

Development of total ASO method in mouse plasma and tissues using LC-FD and LC-MS platforms

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INTRODUCTION

Bioanalytical methods are needed to analyze protein conjugated antisense oligonucleotides (POCs) to accurately quantify the active antisense oligonucleotide (ASO) payloads, assess its pharmacokinetics and biodistribution in plasma and tissues, and ensure patient safety by evaluating potential immunogenicity and toxicity. Because the conjugate, the free ASO, and the linked ASO fragment can all be present, specialized techniques are required to differentiate and quantify these components, which is essential for supporting the development of these complex biotherapeutics (Figure 1).

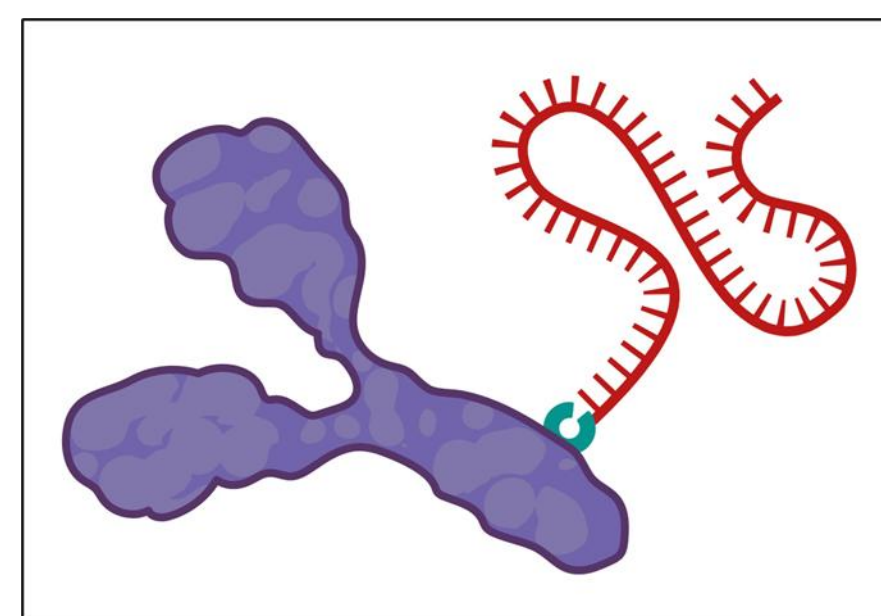


Figure 1: POC

The unique properties of POCs present significant analytical challenges that necessitate specialized methods.

- **Instability and degradation:** Both the ASO payload and the antibody can degrade in biological systems or during the extraction conditions which can affect the accuracy of bioanalytical tests if not properly managed during sample and extraction handling.
- **Metabolite interference:** ASO metabolites often have very similar structures and molecular weights to the parent ASO, making it difficult to distinguish and quantify them accurately.
- **Non-specific binding:** ASOs can bind to plasma proteins and surfaces during analysis. This non-specific binding can cause analyte loss and lead to high variability in results.
- **Need for high sensitivity:** POCs and their various components circulate at very low concentrations. This requires bioanalytical methods with high sensitivity to accurately quantify the different forms of the drug.
- **Multiple detection techniques:** No single method can provide all the necessary information for POC bioanalysis. A combination of techniques is required to analyze the different parts of the molecule, from the large antibody to the smaller ASO and its metabolites.

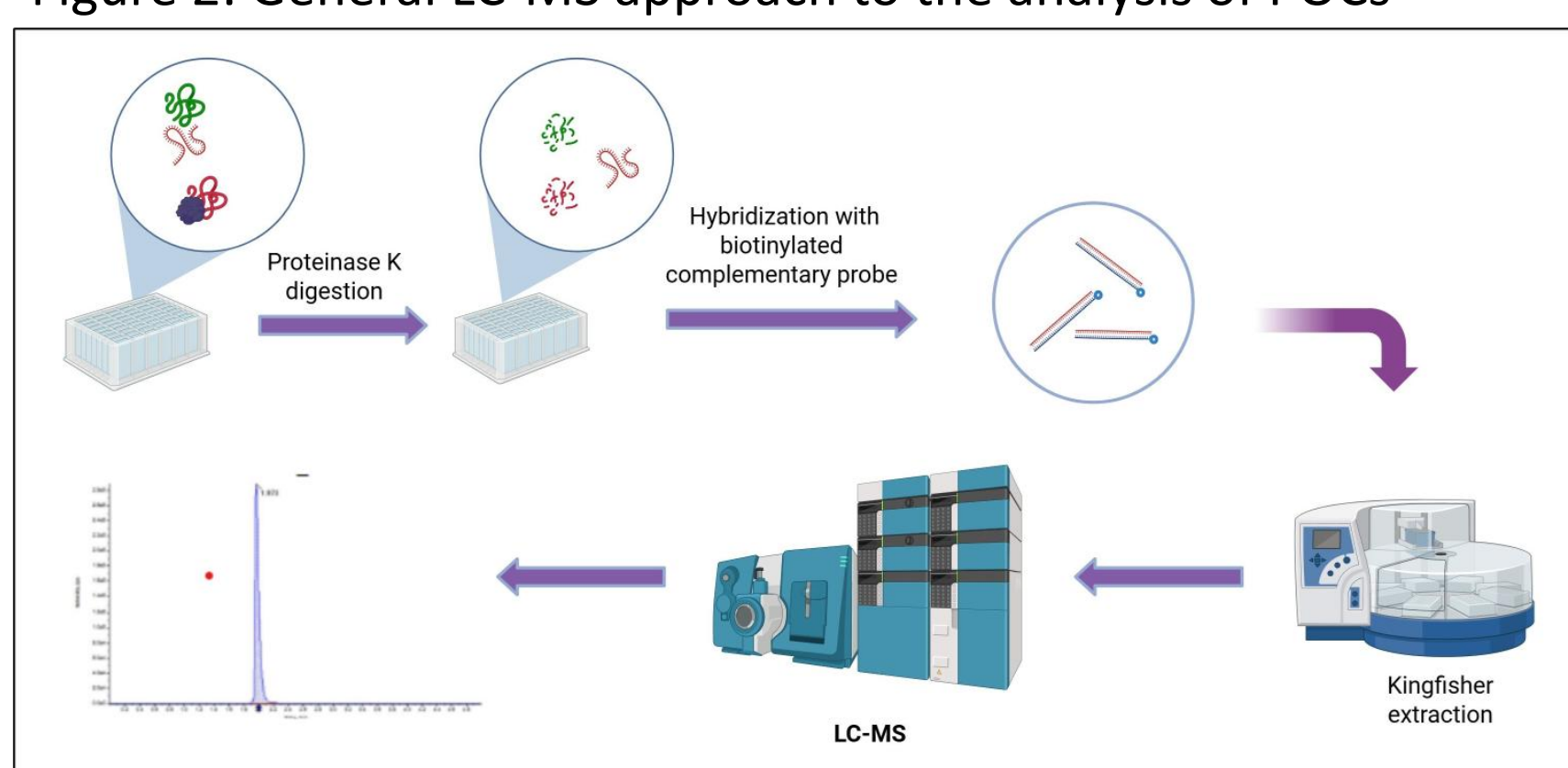
OVERVIEW AND METHODOLOGY

To develop a methodology for quantifying total ASO in POCs that could be universally applied across similar POCs. The study compared mass spectrometry and fluorescence detection platforms to determine optimal sensitivity, selectivity, and adaptability. Additionally, it aimed to establish a single extraction method suitable for both plasma and tissue analysis.

General Workflow

- **Sample preparation:** Given that POCs are typically large and exist within complex biological matrices (e.g., plasma or tissue), the sample must first be processed. Solid-phase extraction (SPE), liquid-liquid extraction (LLE), and hybridization methods can be used to separate the POC from these matrices.
- **Enzymatic cleavage:** To simplify the complex molecule for analysis, the POC may be digested. A protease (e.g., proteinase K) can be used to cleave the protein, leaving the intact oligonucleotide still attached to a smaller peptide fragment.
- **LC-MS analysis:** The pre-treated sample is separated by liquid chromatography (LC) and then analyzed by mass spectrometry (MS). LC: Ion-pairing reversed-phase liquid chromatography (RP-IP) is the most common technique.
- **MS:** High-resolution mass spectrometers (HRMS), such as Orbitrap systems, are used to accurately measure the mass-to-charge (m/z) ratio of the molecules. Triple quadrupole mass spectrometers (LC-MS/MS) systems using Multiple Reaction Monitoring (MRM) are used to achieve the specificity and sensitivity required for POC analysis (Figure 2).
- **The LC/FD (liquid chromatography/fluorescence detection) approach for analyzing POCs** relies on a hybridization-based assay of a specific fluorescence probe to the ASO to achieve high specificity and sensitivity (Figure 3)

Figure 2: General LC-MS approach to the analysis of POCs



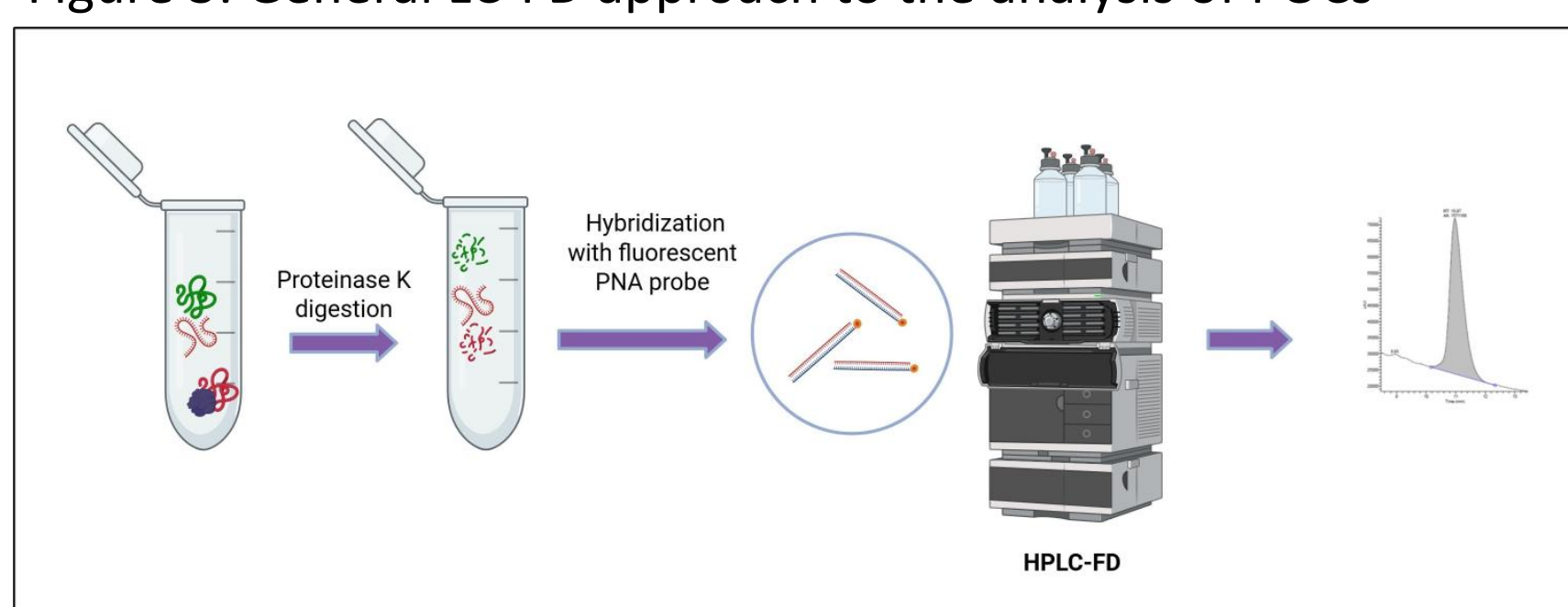
- The study evaluated two mass spectrometry platforms LC-HRMS using a Thermo QExactive (QE) and a LC-MS/MS using a Sciex 7500, along with LC/FD.

- For LC-HRMS analysis, the analyte was extracted using Phenomenex Clarity SPE followed by separation with RP-IP mobile phases.

- For LC-MS/MS analysis, the analyte was extracted by ASO hybridization with peptide nucleic acid (PNA) and locked nucleic acid (LNA) probes followed by RP-IP based separation and detection.

- For LC/FD analysis, the method incorporated proteinase K digestion, PNA probe hybridization, and strong anion exchange (SAX) column separation with a salt gradient.

Figure 3: General LC-FD approach to the analysis of POCs



RESULTS

Early-stage Method Development on LC-MS

1. Unconjugated ASO analyzed with SPE & LC-HRMS: Two abundant parent ions were observed from the unconjugated oligo at 840 m/z and 842 m/z (Figure 4).
2. POC in matrix: Mouse plasma was then fortified with POC to make a calibration curve ranging from 0.145 to 290 nM. Proteins were digested with proteinase K.
3. POC analyzed with SPE & LC-HRMS: Ion with 841 m/z was detected and after quantitation, a limit of sensitivity at 2.90 nM was determined.
4. POC analyzed with SPE & LC-MS/MS: Two peaks were observed from the digested extracts (Figure 5). The two peaks were thought to be a byproduct of the observed two masses that differed from each other by 18 Daltons and could be from hydrolysis that may occur during the Clarity extraction.

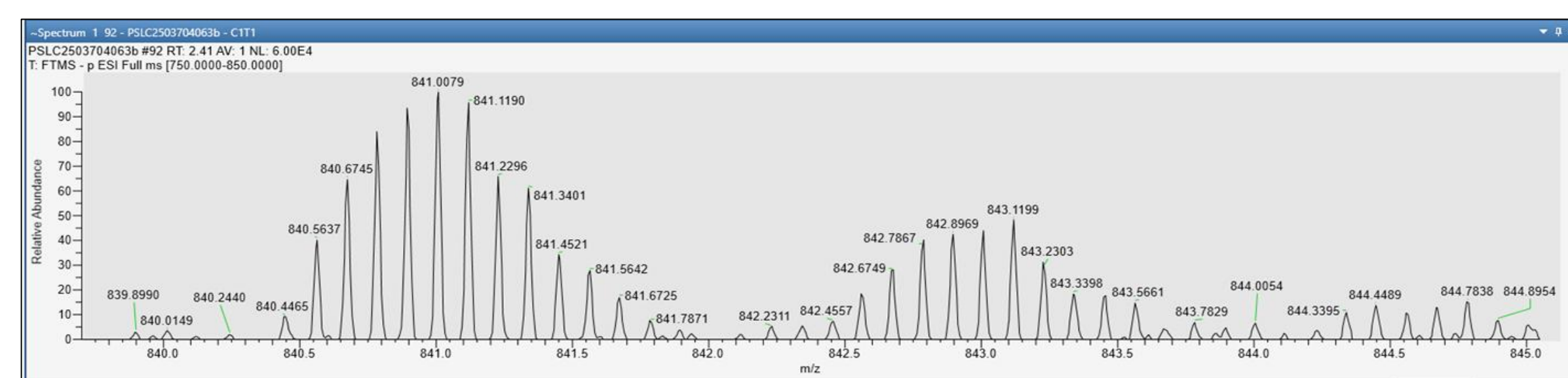


Figure 4: HRMS data of the extracted unconjugated ASO

Figure 5: LC-MS (QE) analysis after SPE extraction

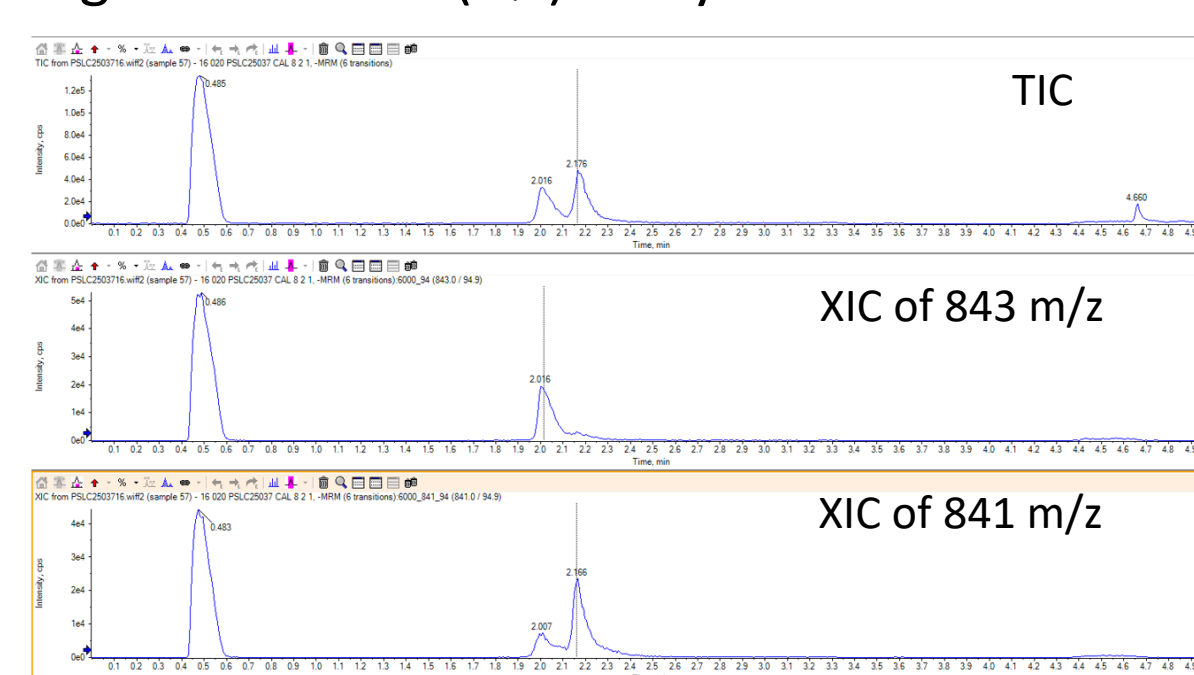
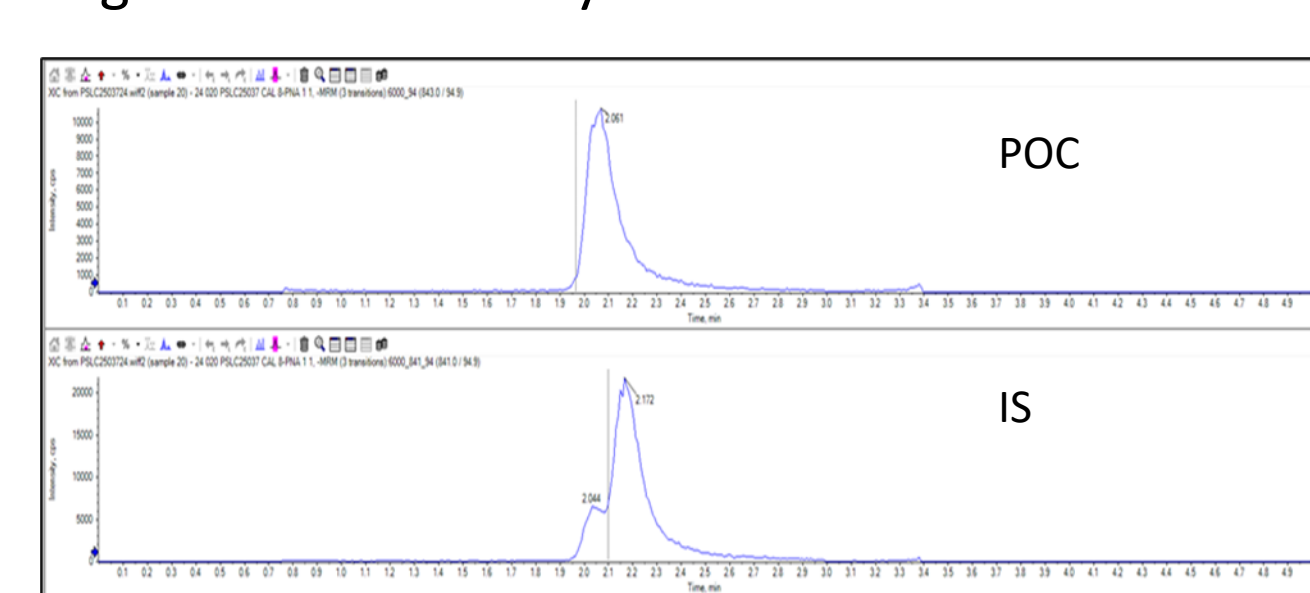


Figure 6: LC-MS of hybridization extraction



Further Method Development on LC-MS: Hybridization & LC-MS/MS

- To overcome issues with SPE and improve sensitivity, a hybridization approach was evaluated using PNA and LNA probes.
- The LNA probe gave superior sensitivity and was chosen for LC/MS optimization (Figure 6). Hybridization eliminated the double peak seen with SPE, likely by preventing ASO hydrolysis.
- Minor unconjugated ASO species were detected, but after switching to 891 m/z (accounted for a few additional amino acids from the protein's proteinase K digestion) and optimizing LC/MS, a single assay for plasma and tissue was achieved with a 0.145–145 nM range and acceptable QC (Tables 1 & 2).
- Full-scan QE analysis confirmed extraction products and m/z values

Table 1

	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8
Theor. Conc. (nM)	0.145	0.291	0.727	2.91	8.72	17.4	26.2	29.1
Found Conc. (nM)	#1	#1	#1	#1	#1	#1	#1	#1
	0.140	0.262	0.713	2.80	9.30	*11.9	*13.6	30.9
	#2	*0.221	0.344	0.721	2.98	8.05	17.8	23.5
Mean	0.140	0.303	0.717	2.89	8.68	17.8	23.5	30.4
%Theoretical	96.6	104.1	98.6	99.3	99.5	102.3	89.7	104.5
%Bias	-3.4	4.1	-1.4	-0.7	-0.5	2.3	-10.3	4.5
n	1	2	2	2	2	1	1	2

Table 2

	LQC-Muscle	LQC-Plasma	MQC-Muscle	MQC-Plasma	HQC-Muscle	HQC-Plasma
Theor. Conc. (nM Plasma; nmol/kg Muscle)	0.436	0.436	2.91	2.91	23.3	23.3
Found Conc. (nM Plasma; nmol/kg Muscle)	#1	#1	#1	#1	#1	#1
	0.512	0.514	2.86	2.41	21.8	22.2
	#2	0.478	0.472	2.79	3.00	21.8
	#3	0.512	0.463	2.70	3.04	21.9
	#4	0.543	0.442	2.69	3.08	22.8
	#5	0.479	0.457	2.71	3.19	27.0
	#6	0.514	0.422	2.59	3.18	24.2
Mean	0.506	0.462	2.72	2.98	23.3	23.5
S.D.	0.0246	0.0311	0.0924	0.291	2.06	0.734
%CV	4.9	6.7	3.4	9.8	8.8	3.1
%Theoretical	116.1	106.0	93.5	102.4	100.0	100.9
%Bias	16.1	6.0	-6.5	2.4	0.0	0.9
n	6	6	6	6	6	6

LC-FD Method Development

- The POC was taken through the typical hybridization protocol that involves a proteinase K digestion, hybridization with the PNA probe, and then injection onto a SAX column running a salt gradient.
- The preliminary analysis demonstrated a high background in the fluorescence trace which limited the sensitivity of the assay.
- Adding a KCl precipitation step after hybridization reduced protein-related background.
- Attempts to merge plasma and tissue methods failed due to background matrix effects (Figure 7), as muscle QCs did not meet plasma-based criteria (Table 3).
- Separate methods were established, each qualified against its own matrix, meeting acceptance criteria at 0.290–58.1 nM for plasma (Table 3) and 5.82–581 nmol/kg for tissue (Table 4 & 5).

Figure 7: LC-FD LLOQ Chromatogram

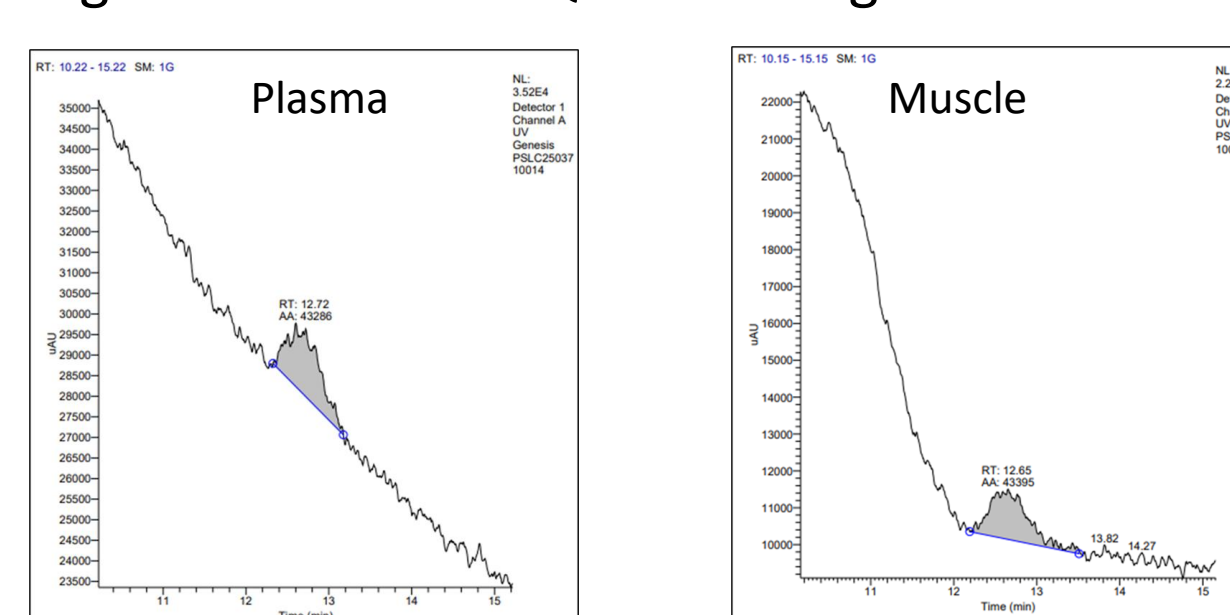


Table 3

	LQC Plasma	LQC Muscle	MQC Plasma	MQC Muscle	HQC Plasma	HQC Muscle
Theor. Conc. (nM Plasma; nmol/kg Muscle)	0.871	8.71	6.97	69.70	46.5	465
Found Conc. (nM Plasma; nmol/kg Muscle)	#1	#1	#1	#1	#1	#1
	0.916	&12.8	6.55	&97.9	46.2	549
	#2	1.04	&13.3	6.64	&98.2	47.9
	#3	1.06	&13.3	6.56	&96.1	48.0
	#4	0.933	&13.0	6.64	&93.0	48.0
	#5	0.971	&13.3	6.51	&97.4	47.7
	#6	0.981	&12.8	6.40	&90.7	47.3
Mean	0.984	13.10	6.55	95.60	47.5	543
S.D.	0.0571	0.0248	0.0899	0.304	0.697	1.01
%CV	5.8	1.9	1.4	3.2	1.5	1.9
%Theoretical	113.0	150.4	94.0	137.2	102.2	116.8
%Bias	13.0	50.4	-6.0	37.2	2.2	16.8
n	6	6	6	6	6	6

Table 4

	CAL 1	CAL 2	CAL 3	CAL 4	CAL 5	CAL 6	CAL 7	CAL 8
Theor. Conc. (nmol/kg Muscle)	2.90	5.81	14.5	58.1	174	349	523	581
Found Conc. (nmol/kg Muscle)	#1	#1	#1	#1	#1	#1	#1	#1
	2.88	5.85	14.3	*39.4	171	349	532	544
	#2	*4.46	5.79	15.0	56.5	183	329	534
Mean	2.88	5.82	14.7	56.5	177	339	533	578
%Theoretical	99.3	100.2	101.4	97.2	101.7	97.1	101.9	99.5
%Bias	-0.7	0.2	1.4	-2.8	1.7	-2.9	1.9	-0.5
n	1	2	2	1	2	2	2	2

Table 5

	LQC Muscle	MQC Muscle	HQC Muscle
Theor. Conc. (nmol/kg Muscle)	8.71	69.7	465
Found Conc. (nmol/kg Muscle)	#1	#1	#1
	8.04	70.0	503
	#2	9.26	72.8
	#3	9.74	72.1
	#4	9.99	70.9
	#5	9.26	70.8
	#6	9.35	73.1
Mean	9.27	71.6	485
S.D.	0.672	1.24	20.1
%CV	7.2	1.7	4.1
%Theoretical	106.4	102.7	104.3
%Bias	6.4	2.7	4.3
n	6	6	6

CONCLUSION

LC-MS/MS and LC-HRMS

- The triple quadrupole platform provided optimal sensitivity, while the orbital trap offered superior selectivity and high-resolution analyte characterization.
- The LC-MS/MS assay using LNA probe demonstrated effectiveness across both plasma and tissue samples with a range of 0.145–145 nM.
- Further optimization of the orbital trap method could potentially improve detection limits and enable comprehensive POC-related compound analysis. Additional development using immunocapture approaches could enhance sample cleanup through a dual hybridization process.

LC-FD

- Fluorescence detection was found to be the most straightforward method to develop.
- The fluorescence detection method achieved ranges of 0.290–58.1 nM for plasma and 5.82–581 nmol/kg for tissue samples.

The %CV and %bias for quality control samples and back-calculated standard curve in both the methods were within the acceptable range of ≤20% in the tissues tested, showing good precision and accuracy.

FUNDING AND CONFLICT OF INTEREST

This work was funded by Sanofi. Padmanabhan Eangoor is a Sanofi employee and may hold shares and/or stock options in the company.