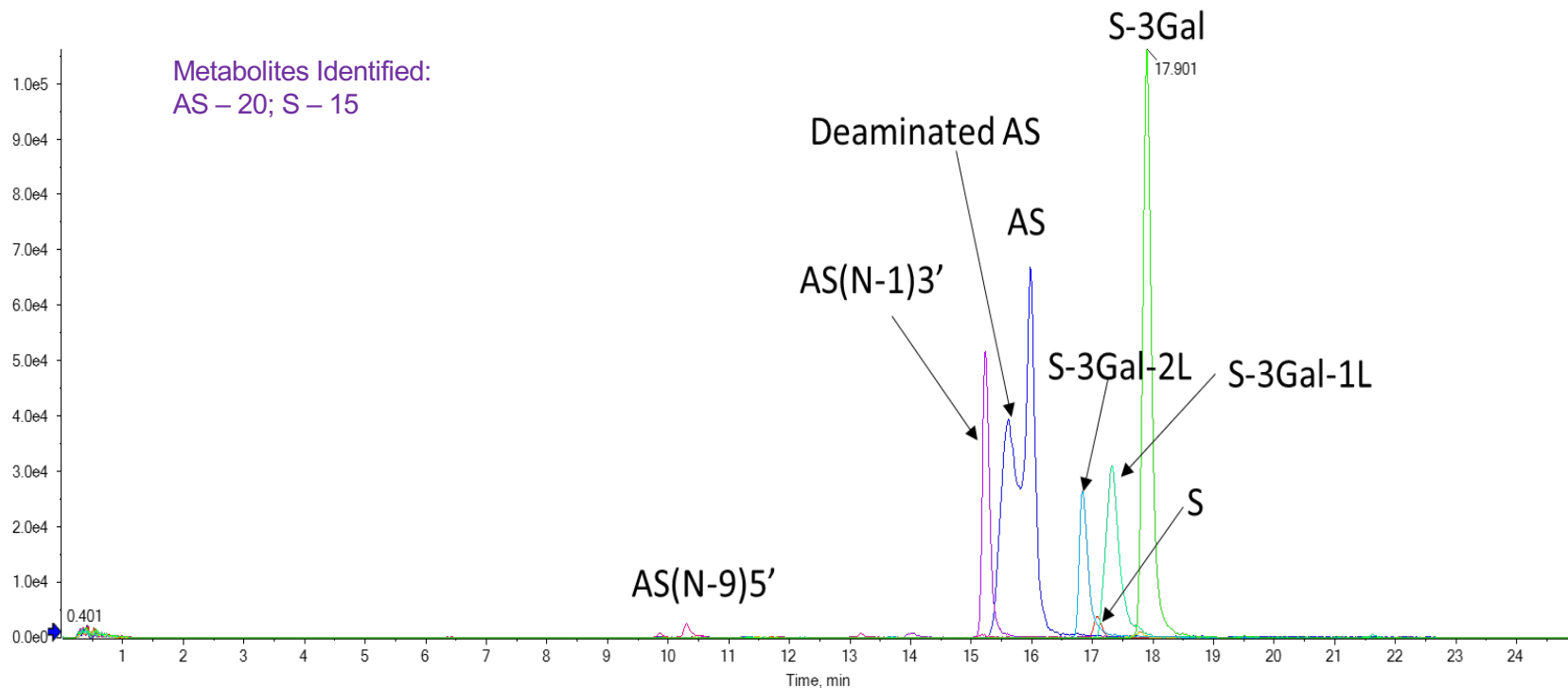
EXAMPLE: RECONSTRUCTED XIC OF siRNA IN PLASMA SAMPLES





siRNA Quantitation by LC-QToF-MS: Power of LC-HRMS

EXAMPLE: RECONSTRUCTED XIC OF siRNA IN LIVER SAMPLES (METABOLISM VIA OXIDATIVE DEAMINATION)





Oligonucleotides Quantitation by LC-HRMS or LC-MS/MS

ACKNOWLEDGEMENT

▶ QPS Delaware, USA

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