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Oligonucleotide Analysis by LC-MS/MS

CASE STUDY

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The Oligonucleotides Analysed

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Two oligonucleotides were custom synthesised:

Oligonucleotide 1 (Analyte):
MW: 6258 Da
18-mer, fully phosphorothioated, 2'-O-methylated modified bases

Oligonucleotide 2 (Internal Standard):
MW:6015.8 Da
19-mer, fully phosphorothioated

Backbone modification by the incorporation of phosphorothioated linkages was selected as this a very common oligonucleotide modification; it confers nuclease resistance and enhances in vivo stability. The modification results in the addition of a chiral centre presenting chromatographic challenges. Extraction challenges due to being highly protein bound.

Second generation antisense oligonucleotides have a ribose sugar modification to improve plasma stability and increase tissue half-lives. Therefore a 2'-O-methyl modification was elected to reflect the generational evolution in synthetic oligonucleotide therapeutics.

Overcoming Challenges of Oligonucleotide Analysis

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Absorption and MS Adducts

- Polypropylene containers, including mobile phase bottles used throughout. No glassware used. Minimising loss due to absorption and reducing Na adducts in the mass spectrometer.
- Serial dilution in plasma from stock solution performed for preparation of calibration standards and QC samples. Diluted precipitated matrix used to prepare post-spike solutions to overcome non-specific binding.

Extraction Recovery

- Optimisation of all steps of the SPE extraction resulting in 70% recovery for both oligonucleotides allowing for a low LLOQ to be achieved.
- Addition of the cysteine to the Lysis-Loading buffer to prevent desulfurization during sample processing.

Chromatography

- Passivation of the LC system and analytical column with ion-pair mobile phase to ensure rapid system equilibration.
- Initial poor peak shape due to the phosphorothioate stereocenter overcome by inclusion of 400 mM HFIP in both LC mobile phases and weak wash solvent.
- Mobile phases prepared daily to maintain peak shape and intensity.

Selectivity

- Selectivity can be challenging due to production of small, non-selective product ions after fragmentation by CID. Numerous charge states and product ions were monitored throughout the development allowing for selection of the optimal MRM to achieve selectivity and sensitivity.

Methodology

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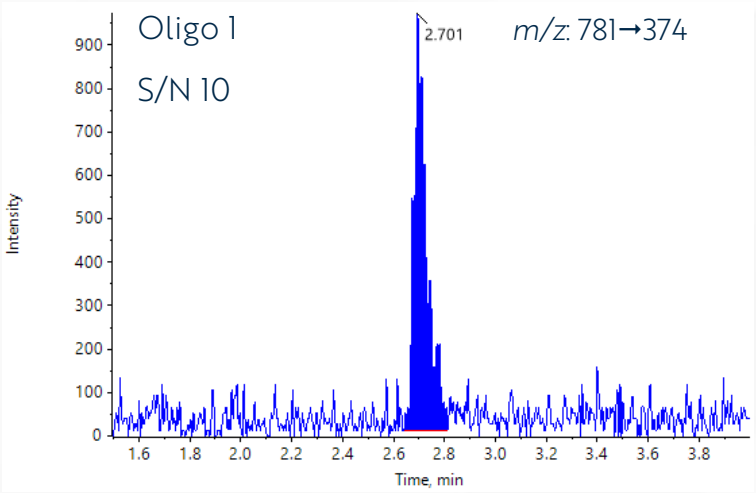
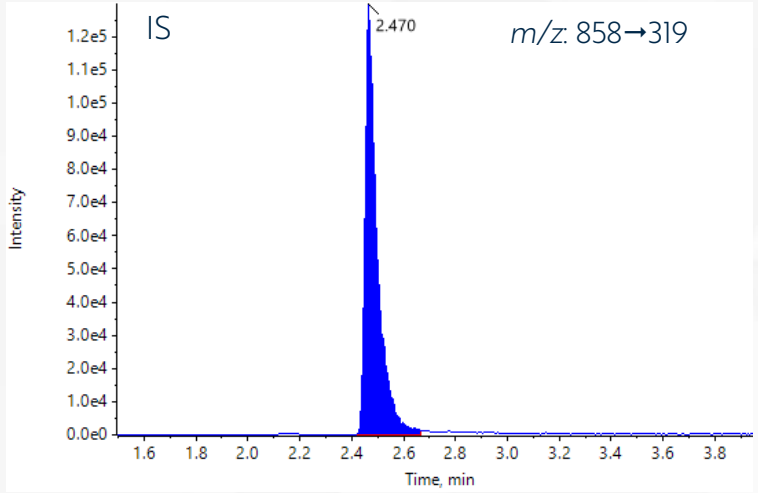
500 µL human plasma

Phenomenex Clarity lysis loading buffer with cysteine

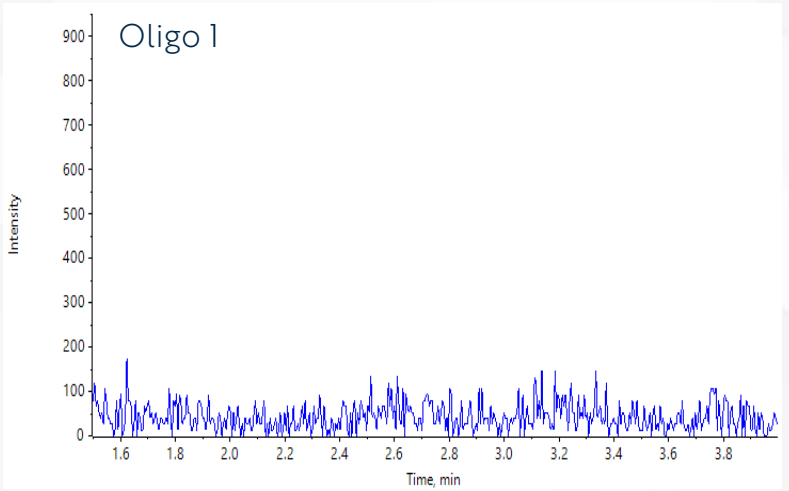
Waters Oasis WAX SPE using Otto Positive Pressure Manifold

Waters Acquity UHPLC coupled to a Sciex 5500 Triple-Quad MS





Extracted 0.25 ng/mL sample



Extracted control blank sample

Performance Data

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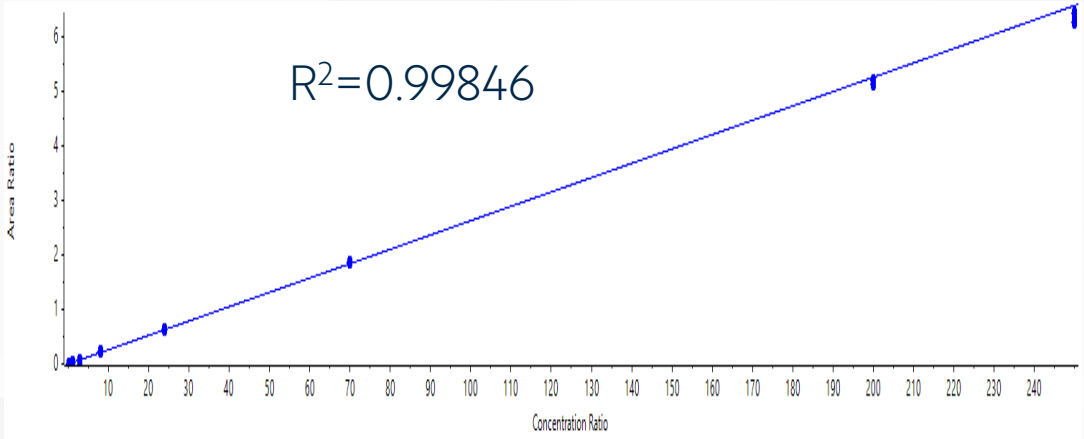
Plasma stability demonstrated after:

- 17 hours at room temperature
- 13 days at -80°C
- 1 freeze-thaw cycle



Additional assessments:

- Selectivity in individuals free of interference
- Matrix effects in individuals acceptable

Table 1: Quality Control Precision and Accuracy

Replicate	QC 1[a]	QC 2[a]	QC 3[a]	QC 4[b]	QC 5[b]	QC 6[b]	QC 7[b]
Mean	0.3	0.5	1.0	4.76	15.6	787	1770
%CV	5.66	3.76	2.68	3.3	4.9	2.0	2.0
Accuracy (%)	0.67	2.4	0.2	-4.83	3.74	-1.59	-5.57

[a] Analytical assay 500 µL sample volume, 0.25-250 ng/mL [b] Analytical assay 200 µL sample volume, 5-2500 ng/mL

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Contact us to discuss how we can support your cutting-edge oligonucleotide therapeutics.

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