High-Sensitivity Workflow for LC-MS Based Analysis of Galnac-Conjugated Oligonucleotides

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Oligonucleotides

Have become popular as drug development candidates due in large part to the (potential) ability to target a single genetic pathway with relatively high specificity.

Oligonucleotides

Primary Challenges and Critical Issues for Use of Oligos as Drug Candidates can include:

Relatively unstable in vivo/in vitro	Highly protein bound (especially for PT-modified)	Very polar	Fragmentation can be a challenge (for MS-based assays)

- Can be addressed via modifications of the structure, for example phosphorothioate (PT) modification, in which a non-bridging oxygen in the phosphodiester linkage is replaced by sulfur.

Oligonucleotides: Platforms

Advances in quantitative bioanalysis of oligonucleotide biomarkers and therapeutics

Technical advances and demands for high-throughput accurate quantification of oligonucleotide therapeutics and biomarkers in pharmaceutical research and clinical diagnosis have aided evolution in quantitative bioanalysis of oligonucleotides. Many bioanalytical methods are available for absolute quantification of oligonucleotides in biological matrices. They can be broadly classified into two categories: hybridization-based assays commonly used by molecular biologists and chromatographic assays

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Oligonucleotides: Platforms

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Technical advances and demands for high-throughput accurate quantification of oligonucleotide therapeutics and biomarkers in pharmaceutical research and clinical diagnosis have aided evolution in quantitative bioanalysis of oligonucleotides. Many bioanalytical methods are available for absolute quantification of oligonucleotides in biological matrices. They can be broadly classified into two categories: hybridization-based assays commonly used by molecular biologists and chromatographic assays routinely used by chemists. Each category has its own advantages and disadvantages for specific applications. This review summarizes the mechanisms and applications of some of the current most commonly used techniques in each category.

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Hybridization-Based Approaches

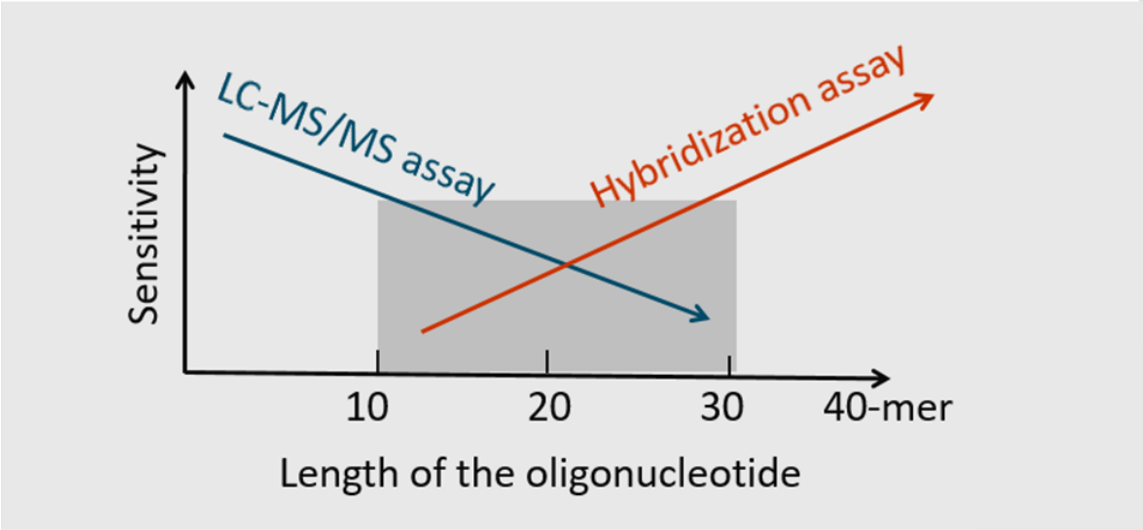
qPCR (many variations)

- Most effective for long sequences

Hybridization LC-fluorescence

- Also more effective for longer sequences; some susceptibility to interference from n-1/n-2 metabolites; may require LC separation of these metabolites

Which Platform?



- LC-MS is good for smaller oligonucleotides; typical LLOQ of ~5 ng/mL
- Hybridization assays are good for larger oligonucleotides; LLOQ sub ng/mL

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Chromatographic-Based Approaches

LC-MS
(triple quadrupole-based MS detection)

LC-MS
(HRMS-based MS detection)

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Mass Spectrometry; Platform to Use?

Triple Quad

Advantages

- Superior absolute sensitivity
- Straightforward data processing

Drawbacks

- Need to fragment; can be difficult to locate fragment ion having sufficient selectivity AND sensitivity
- Need for highly optimized extraction
- Potential need for shallow gradient

HRMS (Q-Exactive or TOF)

Advantages

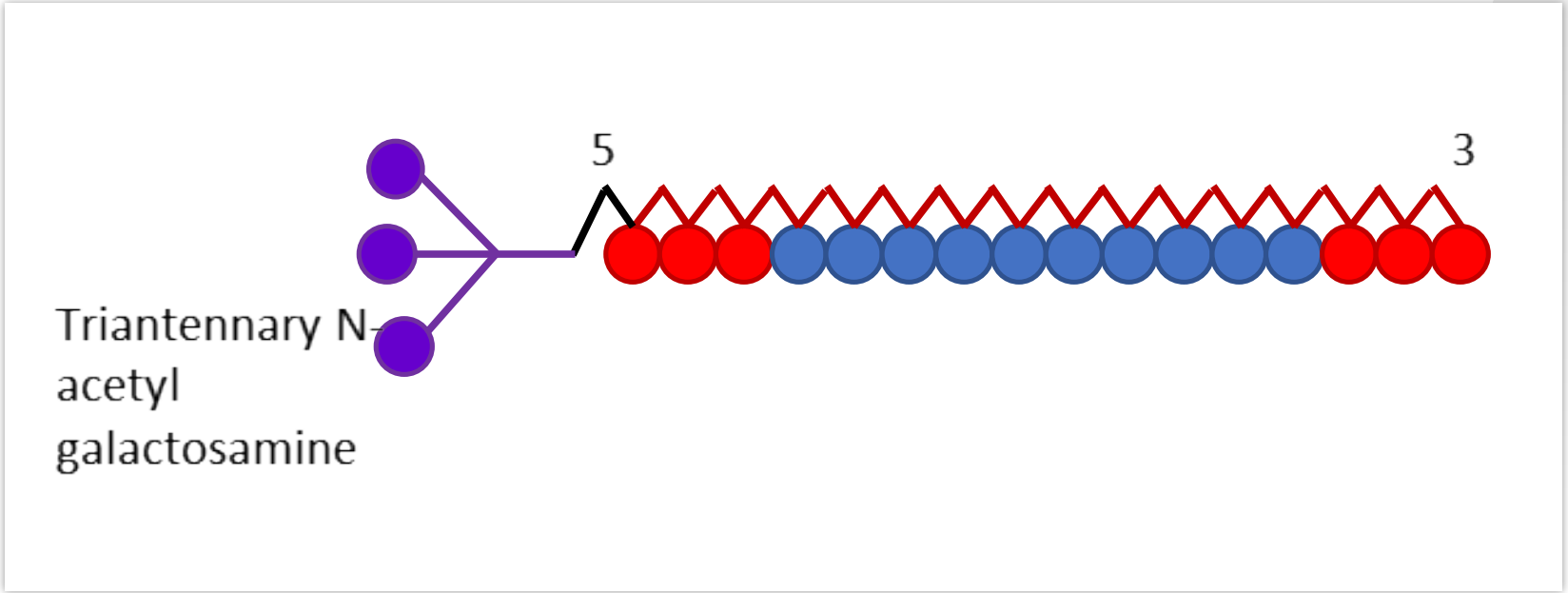
- High Selectivity (no need to fragment)
- Potential for retrospective data analysis

Drawbacks

- Complex data processing
- Additional MS parameters to consider (e.g., AGC target, maximum injection times)

A Test Case: The Analyte

AZD8233; a GalNAc-conjugated Anti-sense Oligonucleotide (16-mer)



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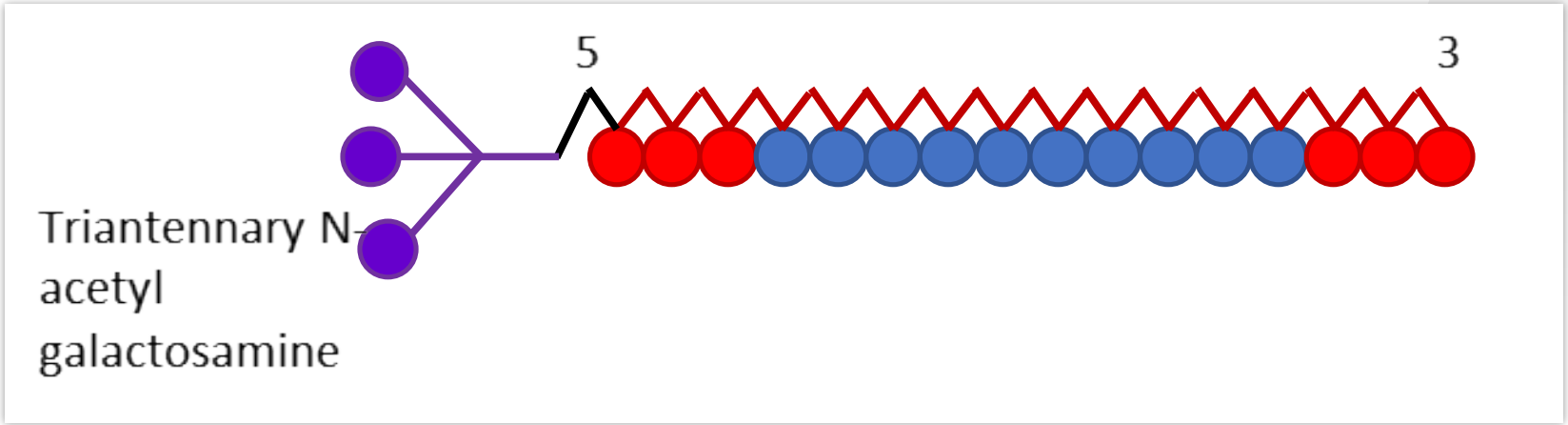
A Test Case: The Analyte

AZD8233: A GalNAc-Conjugated Oligonucleotide (16-mer)

- Need to be able to selectively measure GalNAc conjugated forms of the oligonucleotide, including in the presence of the non-conjugated form.
- LLOQ of 0.1 ng/mL requested



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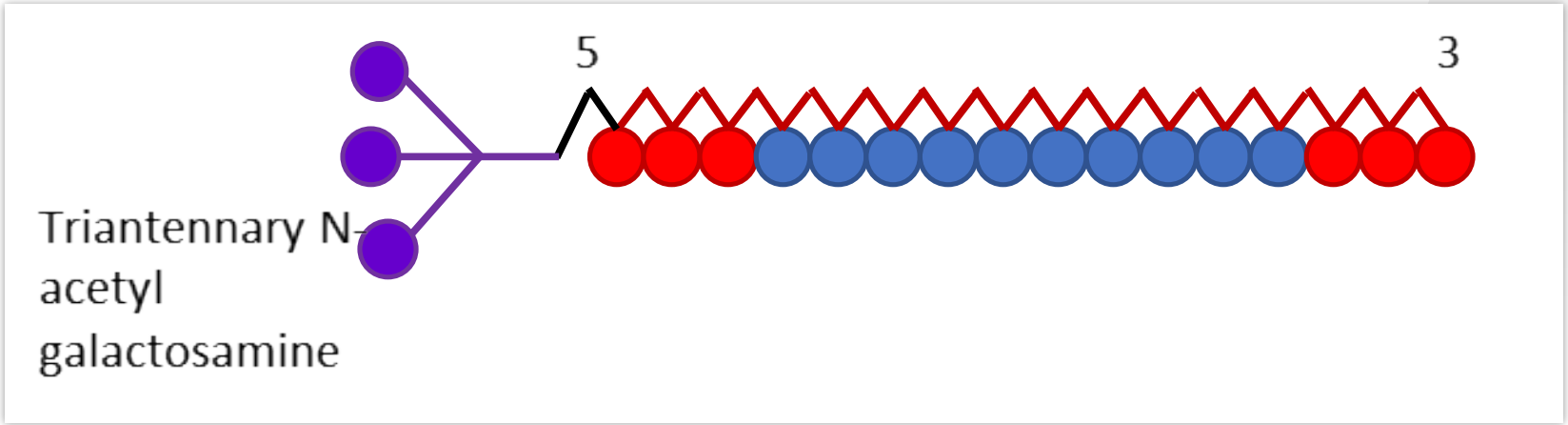
A Test Case: The Analyte

AZD8233: A GalNAc-Conjugated Oligonucleotide (16-mer)

- Need to be able to differentiate GalNAc conjugated vs. non-GalNAc conjugated forms of AZD8233
- **LLOQ of 0.1 ng/mL requested**



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Strategy

In order to obtain sufficient signal to achieve sub-ng/mL LLOQ, we made the determination to utilize an API 6500+ mass spectrometer, operating in negative ESI mode.

In order to achieve sufficient sensitivity, phosphorothioate and phosphite fragment ions (m/z 95 and 79 respectively) were optimized/evaluated. Ultimately, only these fragment ions offer sufficient sensitivity, although both resulted in relatively high baseline/interference peaks.

Additionally, the need for a fairly high aliquot volume (250 µL) resulted in relatively large concentration of extracted matrix components.

To mitigate high background, a combination of a fairly thorough sample cleanup (LLE-SPE) as well as a shallow/optimized LC gradient was needed.

Extraction

BSA Used to Mitigate Non-Specific Binding

1) Aliquot 250 µL plasma.
2) **Add working internal standard (diluent used was BSA [20 mg/mL]:DMF (9:1)).**
3) Vortex-mix thoroughly.
4) Add dilute ammonium hydroxide to each well.
5) Vortex-mix thoroughly.
6) Add phenol/chloroform/isoamyl alcohol mixture to each well.
7) Vortex-mix thoroughly.
8) Centrifuge the plate.
9) Transfer top (aqueous) layer to a clean, pre-labeled plate.
10) Dilute with water:HFIP:DIPEA mixture.
12) Equilibrate SPE plate (C18 chemistry).
13) Load sample to the HLB plate.
14) Wash each well with water:HFIP:DIPEA mixture.
15) Elute each well with Acetonitrile:Water:TEA mixture.
16) Dry under nitrogen at 50 C (total dry-down time ~45 minutes).
17) Reconstitute each well in mixture mimicking initial LC conditions.

Extraction

Added to Ensure AZD8233 Molecules are Charged Prior to Next Step

1) Aliquot 250 µL plasma.
2) Add working internal standard (diluent used was BSA [20 mg/mL]:DMF (9:1)).
3) Vortex-mix thoroughly.
4) **Add dilute ammonium hydroxide to each well.**
5) Vortex-mix thoroughly.
6) Add phenol/chloroform/isoamyl alcohol mixture to each well.
7) Vortex-mix thoroughly.
8) Centrifuge the plate.
9) Transfer top (aqueous) layer to a clean, pre-labeled plate.
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13) Load sample to the HLB plate.
14) Wash each well with water:HFIP:DIPEA mixture.
15) Elute each well with Acetonitrile:Water:TEA mixture.
16) Dry under nitrogen at 50 C (total dry-down time ~45 minutes).
17) Reconstitute each well in mixture mimicking initial LC conditions.

Extraction

Needed to Disrupt Extensive Protein Binding and Ensure Good Recovery

1) Aliquot 250 µL plasma.
2) Add working internal standard (diluent used was BSA [20 mg/mL]:DMF (9:1)).
3) Vortex-mix thoroughly.
4) Add dilute ammonium hydroxide to each well.
5) Vortex-mix thoroughly.
6) **Add phenol/chloroform/isoamyl alcohol mixture to each well.**
7) Vortex-mix thoroughly.
8) Centrifuge the plate.
9) Transfer top (aqueous) layer to a clean, pre-labeled plate.
10) Dilute with water:HFIP:DIPEA mixture.
12) Equilibrate SPE plate (C18 chemistry).
13) Load sample to the HLB plate.
14) Wash each well with water:HFIP:DIPEA mixture.
15) Elute each well with Acetonitrile:Water:TEA mixture.
16) Dry under nitrogen at 50 C (total dry-down time ~45 minutes).
17) Reconstitute each well in mixture mimicking initial LC conditions.

Extraction

Addition of Ion-Pairing Reagent to Ensure Adequate Retention of AZD8233 During Subsequent SPE

1) Aliquot 250 µL plasma.
2) Add working internal standard (diluent used was BSA [20 mg/mL]:DMF (9:1)).
3) Vortex-mix thoroughly.
4) Add dilute ammonium hydroxide to each well.
5) Vortex-mix thoroughly.
6) Add phenol/chloroform/isoamyl alcohol mixture to each well.
7) Vortex-mix thoroughly.
8) Centrifuge the plate.
9) Transfer top (aqueous) layer to a clean, pre-labeled plate.
10) **Dilute with water:HFIP:DIPEA mixture.**
12) Equilibrate SPE plate (C18 chemistry).
13) Load sample to the HLB plate.
14) Wash each well with water:HFIP:DIPEA mixture.
15) Elute each well with Acetonitrile:Water:TEA mixture.
16) Dry under nitrogen at 50 C (total dry-down time ~45 minutes).
17) Reconstitute each well in mixture mimicking initial LC conditions.

Extraction

SPE Cleanup

1) Aliquot 250 µL plasma.
2) Add working internal standard (diluent used was BSA [20 mg/mL]:DMF (9:1)).
3) Vortex-mix thoroughly.
4) Add dilute ammonium hydroxide to each well.
5) Vortex-mix thoroughly.
6) Add phenol/chloroform/isoamyl alcohol mixture to each well.
7) Vortex-mix thoroughly.
8) Centrifuge the plate.
9) Transfer top (aqueous) layer to a clean, pre-labeled plate.
10) Dilute with water:HFIP:DIPEA mixture.
12) **Equilibrate SPE plate (C18 chemistry).**
13) **Load sample to the HLB plate.**
14) **Wash each well with water:HFIP:DIPEA mixture.**
15) **Elute each well with Acetonitrile:Water:TEA mixture.**
16) Dry under nitrogen at 50 C (total dry-down time ~45 minutes).
17) Reconstitute each well in mixture mimicking initial LC conditions.

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LC Conditions

- Mobile Phase A: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: water (2.5: 0.25: 1.25: 100)
- Mobile Phase B: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: methanol (2.5: 0.25: 1.25: 100)

- Port wash: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: methanol: water (2.5: 0.25: 1.25: 10: 90)
- Pump wash: Acetonitrile: Water: TEA (60:40:1)

- Column: Waters BEH Acquity C18, 2.1 x 50 mm, 1.7 um particle size
- Flow rate: 0.30 mL/min
- Column T: 60 C

- Gradient (initial %B is 10%; initial flow rate is 0.300 mL/min):

Time Program

Time	Module	Events	Parameter
3.00	Pumps	Pump B Conc.	50
3.01	Pumps	Total Flow	0.30
3.05	Pumps	Pump B Conc.	55
3.10	Pumps	Total Flow	0.50
3.60	Pumps	Pump B Conc.	55
3.80	Pumps	Pump B Conc.	95
3.90	Autosampler	Rinse	
4.50	Pumps	Pump B Conc.	95
4.75	Pumps	Pump B Conc.	10
5.05	Pumps	Total Flow	0.50
5.10	Pumps	Total Flow	0.30
5.40	System Controller	Stop	

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LC Conditions

- Mobile Phase A: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: water (2.5: 0.25: 1.25: 100)
- Mobile Phase B: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: methanol (2.5: 0.25: 1.25: 100)

- Port wash: [EDTA 1 mM (aq.):TEA, 100:1]: TEA: HFIP: methanol: water (2.5: 0.25: 1.25: 10: 90)
- Pump wash: Acetonitrile: Water: TEA (60:40:1)

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Time Program

Time	Module	Events	Parameter
3.00	Pumps	Pump B Conc.	50
3.01	Pumps	Total Flow	0.30
3.05	Pumps	Pump B Conc.	55
3.10	Pumps	Total Flow	0.50
3.60	Pumps	Pump B Conc.	55
3.80	Pumps	Pump B Conc.	95
3.90	Autosampler	Rinse	
4.50	Pumps	Pump B Conc.	95
4.75	Pumps	Pump B Conc.	10
5.05	Pumps	Total Flow	0.50
5.10	Pumps	Total Flow	0.30
5.40	System Controller	Stop	

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MS Conditions

Source Conditions:

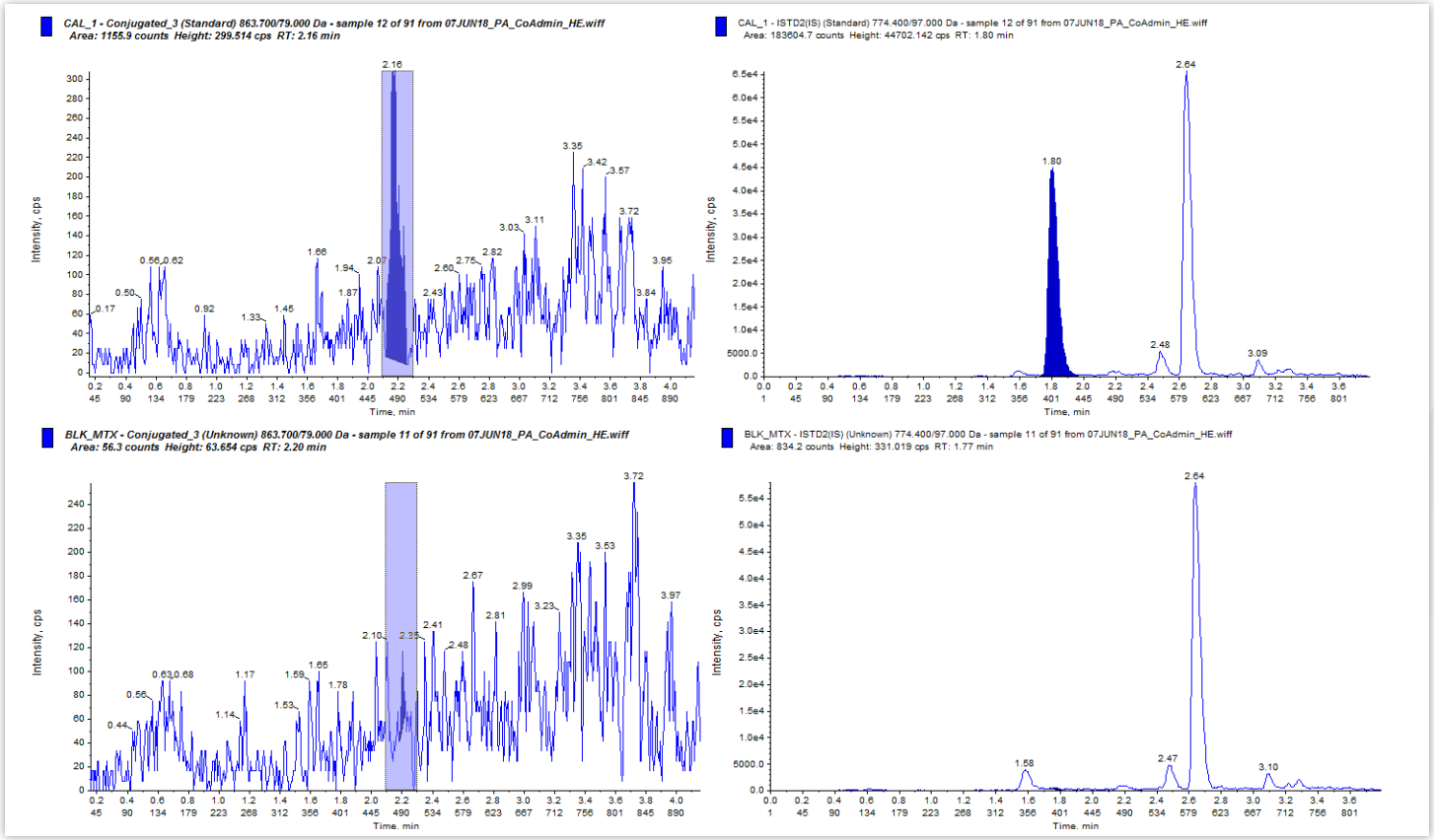
Parameter Table(Period 1 Experiment 1)	
CUR:	40.00
TEM:	500.00
GS1:	50.00
GS2:	55.00
CAD:	11.00
IS:	-4500.00
DP	-50.00
EP	-10.00
CE	-150.00

MRM Conditions:

Q1 Mass (Da)	Q3 Mass (Da)	Dwell(msec)	Param	Start	Stop	ID
863.700	79.000	60.00	CXP	-12.00	-12.00	AZD8233

Q1 Mass (Da)	Q3 Mass (Da)	Dwell(msec)	Param	Start	Stop	ID
774.400	97.000	40.00	CXP	-26.00	-26.00	ISTD1

LLOQ (Top) and Matrix Blank (Bottom)



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AZD8233 Precision/Accuracy:

Overall Linearity was Adequate. %CV is ~17% At LLOQ, ~13% at LQC, and 1-2% at HQC Level. The Low Variability at the HQC Level Indicates Good Overall Technical Reproducibility. The Higher Variability at Lower Levels is Rationalized as Being Due to Marginal S/N.

Sample	Conc. (ng/mL)	Accuracy	%CV
CAL-1 (n=2)	0.10	95.6	19.3
CAL-2 (n=2)	0.20	106.1	9.45
CAL-3 (n=2)	0.50	104.8	2.78
CAL-4 (n=2)	1.00	94.2	1.61
CAL-5 (n=2)	5.00	98.1	2.91
CAL-6 (n=2)	20.0	100.7	3.61
CAL-7 (n=2)	82.0	101.2	1.18
CAL-8 (n=2)	100	98.4	0.09
LLOQ (n=6)	0.10	100.0	17.4
LQC (n=6)	0.30	104.4	12.7
HQC (n=6)	80.0	99.9	1.67

Raised LLOQ to 0.20 to Ensure good ruggedness of the assay

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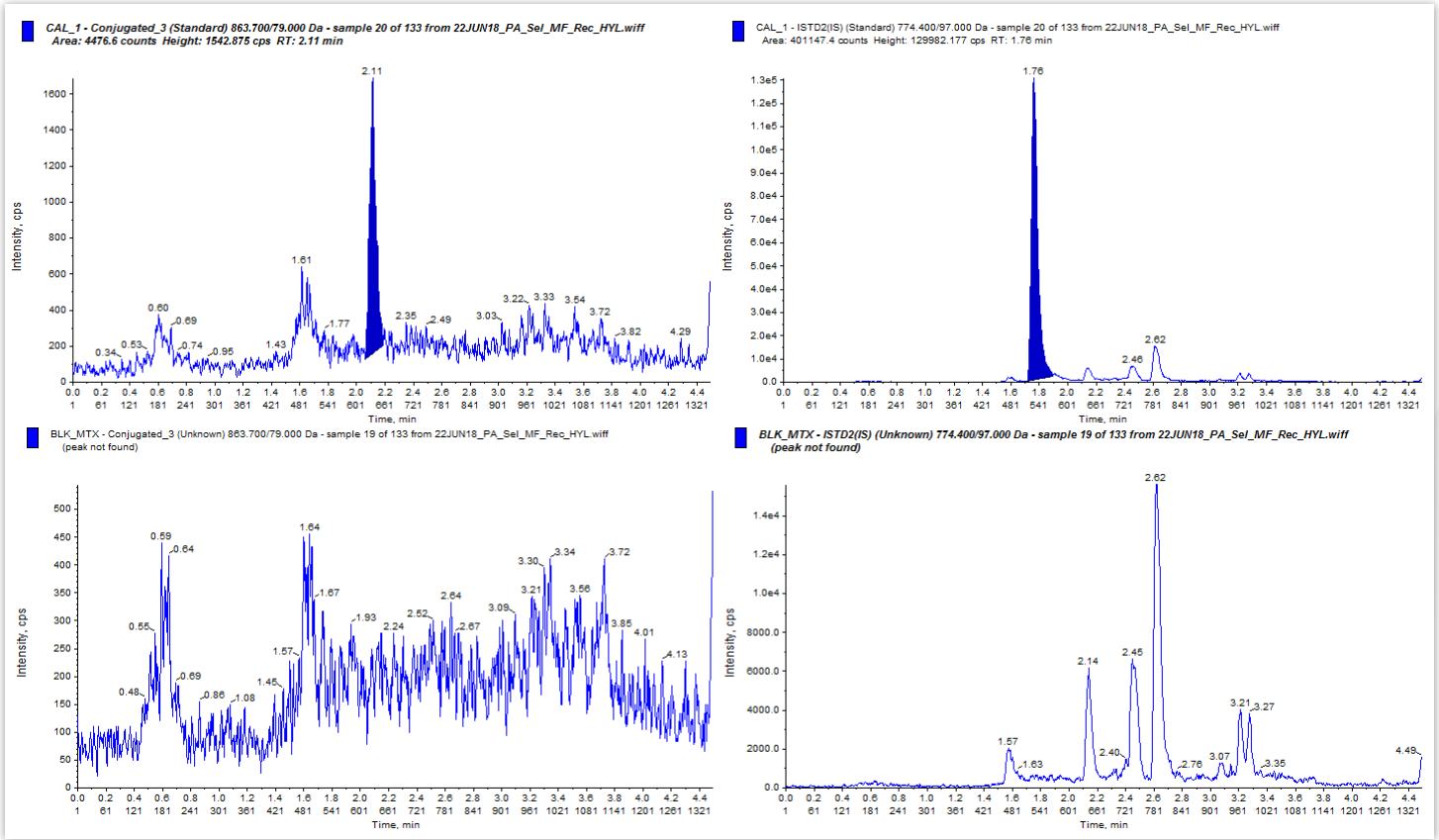
LLOQ (Top) and Matrix Blank (Bottom); Inject 30 µL



Overall S/N looks good.

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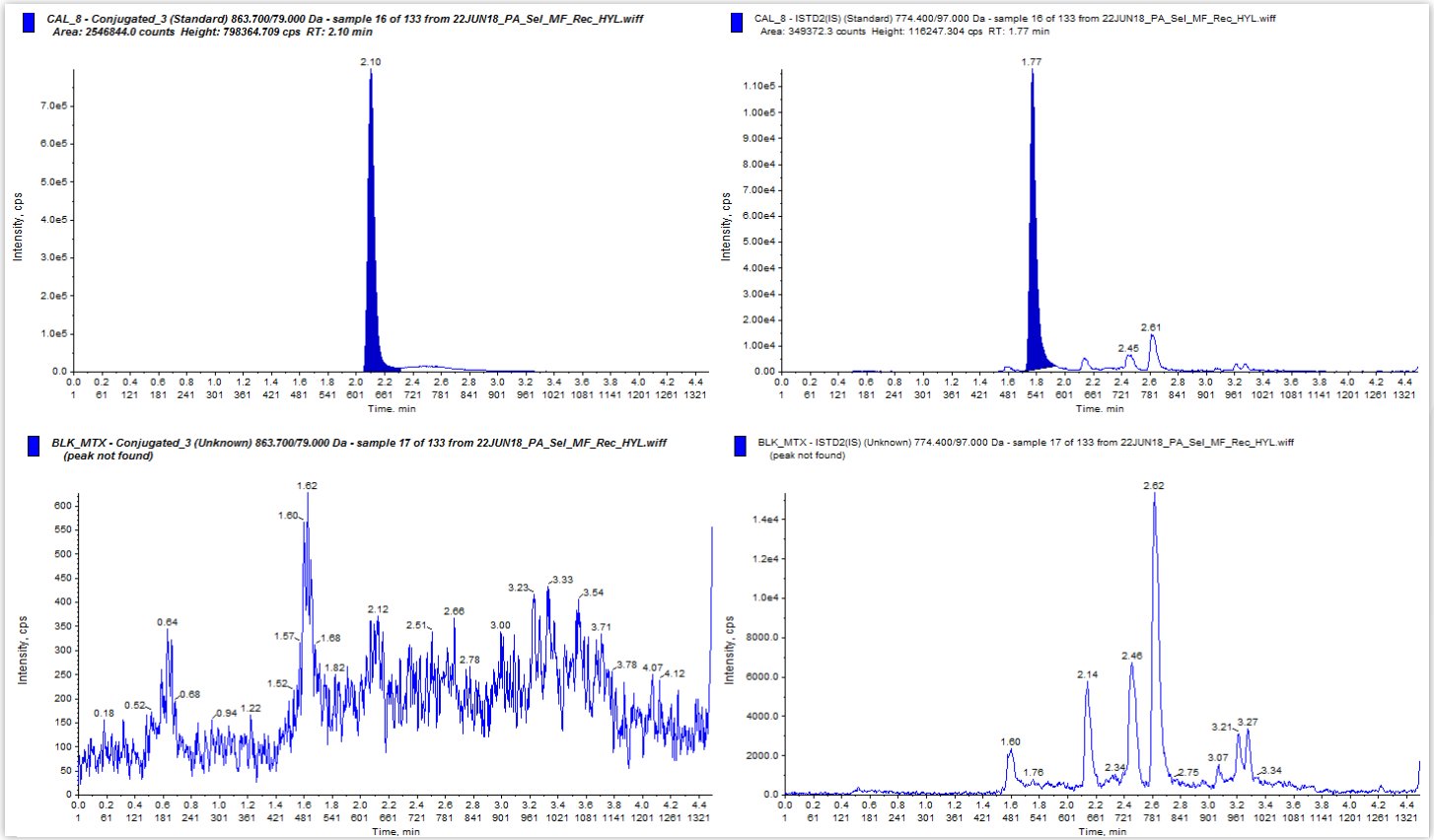
ULOQ (Top) and Matrix Blank Following ULOQ (Bottom); Inject 30 µL



Carryover of ~10-15% of LLOQ

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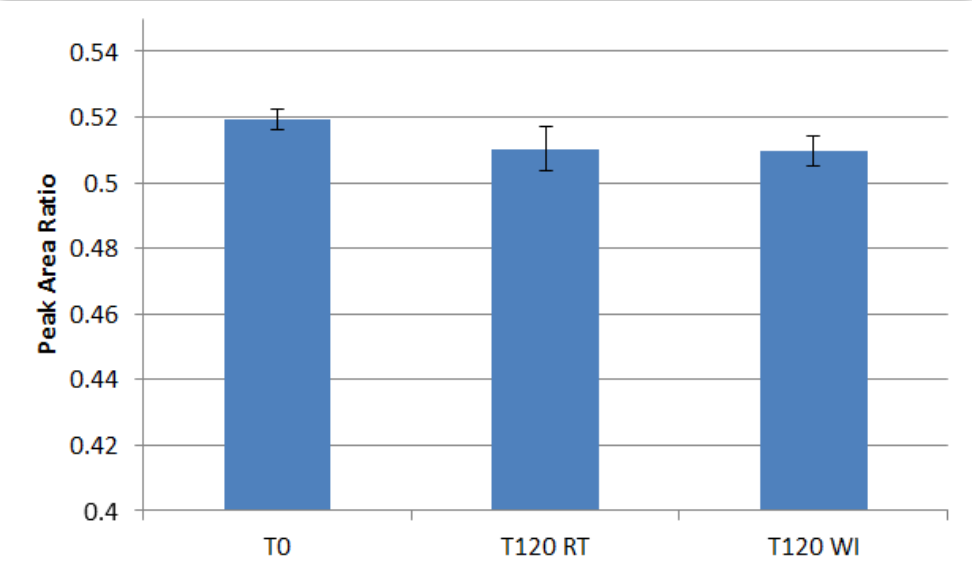
Precision/Accuracy

Precision/Accuracy Comfortably Meets Criteria

	LLOQ	LQC	LMQC	MQC	HQC	
Concentration (ng/mL)	0.20	0.60	5.00	50.0	80.0	
Accuracy (%)	102.0	109.0	102.0	100.0	104.4	PA Batch 1
Variability (%CV)	6.1	7.2	5.1	2.8	1.9	
Accuracy (%)	96.0	109.5	108.0	108.0	107.0	PA Batch 2
Variability (%CV)	2.7	2.6	2.4	3.0	2.8	
Accuracy (%)	100.8	102.5	98.0	95.8	97.6	PA Batch 3
Variability (%CV)	5.9	2.1	3.0	1.5	2.4	

Sample Collection (Whole Blood) Stability Looks Good

Both Room Temperature and Wet Ice Whole Blood Handling Looks Good Over at Least 2 Hours Ex Vivo



	Peak Area Ratio	%CV	% rel. to T0
T0	0.519	0.62	n/a
T120 RT	0.510	1.33	98.3
T120 WI	0.510	0.91	98.1

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Short-term Plasma Stability

AZD8233 Demonstrates Good Stability in Plasma Over Multiple FT Cycles and 24-Hour Room Temperature or Wet Ice Exposure

	Conc. (ng/mL)	%CV	Accuracy (%)
LQC (n=6)	0.294	2.49	98.0
LQC 24 hour RT (n=6)	0.299	3.86	99.7
LQC 24 hour WI (n=6)	0.302	1.83	100.5
LQC 4x FT (n=6)	0.289	3.07	96.5
HQC (n=6)	40.5	1.28	101.2
HQC 24 hour RT (n=6)	39.7	1.17	99.2
HQC 24 hour WI (n=6)	40.4	1.43	100.9
HQC 4x FT (n=6)	40.1	0.80	100.1

Selectivity

Individual LLOQ Samples (from Six Individual Lots) Look Similar to Batch LLOQ and All Pass

Sample	Conc. (ng/mL)	Accuracy (%)	%CV
Lot 1 (n=3)	0.1943	97.2	9.89
Lot 2 (n=3)	0.1900	95.0	3.68
Lot 3 (n=3)	0.1840	92.0	8.75
Lot 4 (n=3)	0.1937	96.8	5.47
Lot 5 (n=3)	0.2013	100.7	10.34
Lot 6 (n=3)	0.1737	86.8	3.83
Average	0.1895	94.7	5.07
Batch LLOQ	0.1897	94.8	8.38

Recovery

Recovery is Generally Consistent Across the Curve Range
Absolute Recovery of ~55-60%

	Analyte	ISTD
LQC (0.60 ng/mL)	61.6%	56.2%
MQC (50.0 ng/mL)	54.5%	52.1%
HQC (80.0 ng/mL)	56.7%	53.6%
Average	57.6%	54.0%

Matrix Factor

Matrix Factor Test Indicates Minimal Suppression in Matrix Extracts

	Analyte peak area	Analyte Matrix Factor	ISTD peak area	ISTD Matrix Factor
Lot 1	28,796	0.955	503,934	1.00
Lot 2	28,605	0.948	524,297	1.04
Lot 3	29,927	0.992	503,750	1.00
Lot 4	29,395	0.974	488,933	0.971
Lot 5	27,443	0.910	477,149	0.947
Lot 6	29,182	0.967	497,256	0.987
Pure Solution (n=3)	30,166		503,701	

A Note on Recovery/matrix Factor Tests

Due to Issues with Non-specific Binding, a Somewhat Unconventional Diluent was Used to Prep Recovery Solutions.

Precipitate 6.00 mL of human plasma with 14.0 mL methanol. Thoroughly vortex-mix and centrifuge at 3,000 rpm for ~5 minutes. Combine 8.50 mL of the resulting supernatant with 51.5 mL of Type 1 water, 1.50 mL of 1 mM EDTA (aq): TEA (100:1), 0.750 1mL of HFIP, and 150 µL of TEA.

A Note on Recovery Test

Due to Issues with Non-Specific Binding, a Fairly Unique Solution Was Used to Prep Recovery Solutions

3.10 Preparation of Recovery and Matrix Effect Samples (for Validation Study Only)

Solution ID	Source Solution Used	Source Solution Concentration (ng/mL)	Source Solution Volume Used (µL)	Reagent/Solvent Volume (mL)	Final Volume (mL)	Final Concentration Analyte/ISTD (ng/mL)*
HRS	V-A V-D	50,000 6,500	50.3 48.5	3.90	4.00	629 10.5
MRS	V-B V-D	30,000 6,500	52.4 48.5	3.90	4.00	393 10.5
LRS	V-C V-D	400 6,500	47.1 48.5	3.90	4.00	4.71 10.5

*For calculation of recovery, it is assumed that the total volume of the aqueous layer is 525 µL.

Container: Polyethylene / Polypropylene

Diluent: Water: Precipitated Plasma supernatant: [1 mM EDTA (aq): TEA (100:1)]: HFIP: TEA (25.75: 4.25: 0.75: 0.375: 0.075)

Storage temperature: Refrigerated

Expiry: Assigned 7 days

Post extraction spiking procedure for Recovery and Matrix Effects samples

Following dry-down (step 17), for post-spiked samples (recovery and matrix effects) add 130 µL of reconstitution solvent and 20.0 µL of LRS, MRS, or HRS.

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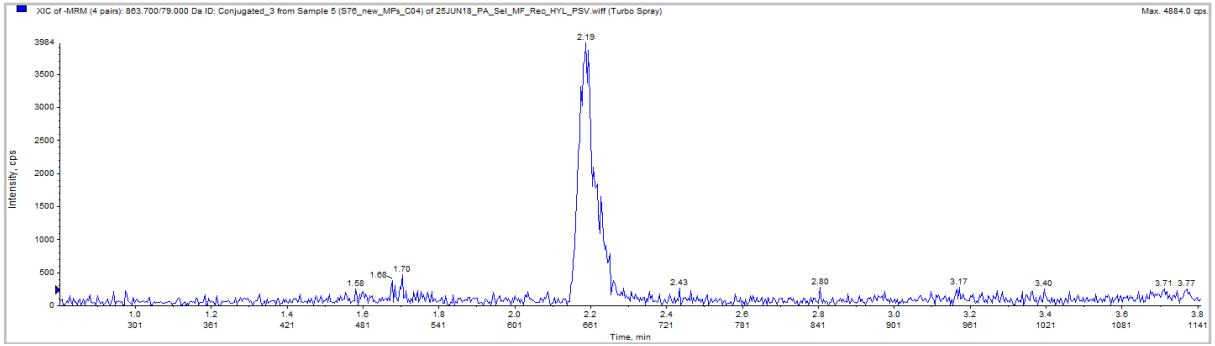
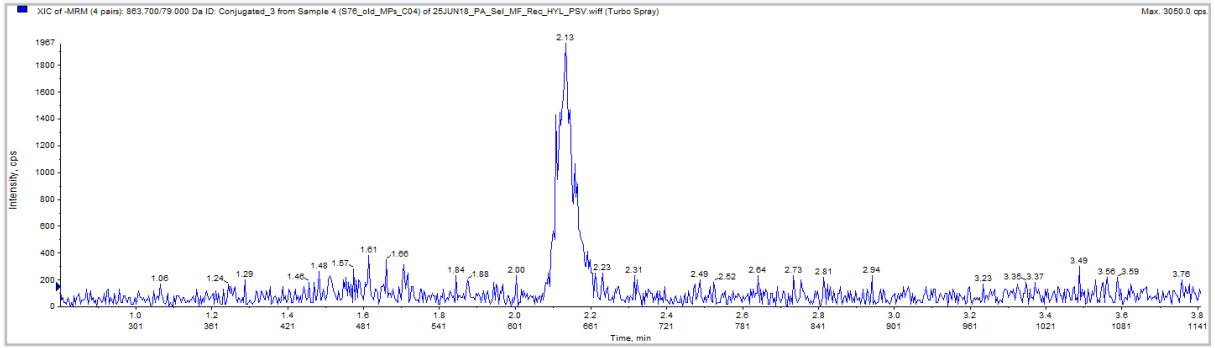
One Additional Test: Importance of Fresh Mobile Phases

Comparison of Fresh-Prepped Mobile Phases (Top Chromatogram) vs. Mobile Phases that were ~60 Hours Old (Bottom Chromatogram) and Fresh-Prepped (Bottom)

Fresh-prepped mobile phases; peak height is ~4,000 CPS



60 hours old mobile phases; peak height is ~1,900 CPS



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Conclusions

A combination of LLE-SPE extraction, optimized LC conditions and 6500+ triple quad mass spectrometer was utilized to fully validate an assay for a GalNAc-conjugated 16-mer oligonucleotide in human plasma having an LLOQ of 0.200 ng/mL

The assay was fully capable of selectively measuring the GalNAc-conjugated form in the presence of the non-conjugated form

This general approach (LLE-SPE, in combination with triple quad detection) has been successfully employed to validate methods for two additional anti-sense oligonucleotides (although the specific concentrations of the mobile phase modifiers has required some optimization to ensure the most robust signal)

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