Development of a sensitive and selective high resolution accurate mass LC-MS for the detection of oligonucleotides in biological matrices

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Overview

- Development of high resolution accurate mass LC-MS for sensitive and selective detection of antisense oligonucleotide (ASO) by
  - optimization of chromatographic separation of ASO and minimization of ion suppression
  - optimization of sample preparation with acceptable recovery
- Establishing the LOD of ASO by narrowbore LC-ESI/MS and capillary LC CaptiveSpray or MicroSpray MS
- Application of this technique to investigation of *in vitro* metabolic stability of ASO

Introduction

The recent successes of ASO biotherapeutics resulted in greater representation in big pharma portfolios to balance out the high risk of drug failure in clinics associated with small drug molecules. ASO is a short strand of a deoxyribonucleotide analogue with a phosphorothioate or thiophosphoramidate linker to improve metabolic stability. The formation of the ASO-mRNA heteroduplex occurs via a Watson-Crick base pairing leading to downregulation of target protein expression. This can occur by either triggering RNase H activity, leading to mRNA degradation (inducing translational arrest by steric hindrance of ribosomal activity followed by interference with mRNA maturation) or by destabilization of pre-mRNA in the nucleus. Hence, ASO is a highly selective therapeutic strategy for diseases presenting dysregulated protein expression.
In this poster, we report the development of a high resolution accurate mass narrowbore and capillary LC-MS for detection of ASO in biological matrix .

Methods

In vitro incubation

The metabolic stability of ASO was conducted at 1000ng/ml, in a total volume of 1 ml, containing 1 mg/mL pooled dog intestinal S9, 1mM NADPH, 1mM NADH, 4mM $MgCl_2$ and 0.1M phosphate buffer, pH 7.4. The mixture was incubated at 37°C for 2h and followed by sample preparation. The residue was reconstituted, filtered by centrifugation and the filtrate was analyzed by LC-MS.

Chromatography

LC: Thermo Scientific Accela or Waters Nano Acquity
Column: Zorbax 300Å Extend-C18 (150 x 2.1 mm ID or 100 x 0.3 mm ID, 3.5 μm)
Column temperature: 40°C or room temperature
Mobile phase: A: water + 10mM TEAB, pH 8.5; B: acetonitrile: isopropanol (1:1) + 10mM TEAB, pH 8.5
Flow rate: 200 or 5 μL/min
Gradient: held at 3% B for 1 min, ramped from 5 to 90% B over 21 min, held at 90% B for 2 min before ramping down to 5% B over 1 min

Thermo Scientific LTQ/Orbitrap XL ESI-MS

The LC eluant was sprayed into MS at 3.5 kV with sheath and auxillary and sweep gas set at 60, 5 and 5 arbitrary units.

Micro ESI-MS (CaptiveSpray or MicroSpray)

No gas was turned on for CaptiveSpray and only sheath gas at 20 arbitrary units was used for MicroSpray.
Solvent droplets were desolvated using a heated capillary at 275°C and a source fragmentation of 15V. Mass axis calibration was performed by infusion of Agilent TOF ESI tuning solution prior to full scan mass analysis at 30,000 resolution from m/z 200 – 2000. SIM was acquired at 30,000 resolution with a scan width of 4 m/z of a center *m/z*. Data acquisition and reduction was conducted using Xcalibur version 2.0

Results

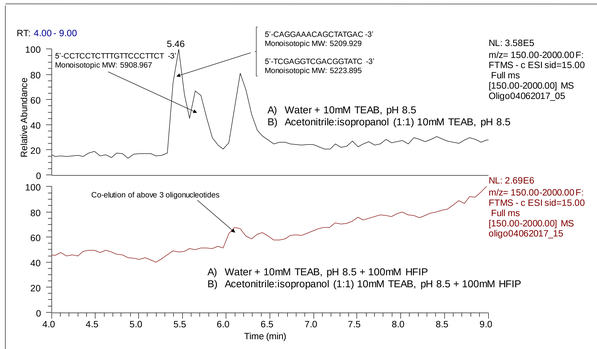


Figure 1: Effect of mobile phase composition on sensitivity of detection as illustrated by the TIC corresponding to a mixture of 3 ASOs. **The above mobile phase with 5 or 10 mM TEAB, pH 8.5 was selected since lower TEAB concentration gave higher S/N**.

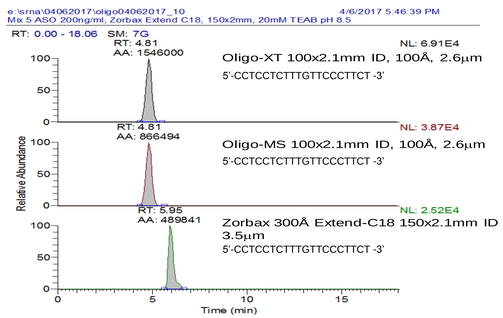
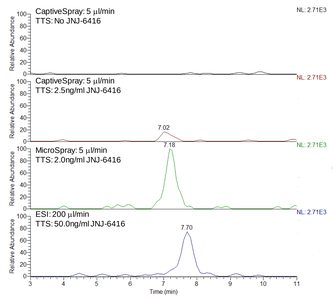


Figure 2: Retention of ASOs using reverse phase ion-pairing chromatography with 10mM triethylammonium bicarbonate buffer (TEAB), pH 8.5. Any of the above column can be used.



Capillary LC gradient	
Time (min)	% B
0	3
1	3
26	95
27	95
28	3
35	3

Flow rate: 5μl/min

Figure 3: Limit of detection of ASO-6416 by narrowbore and capillary LC-MS. Capillary LC system consisted of a Waters NanoAcquity fitted with a Zorbax 300Å Extend-C18 (100 x 0.3mm ID, 3.5μm) coupled to a CaptiveSpray or MicroSpray.

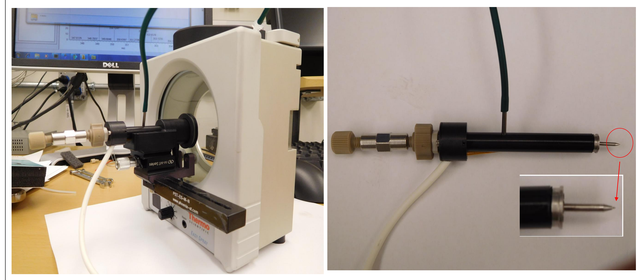


Figure 4: Modification of EASYSPRAY to accommodate a MicroSpray probe with low sheath gas to assist nebulizing of capillary flow during gradient elution from 3 – 95% organic at 5 μl/min. (collaboration with Dr. Sau Lan Tang Staats, Phoenix S&T)

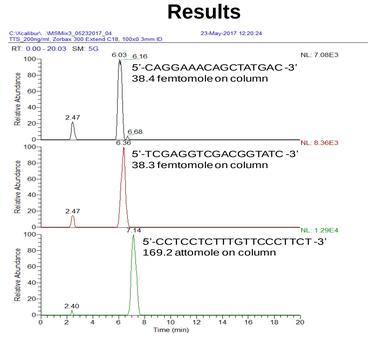


Figure 5: Separation of a mixture of 3 ASOs, each at 200 ng/ml, by linear gradient capillary LC flow at 5 μl/min and nebulizing gas-assisted spraying into MicroSpray ESI source interfaced to LTQ-Orbitrap XL (scanned from *m/z* 200-2000 at 30,000 resolution)

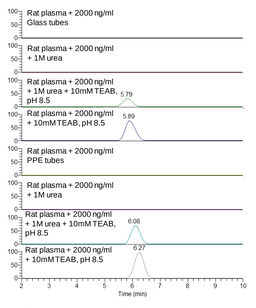


Figure 6: Evaluation of various sample preparation procedures for recovery of ASO-6416 (5'-CCTCCTCTTTGTTCCCTTCT -3') spiked at 2000ng/mL in rat plasma. Similar recovery of ASO-6416 spiked in rat liver S9 was also evaluated.

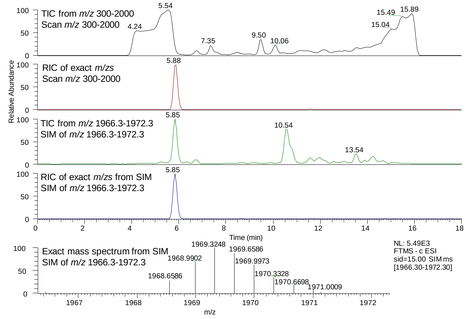


Figure 7: Selectivity of detection from incubation of 1000ng/ml ASO-6416 in 1mg/ml dog intestinal S9 at 37°C for 2h.

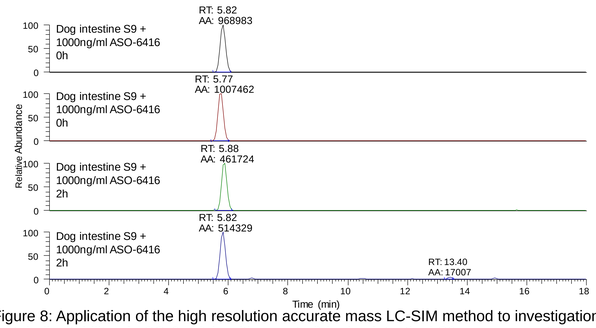


Figure 8: Application of the high resolution accurate mass LC-SIM method to investigation of metabolic stability of ASO-6416 (positive control) in dog intestinal S9. An average turnover of 49.4% was obtained.

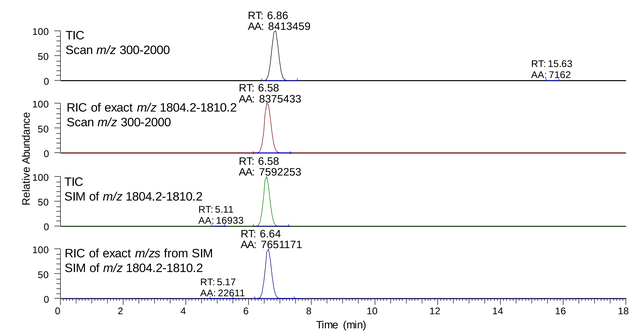


Figure 9: Application of the high resolution accurate mass LC-SIM method to investigation of metabolic stability of modified ASO in dog intestinal S9. An average turnover of 90.8% was obtained and consistent with improved stability of modified ASO.

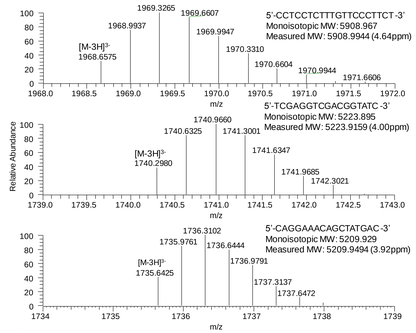


Figure 10: Utilization of monoisotopic peak to determine the molecular weight of the ASO with mass accuracy of <5ppm

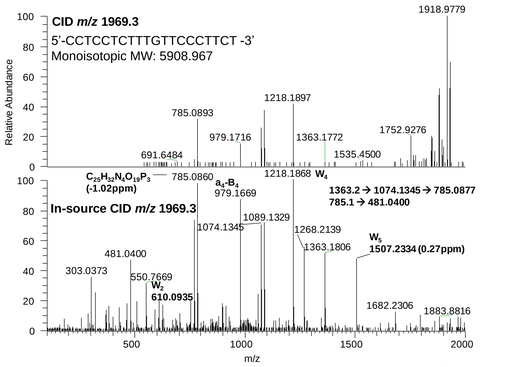


Figure 11: Comparison of CID and in-source CID in fragmentation of $[M-3H]^{3-}$ ASO-6416 for structural elucidation of metabolites

Conclusions

- A high resolution, accurate mass and narrowbore LC-MS method was developed with sufficient selectivity and sensitivity for detection of spiked ASO in biomatrices
- A sample preparation procedure based on protein precipitation with 3-fold mixture of methanol:acetonitrile (85:15) containing 1M urea and 10mM TEAB, pH 8.5. The residue was reconstituted with water prior to analysis.
- The LC-MS method was successfully applied to determination of the metabolic stability of ASO in dog intestine S9.
- The phosphorothioate-based ASO is metabolically more stable than the phosphodiester-based ASO in dog intestinal S9
- A lower limit of detection was obtained using capillary LC interfaced with CaptiveSpray or MicroSpray MS