Metabolite profiling of the antisense oligonucleotide Tofersen using UHPLC-HRAM mass spectrometry

Robert L. Ross, Stephane Houel, Roberto Gamez, Keeley Murphy, Min Du, Thermo Fisher Scientific, Lexington, MA

Introduction

Oligonucleotide metabolite identification (MetID) is the process of detecting the products of an oligonucleotide that has been degraded by cellular metabolism. In the context of RNA therapeutics, identification and characterization of exogenous metabolites is crucial to understanding how the drug has been processed by the cell. This knowledge is used to understand the drug's efficacy as well as toxicity. Without question, the gold standard for performing pharmacokinetic studies of oligonucleotide MetID is Ultra High-Performance Liquid Chromatography coupled to High-Resolution Accurate Mass Tandem Mass Spectrometry (UHPLC-HRAM-MS/MS). The use of mass spectrometry for the measuring an oligonucleotide drug for MetID is essential as the accuracy of Orbitrap technology used concomitantly with linear ion trap technology gives the highest degree of accuracy and precision for monitoring the changes of the drug within a biological matrix over time. A challenge with MetID is the measured degradation of the therapeutic is performed in a background matrix, usually a biofluid such as urine, plasma, or liver homogenate. Salt, lipids, proteins and endogenous nucleic acids within the background matrix need to be removed before LC separation and mass spectrometric acquisition of the therapeutic. Two methods for sample clean up are routinely employed, liquid-liquid extraction and offline solid phase extraction (SPE). Both can lead to systematic errors in the measurement as excessive handling or inefficient extraction results in sample loss. The use of spiked controls can reduce the impact of systematic error yet add to the cost of performing the assay. Furthermore, SPE cleanup using cartridges, either vacuum or gravity, are expensive, time-consuming, and usually need to be performed offline. Repeated handling, even with spiked controls, can result in loss of low abundant metabolites. In this work, we applied a Thermo Scientific™ AcquireX™ Ab workflow, in conjunction with an online SPE approach to measure the degradation products of the ASO Tofersen using UHPLC separations and accurate mass tandem mass spectrometry.

Materials and methods

Mass spectrometry

HRAM analyses were performed on a Thermo Scientific™ Orbitrap™ Ascend Tribrid™ mass spectrometer interfaced with a heated electrospray (H-ESI) source in negative polarity. Full scan data was acquired in Peptide mode at 60K resolution, mass range 800-3000 m/z, automatic gain control (AGC) 400, and max injection time (IT) 500 ms. Tandem mass spectrometry was performed in the linear ion trap (LIT) with a NCE% of 27, an activation time of 15 ms and a Q of 0.25. Detection was performed in the orbitrap at 30K resolution.

Data acquisition was accomplished with an AcquireX Ab workflow performed running Thermo Scientific™ Xcalibur™ 4.7 data system. Data was processed using Thermo Scientific™ Freestyle™ 1.8 and BioPharma Finder™ software.

Chromatography

Chromatography was performed using a Thermo Scientific™ Vanquish™ Horizon Quaternary UHPLC platform with a 6-port/2-valve switch in the column compartment. Samples were separated on a Thermo Scientific™ DNAPac™ RP column (2.1 x 50 mm, 4 µm). Utilizing ion pairing chromatography with a dibutylamine/HFIP mobile phase (15 mM:25 mM, respectively) for MPA and 100% acetonitrile for MPB. An initial gradient of 2%B was held for 2 minutes with the first minute diverted to waste. MPB was increased to 30% at 9.5 min and increased to 90% for 1 minute before returning to initial conditions and re-equilibrating for 5 minutes. Flow rate was 400 µL/min with a column compartment and preheater temperature of 75 degrees.

Table 1. Consolidated results of metabolic products detected in this study. Table shows all detected features having MS/MS with a confidence score of >50%. Highlighted feature is the full-length product (FLP) with identified metabolites aligned against the intact sequence.

Identification	Oligo Sequence	Mod	Δ ppm	Conf. Score	RT	M/Z	Charge St.
S1-A5 = 1927.4647	Se-sAe-pGe-sGe-pAe	(3')n-15	6.59	94.4	3.72	962.731	-2
S1-T6 = 2247.4879	Se-sAe-pGe-sGe-pAe-sTd	(3')n-14	3.97	99.3	4.57	1122.741	-2
S1-A7 = 2576.523	Se-sAe-pGe-sGe-pAe-sTd-sAd	(3')n-13	7.03	96.8	5.10	1287.764	-2
S1-A9 = 3224.597	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd	(3')n-11	5.25	96.5	5.92	1611.804	-2
S1-T10 = 3544.620	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd	(3')n-10	3.53	92.8	6.35	1771.817	-2
S1-T12 = 4184.666	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd	(3')n-8	3.46	98.9	7.01	1394.558	-3
S1-S13 = 4503.705	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd	(3')n-7	6.77	99.9	7.17	1500.906	-3
S1-T14 = 4823.729	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd	(3')n-6	9.71	100.0	7.41	1607.583	-3
S1-A15 = 5152.763	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd	(3')n-5	1.21	99.1	7.60	1717.594	-3
S1-A17 = 5932.934	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe	(3')n-3	5.91	100.0	7.91	1977.651	-3
S1-T20 = 7123.159	Se-sAe-pGe-sGe-pAe-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	FLP	5.59	100.0	8.26	1017.162	-7
A5-T20 = 5520.833	Ae-sTd-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-4	8.62	100.0	7.88	1840.282	-3
T6-T20 = 5117.761	Td-sAd-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-5	9.92	100.0	7.62	1705.926	-3
A7-T20 = 4797.738	Ad-sSd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-6	3.30	99.8	7.41	1599.253	-3
S8-T20 = 4468.703	Sd-sAd-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-7	7.17	97.3	7.21	1489.237	-3
A9-T20 = 4149.664	Ad-sTd-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-8	9.75	89.9	7.06	1383.229	-3
T10-T20 = 3820.629	Td-sTd-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-9	6.91	100.0	6.84	1909.814	-2
T11-T20 = 3500.606	Td-sTd-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-10	5.73	100.0	6.48	1750.306	-2
T12-T20 = 3180.583	Td-sSd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-11	5.69	100.0	6.04	1589.799	-2
S13-T20 = 2860.560	Sd-sTd-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-12	8.80	98.9	5.52	1429.784	-2
T14-T20 = 2541.521	Td-sAd-sSe-pAe-sGe-pSe-sTe	(5')n-13	6.21	98.8	5.06	1270.262	-2
S16-T20 = 1892.4626	Se-pAe-sGe-pSe-sTe	(5')n-15	5.93	99.7	3.55	945.230	-2

AcquireX Ab workflow

The AcquireX Ab intelligent data acquisition workflow lets you streamline the development and optimization of your mass spectrometry experiments. When an AcquireX Ab experiment is submitted, the Xcalibur 4.7 data system can create an exclusion list through iterative injection. An initial exclusion list is created from a blank sample. With each new injection, masses detected from the previous injection are added to the exclusion list and are excluded from fragmentation in the next acquisitions. The process is continued until every mass in the sample(s) have undergone fragmentation, or until a user defined number of injections is reached. This process allows for detection and identification of features in the sample that would otherwise be missed. When applied to a MetID experiment, we can detect and monitor the degradation products over the time course. This approach has the benefit of detection of predicted targets but also unknown metabolites that may arise through the time course.

DNAPac RP column

The use of offline SPE for sample cleanup generally involves the same substrate, polystyrene-divinylbenzene, as the DNAPac RP chromatography column. We sought to use the chromatography column for online SPE. Before injecting onto the chromatography column, we first centrifuged the sample at 12K g for 20 minutes. The supernatant was then removed from the microcentrifuge tube into a new tube, diluted by 1/3 with water, spiked with the ASO, and placed in the heat block for the study. Once removed from the block, the sample is subjected a second time to centrifugation (12K g for 20 min), the supernatant is removed to a sample vial and injected onto the column.

Consumables

Tofersen ASO standard was purchased from IDT-DNA. Liver S9 Homogenate was purchase from MP Biomedicals. Homogenate was centrifuged at 10K RPM for 20 min. Supernatant was separated into 100 µL aliquots then spiked with one microgram of Tofersen. Samples were incubated at 30 degrees for 0, 1, 3, 5, 7, and 9 days.

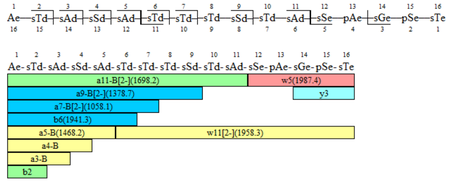

Figure 1. Fragment coverage map for the Tofersen metabolite (5') n-4 showing structural deconstruction from LIT fragmentation. Ion intensity in the spectra are represented by color gradients with red being the most intense.

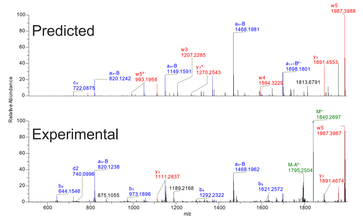

Figure 2. Tandem mass spectra of the Tofersen metabolite (5') n-4. Fragmentation is generated in the LIT. Top pane shows predicted fragmentation matched against observed (bottom pane).

Results

Table 1 shows the resultant identification of metabolites detected in this study. All reported products have a Δ ppm of <10 with a confidence score of 90 or greater for 90% of the detected products. The Confidence Score indicates how closely the predicted and experimental MS spectra match for an identified component using a percentage scale (between 0 and 100%). The average structural resolution (ASR) is also a confidence metric; this value is determined through the number of bonds broken within the oligonucleotide**.** Figure 1 shows this for the (5') n-4 metabolite, The Fragment Coverage Map shows the number of identified ions detected through MS/MS fragmentation and generates the (ASR) value used in confidence scoring. The intensity on the detected ion is color coded. Figure 2 shows the spectral match against the predicted spectra (top pane).

BioPharma Finder software uses a kinetic model to predict oligonucleotide MS2 (MS/MS) spectra. Following known fragmentation channels for oligonucleotides, a list of predicted masses can be generated and matched against detected ions in acquired spectra. Figure 3 shows the Trend Ratio of the area abundance of the (5') n-4 metabolite over the course of the 9-day study. The presence in the sample of the larger (16mer) would be expected for the early samples (days 1-5), and gradually disappearing in the later sample sets (days 7-9). The opposite trend would be expected for short-mers present in the sample. Figure 4 shows the opposite trend, where the peak areas for the (5') n-15 metabolite increases in abundance over the course of the study. Confident verification of identity is obtained through LIT-CID fragmentation, Figure 5.

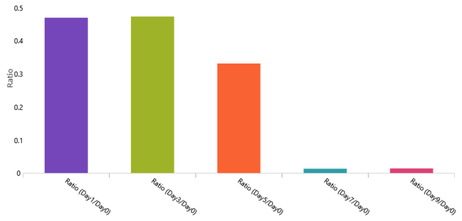

Figure 3. Trend Ratio of the detection of the (5') n-4 metabolite over the course of the 9-day study.

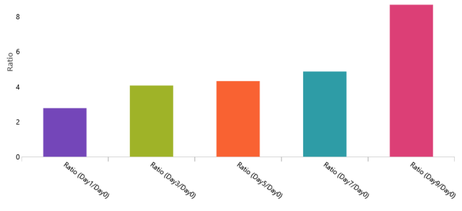

Figure 4. Trend Ratio of the detection of the (5') n-15 metabolite over the course of the 9-day study.

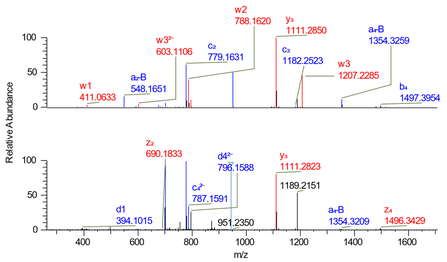

Figure 5. Tandem mass spectra of the Tofersen metabolite (5') n-15. Fragmentation is generated in the LIT. Top pane shows predicted fragmentation matched against observed (bottom pane).

Discussion

Identification through fragmentation

Current approaches for performing MetID of oligonucleotides utilize fragmentation of a single charge state after performing a lengthy sample cleanup. This approach does not provide a comprehensive knowledge of the sample. Most commercial MS systems rely on beam or high energy dissociation which leads to dramatic over-fragmentation, resulting in fragmentation identification ambiguity. By utilizing a linear ion trap and resonance dissociation, we can obtain a high confidence in our identification. This approach yields predictable fragmentation and results in a higher confidence when assigning ions. Furthermore, current approaches are time restricted chromatographically, where the number of fragmentation events are restricted by the chromatographic peak width and duty cycle of the instrument. The use of **AcquireX Ab workflow** in the method presented here provided high quality MS/MS for low level metabolites at 5 orders of magnitude less than the FLP and resulted in a MetID coverage of 100%.This newly developed approach, combining the fragmentation of the **Orbitrap Ascend Tribrid MS linear ion trap**, in conjunction with the use of **AcquireX Ab workflow** and **BioPharma Finder software**, results in easy acquisition, automated detection and identification of the low abundance metabolites of therapeutic oligonucleotides.

Conclusions

- The use of online SPE using the **DNAPac RP column** presents an approach that limits sample loss through handling error.
- The use of the **Orbitrap Ascend Tribrid mass spectrometer** presents a fragmentation profile with a greater degree of confidence due to kinetic modeling and known fragmentation channels using resonance activated collisional induced dissociation in the linear ion trap.
- The **AcquireX Ab workflow** allows for a more thorough understanding of the sample through an exclusion list strategy where more features are detected and fragmented.
- Data processing and reporting is quick and thorough using the **BioPharma Finder software**. Feature detection algorithms as well as fragment ion spectral matching increases confidence in assignation of metabolites in MetID experiments.

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