A highly sensitive and automated workflow for the ID and relative quantification of oligonucleotide impurities and metabolites

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INTRODUCTION

- Synthetic oligonucleotides, e.g., small interfering RNAs (siRNAs), and antisense oligonucleotides (ASOs), continue to grow as viable therapeutic modalities.
- Synthesis often results in various impurities present in the final product, resulting in the need for methods to detect these impurities and quantify their presence relative to the main product.
- Metabolite identification and relative quantification of metabolites require similar methods for plasma, blood, and tissue samples as part of biotransformation studies.
- LC-MS/MS using quadrupole time-of-flight (QTOF) mass spectrometry is most suitable for this task, as its high-resolution spectra coupled with high acquisition speeds allow for untargeted identification of impurities and metabolites with high confidence.
- In this presentation the analysis of both an ASO sample spiked with three of its 5′ shortmers at different levels (to mimic impurity analysis) and a ASO with three 5′ shortmers and three 3′ shortmers spiked into extracted rat plasma sample (to mimic metabolite analysis) is shown, using:
  - The ZenoTOF 7600 system (SCIEX) with a novel ion trap (Zeno trap) to achieve significantly improved MS/MS fragment ion spectra, allowing for improved sequence coverage
  - The Molecule Profiler application, which leverages both MS and MS/MS data for identification and relative quantification of impurities and metabolites.

WORKFLOW

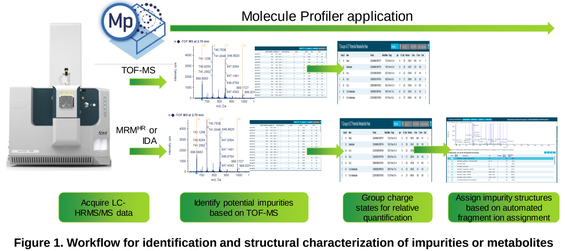


Figure 1. Workflow for identification and structural characterization of impurities or metabolites using the Molecule Profiler application.

MATERIALS AND METHODS

- A 10 µg/mL 18-mer 2′-O-methoxyethyl phosphorothioated RNA (same structure as the drug nusinersen) was spiked in mobile phase A containing 100 µM EDTA together with its 5′(*n*-1, 2, and 3) shortmers at levels from 0.1 -10% to simulate a sample for impurity analysis and spiked in extracted rat plasma together with its 5′(*n*-1,2, and 3) and its 3′ (*n*-1, 2 and 3) shortmers at levels from 0.1 -10% to simulate a sample for metabolite analysis. For the metabolite sample, 1 mL rat plasma was extracted using Clarity OTX solid phase extraction (SPE) cartridges (Phenomenex) following the manufacturer's protocol for extracting oligo therapeutics from biological samples. After drying with nitrogen gas at 40ºC, the plasma extract was reconstituted in 1 mL mobile phase A containing 100 µM EDTA.
- Gradient LC was performed at 0.25 mL/min using water and methanol with the ion-pairing reagents N,N diisopropylethylamine (15 mM) and hexafluoroisopropanol (35 mM) and a 2.1 x 50 mm ACQUITY PREMIER Oligonucleotide C18 column (Waters) at 70ºC. The gradient used was from 20-40% B in 5 min with a 1.5 min wash step at 90% B.
- MS and MS/MS data were acquired using a SCIEX ZenoTOF 7600 system in negative ionization mode. MS/MS data was acquired in MRM^{HR} mode for the most abundant charge states of the main product and spiked in impurities/metabolites
- Data was processed using the Molecule Profiler application (Figure 1). Identification is based on accurate mass and a comparison between measured and theoretical isotope patterns. Processing parameters were selected so that the software considered possible impurities based on a list of 83 potential modifications and the breaking of one internal bond. Charge states from -6 to -10 and a 20 ppm max. mass tolerance were used for the identification of potential impurities. Relative quantification based on TOF data was performed automatically by grouping all charge states of an identified impurity. Terminal fragments (*a, w, c, y* and *d*) were automatically assigned in the MS/MS spectra using a 10 ppm tolerance and allowing for a maximum of one bond to break, and a maximum of one water or base loss.

RESULTS

Impurity ID

For the impurity sample the Molecule Profile application identified the main full-length product (FLP) and the spiked-in impurities at all spike-in levels.

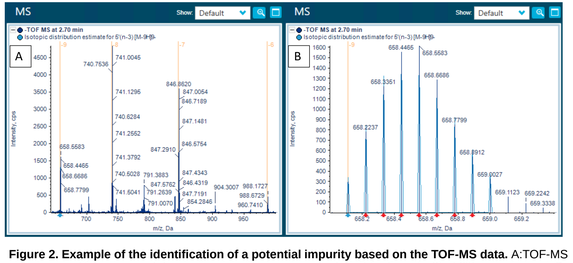


Figure 2. Example of the identification of a potential impurity based on the TOF-MS data. A:TOF-MS showing identified charge states (yellow labelling) and suggested ID for the 5′(*n*-3) at 3% spike-in level. B: Zoom-in to TOF-MS data of charge state -9 for 5′(*n*-3). Blue arrow indicates the theoretical monoisotopic *m/z* and the first seven isotopes are indicated with red arrows. An overlay of the theoretical isotopic distribution (light blue triangles) can be used for further confidence in the correct assignment.

For the *n*-1 and *n*-3 shortmers good correlation was found between spike-in levels and the reported relative areas of the TOF-MS extracted ion chromatograms of isotope series of several charge states (Table 1) from the software. Because of an overlap of the -8 charge state isotopes of the 5′(*n*-2) impurity with the -9 charge state of the FLP (no chromatographic resolution), the charge state was excluded for relative quantification, leading to lower %area values for the 5′(*n*-2) impurity.
Plots for the 5′(*n*-1) and 5′(*n*-3) impurities (Figure 3) show good linearity across the spike-in levels. The FLP itself (control sample) was found to already contain some of the shortmers (Table 1).

Table 1. Relative quantification results for identified analytes.

Spike-in (% w/w of FLP)	Spike-in (% w/w of total)	FLP (%area)	*n*-1 (%area)	*n*-2 (%area)	*n*-3 (%area)
0	*0.00*	*83.01*	*0.11*	*NA*	*0.26*
0.10	*0.10*	*82.50*	*0.17*	*0.02*	*0.34*
0.30	*0.30*	*80.99*	*0.34*	*0.11*	*0.50*
1.00	*0.97*	*81.34*	*0.97*	*0.37*	*1.08*
3.00	*2.75*	*75.48*	*2.89*	*1.12*	*2.68*
10.00	*7.69*	*63.35*	*8.73*	*6.39*	*8.73*

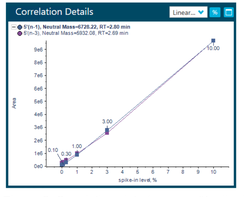


Figure 3. Correlation plot of area vs. %spike-in.

Biotransformation ID

Great correlation was observed between the spiked amounts and the reported areas for the 5′(*n*-1), 5′(*n*-3), 3′(*n*-1) and 3′(*n*-3) metabolites, with small amounts of shortmers found in the control sample (Table 2). For the 5′(*n*-2) metabolite the correlation was found to be poor. For the impurities sample, this could be attributed to an overlap of the -8 charge state isotopes of the metabolite with those of the -9 charge state of the FLP. For relative quantification of this compound based on MS data, a better chromatographic separation will be required. Alternatively, the Analytics module in SCIEX OS software can be used to perform quantification based on reconstruction of the TOF-MS data or based on fragment masses using MRM^{HR} data.
No signal was found for any of the charge states of the 3′(*n*-2) metabolite at the 0.1 and 0.3% spike-in levels. Possible causes for this could be sequence-specific adsorption or ion suppression.

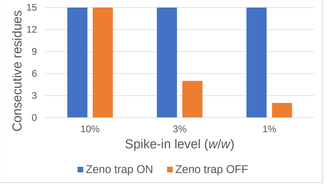
Table 2. Relative quantification of the spiked-in metabolites.

Spike-in (% w/w of FLP)	Spike-in (% w/w of total)	FLP (% area)	5′(*n*-1) (%area)	5′(*n*-2) (%area)	5′(*n*-3) (%area)	3′(*n*-1) (%area)	3′(*n*-2) (%area)	3′(*n*-3) (%area)
0	*0.00*	*81.5*	*NA*	*NA*	*0.26*	*NA*	*NA*	*0.020*
0.10	*0.10*	*79*	*0.12*	*NA*	*0.31*	*0.080*	*NA*	*0.080*
0.30	*0.29*	*79*	*0.25*	*0.10*	*0.46*	*0.11*	*NA*	*0.18*
1.00	*0.94*	*75*	*0.69*	*0.27*	*0.99*	*0.52*	*0.38*	*0.56*
3.00	*2.54*	*70*	*2.2*	*0.88*	*2.5*	*1.7*	*1.5*	*1.7*
10.00	*6.25*	*52*	*6.1*	*4.6*	*6.1*	*5.1*	*5.5*	*4.8*

Structural confirmation

The potential metabolites suggested by the software are based on accurate mass and isotope pattern matching of the TOF-MS data. MS1 data does not provide information for the localization of a modification, or the sequence of a potential metabolite. However, the Molecule Profiler application can help in confirming the structures of each of the potential metabolites by annotating the MS/MS spectra. Allowing for *a*, *w*, *c*, *y* and *d* terminal fragments, and allowing for the loss of one base or water molecule, the software was used to determine consecutive sequence coverages. As an example, the number of consecutive residues covered in the MS/MS spectra of the -9 charge state for the 3′(*n*-3) metabolite at various spike-in levels is shown in Figure 4. Consecutive coverage was found down to the 1% level when the Zeno trap was used. Without the Zeno trap, the coverage was significantly lower, as expected. When allowing for two bonds to break, consecutive coverage was seen at the 0.3% and 0.1% levels as well, with the Zeno trap on (data not shown). Figure 5 illustrates how much more information-rich MS/MS spectra with higher S/N can be acquired using the Zeno trap compared to traditional MS/MS analysis. The higher quality data enabled the assignment of significantly more fragments with great S/N by the software. The high-quality MS/MS information can be used to verify/confirm the identification based on MS further.

Figure 4. Consecutive residues for different spike levels of the 3′(*n*-3) metabolite, with and without use of the Zeno trap. The metabolite has a length of 15 nucleotides.



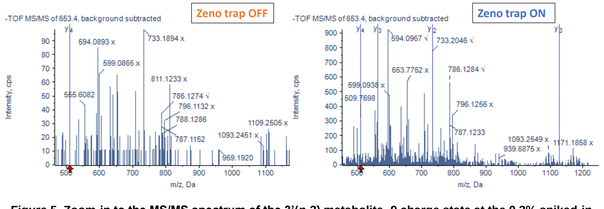


Figure 5. Zoom-in to the MS/MS spectrum of the 3′(*n*-3) metabolite -9 charge state at the 0.3% spiked-in level with *y* fragment annotation. Data was acquired without using the Zeno trap (left) and with using the Zeno trap (right). Just one *y* fragment ion was found in the MS/MS spectrum acquired without using the Zeno trap, while four were found when the Zeno trap was used. S/N of the spectrum with Zeno trap ON was approximately 10x better showing significantly more automatically assigned fragment ions in Molecule Profiler application.

CONCLUSIONS

- Excellent MS data quality and mass accuracy were leveraged for the identification of FLP and spiked-in impurities/metabolites down to a 0.1% level
- A fast and accurate relative quantification of oligonucleotides and their metabolites based on MS can be performed in Molecule Profiler application enhancing product understanding and PK/PD and toxicology studies
- The annotation of MS/MS with the most commonly found fragment ions based on a proposed structures in Molecule Profiler application greatly reduces manual workload and speeds up the correct identification of oligonucleotide-based drugs, their impurities and biotransformation products
- MS/MS data quality and fragment assignment can significantly be enhanced using the Zeno trap with the ZenoTOF 7600 system, which allows for the identification of even very low-abundance metabolites

TRADEMARKS/LICENSING