

Identification and relative quantification of oligonucleotide metabolites from extracted rat plasma

Identify and confirm the structure of oligonucleotide metabolites using the X500B QTOF system and Molecule Profiler software from SCIEX

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This technical note describes the identification, relative quantification and structural confirmation of 9 metabolites of an oligonucleotide spiked in a rat plasma extract.

Oligonucleotide therapeutics and gene therapies are rapidly gaining attention as their potency improves and delivery challenges are addressed. Modalities such as antisense oligonucleotides (ASOs) are becoming more important due to their high specificity and ability to reach formerly undruggable targets. To ensure safe drugs, methods for the identification and characterization of the full-length product (FLP) and metabolites are critical. Accurate mass spectrometry (MS) can be used for the identification of potential metabolites, by comparing the measured *m/z* and isotope patterns with those calculated. However, there is a lack of powerful, yet intuitive processing software and manual interpretation is cumbersome and time consuming. Furthermore, structural confirmation leveraging MS/MS adds an additional level of complexity.

Using the Molecule Profiler software to overcome these challenges, this technical note shows the identification and relative quantification of 9 potential metabolites of a fully

phosphorothioated and partially 2'-O-(2-methoxyethylated) and 5-methylated FLP spiked into a plasma extract at 3% (w/w). The software can perform relative quantification based on TOF MS and assign MS/MS fragment ions of potential metabolites to confirm their structures, facilitating the characterization of drugs in development (Figure 1).

Key features of the SCIEX X500B QTOF system and Molecule Profiler software for oligonucleotide metabolite analysis

- Excellent data quality and high mass accuracies for TOF MS and TOF MS/MS data allow for confident assignment of oligonucleotide FLP and metabolites using the Molecule Profiler software
- Straightforward relative quantification based on TOF MS peak areas is achieved by grouping charge states considering all isotopes for highest accuracy
- Fragment coverage displays of proposed sequences based on MS/MS data enable confident identification of metabolites based on user selectable fragment ion types

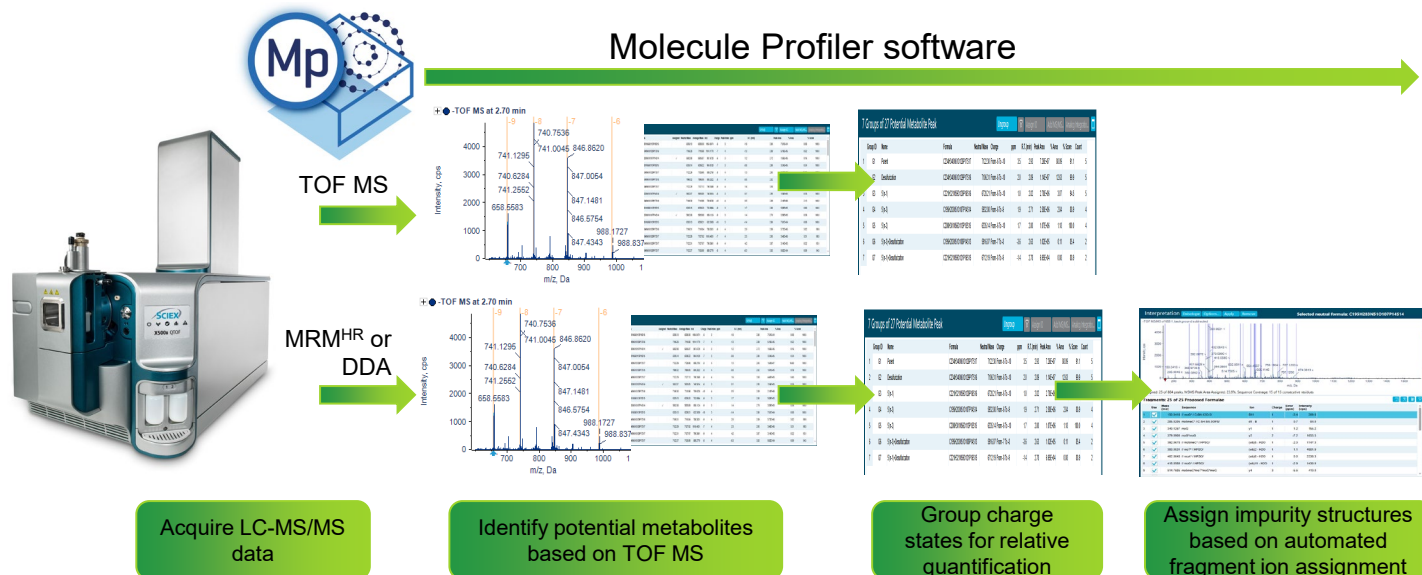


Figure 1. Workflow representation for relative quantification and structural confirmation of metabolites using the Molecule Profiler software.

Methods

Samples and reagents: A 20-mer fully phosphorothioated oligonucleotide with 2'-O-methoxyethyl and 5-methyl modifications (ASO-20PS2MOE-FLP) that has the same sequence as mipomersen, a drug developed for the treatment of familial hypercholesterolemia via RNaseH-mediated degradation of apoB-100 mRNA, was used as the FLP. To simulate metabolites, the 5'(n-1), (n-2) and (n-3) shortmers and 6 metabolites identified in the literature (see Table 1) were used.¹ All oligonucleotides were ordered (desalted) from IDT. The ion-pairing reagents 1,1,3,3,3-hexafluoroisopropanol (HFIP, ≥99.8%) and diisopropylethylamine (DIEA, ≥99.5%,) were purchased from Sigma Aldrich.

Sample preparation: 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 40°C, the plasma extract was reconstituted in 1 mL mobile phase A. Samples of 20 µg/mL FLP and of the FLP plus the 9 metabolites at 3% (w/w relative to a total amount of 20 µg/mL) were prepared in the rat plasma extract.

Table 1. Metabolites used for the simulated metabolite sample.

Analyte	Molecular weight (Da)	Description in literature reference ¹
FLP	7,177.1	-
5'(n-1)	6,757.8	-
5'(n-2)	6,364.4	-
5'(n-3)	5,971.1	-
5'(n-6)	4,854.1	A
5'(n-7)	4,508.8	B
5'(n-9)	3,869.3	C
5'(n-10)	3,549.0	E
3'(n-11)	3,229.8	G
3'(n-12)	2,910.5	H

Chromatography: A Shimadzu LC-20 series HPLC system was used with water containing 15mM N,N-diisopropylethylamine and 35mM hexafluoro-isopropanol as mobile phase A and methanol as mobile phase B. A gradient from 10–40% B in 9 min with a 2 min wash step at 95% B was used at a flow rate of 0.3 mL/min. The column was a Waters ACQUITY PREMIER Oligonucleotide C18 (2.1 × 150 mm, 1.7 µm, 130 Å) kept at 70°C. The injection

volume was set to 10 µL, resulting in a total amount of 200 ng of oligonucleotides on column.

Mass spectrometry: A SCIEX X500B QTOF system was used in negative polarity using an MRM^{HR} method for the 3 most intense charge states of the FLP and each of the spiked-in metabolites (method details available on request). Collision-induced dissociation (CID) was used and collision energies (CE) were selected that ensured the generation of fragment-rich MS/MS spectra. The parameters for the final MRM^{HR} method are summarized in Table 2.

Table 2. MS parameters.

Parameter	MS	MS/MS
Scan mode	TOF MS	MRM ^{HR}
Polarity		negative
Gas 1		60 psi
Gas 2		60 psi
Curtain gas		30 psi
Source temperature		350°C
Ion spray voltage		-4500 V
Declustering potential		-80 V
CAD gas		7
Start mass	600 m/z	150 m/z
Stop mass	2,000 m/z	3,000 m/z
Q1 resolution	N/A	Low
Accumulation time	0.2 s	0.03 s
Collision energy	-10 V	available upon request
CE spread	0 V	5 V
Time bins to sum	6	12
QJet RF amplitude	190 V	190 V

Data processing: The molecular weight (MW) of the FLP and metabolites was confirmed by reconstruction using the Bio Tool Kit in the SCIEX OS software. Data were processed using the SCIEX Molecule Profiler software (v.1.2) for identification and relative quantification of the FLP and metabolites. Considering the structure of the oligonucleotide, the number of bonds to break in the parent structure and a comprehensive list of possible transformations (77 for the work presented), the software identifies the different charge states of potential metabolites. This assignment is based on the m/z match, a 10 ppm tolerance was used for the work presented in this technical note and the match between the measured and

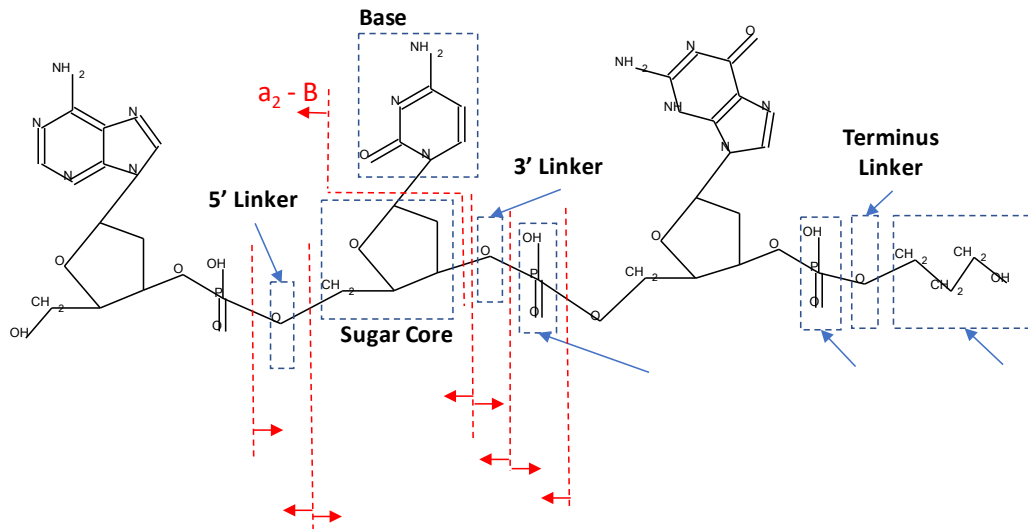


Figure 2. Representation of an oligonucleotide showing the different groups that can be defined for custom nucleotides. Also, the nomenclature of the MS/MS fragments used for sequence confirmation is shown in this figure, taken from the Molecule Profiler software.

theoretical isotope patterns. Additional transformations can be added, while also custom nucleotide residues can be used by defining the 5' and 3' linkers, sugar, base and phosphate groups (Figure 2). The list of found potential metabolites can be manually curated, and the different charge states of each potential impurity are grouped together for relative quantification based on the TOF MS data.

For confirmation of the structure of each potential impurity, MS/MS spectra can be automatically annotated with *a*, *b*, *c*, *d*, *w*, *x* and *y* fragments, including allowing for possible water and base losses (see Figure 2). Sequence coverage is automatically determined by the software, considering user selectable fragment types, signal-to-noise ratio and number of bonds to break. A map of the proposed sequence indicating the fragments that MS/MS evidence was found for is shown, facilitating an easy view of sequence coverage.

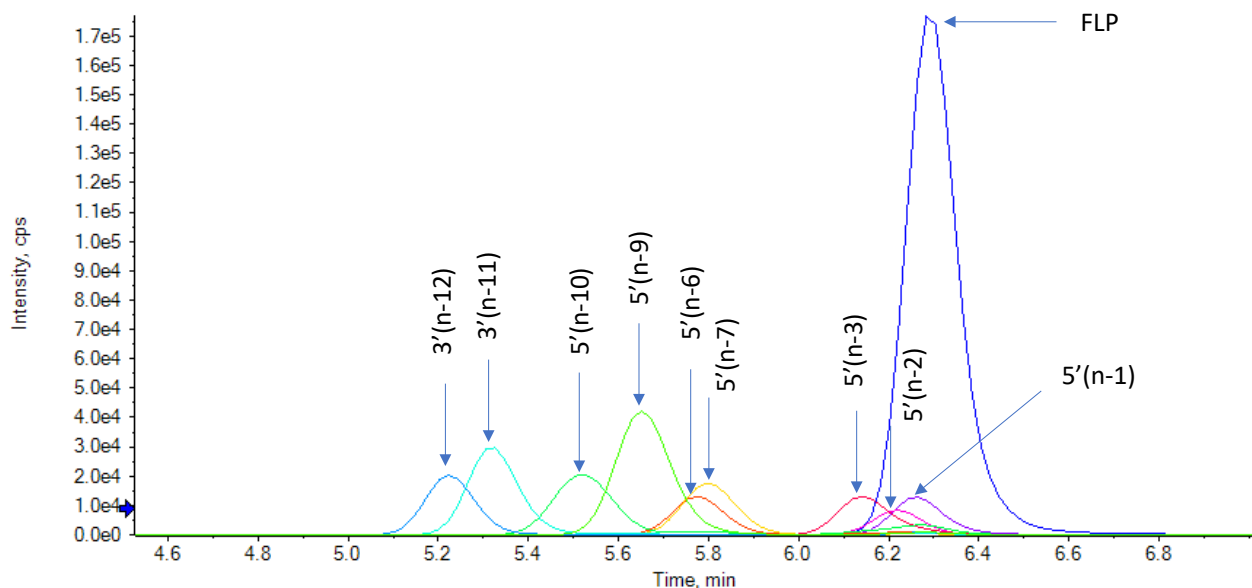


Figure 3. Extracted ion chromatograms (XIC) of the FLP and all spiked-in metabolites. One charge state for each of the analytes from the TOF MS data were used for XIC generation. Good separation from the FLP was achieved for most of the metabolites.

Chromatography

While chromatographic separation between the FLP and metabolites, which are only 1 or 2 nucleotides shorter in length is difficult, most of the metabolites could be separated from the FLP (Figure 3). Separation from the FLP is important, as the multiple charge states from both the FLP and metabolites can result in overlap on the m/z axis, interfering with correct identification and relative quantification.

MW determination using SCIEX OS software

As an example, Figure 4 shows the TOF MS spectrum of the C metabolite ($5'(n-9)$) acquired with the X500B QTOF system, a zoom-in of the -6-charge state and the reconstructed mass as calculated by the Bio Tool Kit software add-on that is part of SCIEX OS software. The high-resolution of the X500B QTOF system allows for baseline separation of the isotopes of each charge state. Excellent mass accuracy was observed. For the -5-charge state the mass accuracy of the monoisotopic isotope was -3.8 ppm and that of the reconstructed monoisotopic mass was -1.7 ppm.

Table 3. Mass accuracies for the FLP and all spiked-in metabolites.

Analyte	Theoretical monoisotopic mass (Da)	Mass error (ppm)
FLP	7,172.09168	-3.86
5'(n-1)	6,753.02523	-3.74
5'(n-2)	6,359.94927	-3.82
5'(n-3)	5,966.87331	-2.57
5'(n-6) (A)	4,850.70262	-3.22
5'(n-7) (B)	4,505.67294	-1.98
5'(n-9) (C)	3,866.61057	-1.70
5'(n-10) (E)	3,546.58737	-1.23
3'(n-11) (G)	3,227.54819	-0.99
3'(n-12) (H)	2,908.50901	-0.35

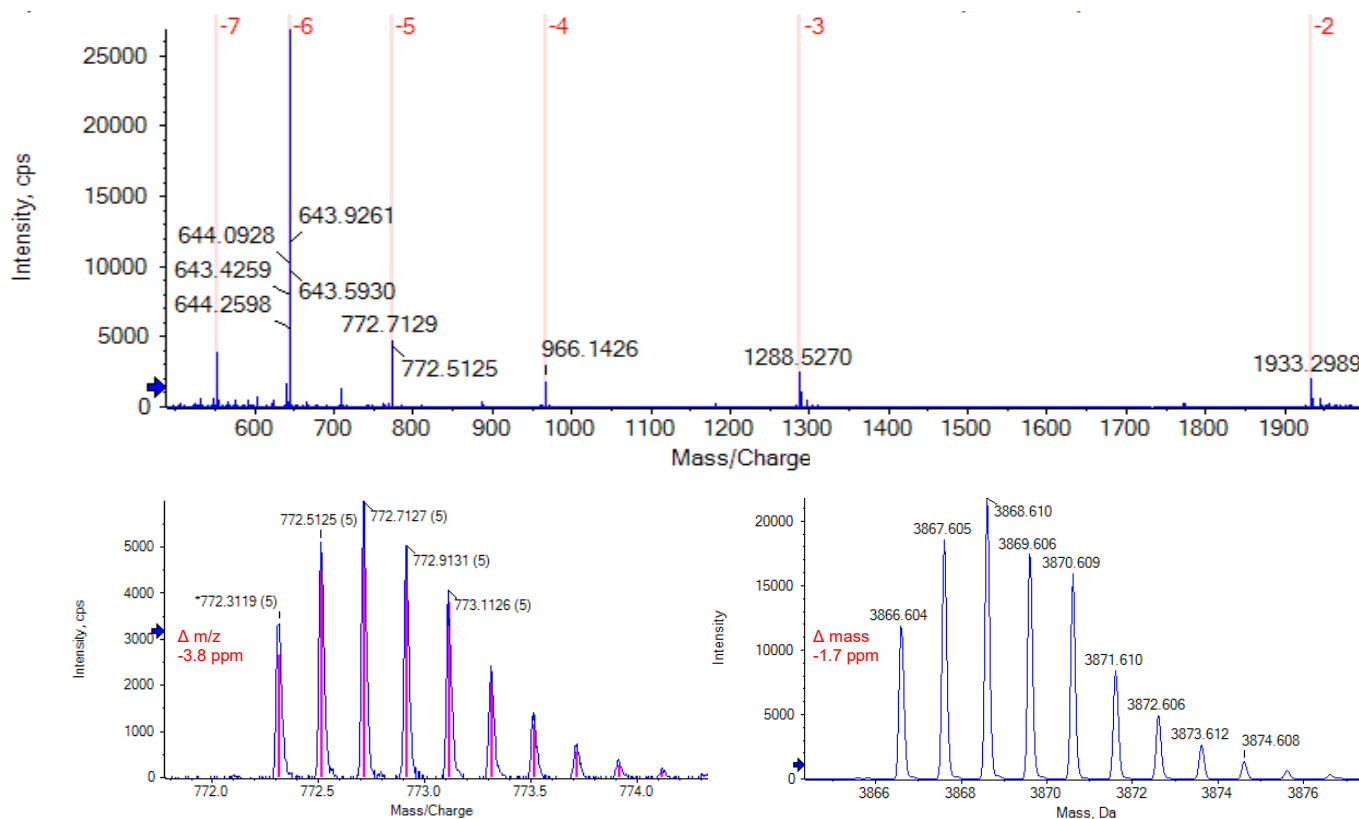


Figure 4. MS spectrum of the $5'(n-9)$ metabolite. Top shows the full spectrum with the charge state envelope from -2 to -7. The bottom shows a zoom-in of the -5-charge state (left) and the isotopically resolved reconstruction with a step mass of 0.03 Da (right). Mass error was -3.8 ppm for the monoisotopic peak of the -5-charge state and -1.7 ppm for the monoisotopic mass in the reconstructed spectrum.

Table 3 summarizes the mass errors of the measured, reconstructed monoisotopic masses for the FLP and the spiked-in metabolites.

Identification and relative quantification of FLP and impurities based on TOF MS data

The Molecule Profiler software was used to identify the FLP and metabolites. Figure 5 shows 2 examples of the match between the measured MS spectrum and the theoretical isotope pattern for the -6-charge state of the 5'(n-9) and -5-charge state of the 3'(n-11) metabolites. The excellent match between theoretical and measured m/z profiles allows for confident identification of these and other metabolites. Figure 6 shows the top 15 of 82 identified potential impurities by %area after grouping charge states for an injection of the ASO-20PS2MOE-FLP spiked with 3% (of total weight) of each metabolite. The scoring was selected to be performed on the isotope ratio matching only, while an m/z tolerance of 10 mDa was used for searching. A 40% cut-off percentage for the score was used to ensure only charges states with a good match of the isotope ratio were reported. Charge states from -3 to -11 were considered and only terminal fragments were allowed for the search. When multiple potential metabolites were listed for a specific peak, the lowest scoring metabolite was manually removed. All 9 spiked-in metabolites were found in the top 15 reported potential metabolites. The high % of the desulfurization product was likely caused during sample storage.

Table 4. Average %areas measured for the different 5'(n-1) spike-in levels. As the spiked-in shortmer was only 76% pure as determined by TOF MS and Molecule Profiler software, the measured values were in very good correlation with the spiked-in amounts.

Metabolite	Average %area in FLP	Average %area in 3% spike-in	RSD %area in 3% spike-in
5'(n-1)	0.21	3.23	9.20
5'(n-2)	0.11	2.94	4.65
5'(n-3)	0.41	4.00	6.34
5'(n-6) (A)	0.79	3.83	5.28
5'(n-7) (B)	0.24	5.07	4.55
5'(n-9) (C)	0.42	7.73	2.79
5'(n-10) (E)	1.30	6.65	6.02
3'(n-11) (G)	0.00	2.71	4.49
3'(n-12) (H)	0.00	2.60	6.17

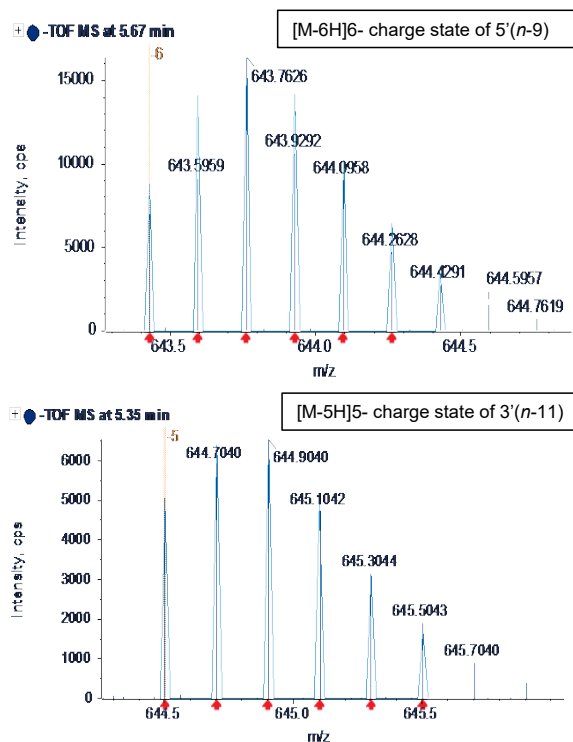


Figure 5. Comparison of the observed m/z and theoretical information for 2 metabolites. An excellent match between the theoretical isotope distribution (light blue) and the centroided MS data (dark blue) of the [M-6H]6- charge state of the 5'(n-9) metabolite (top) and [M-5H]5- charge state of the 3'(n-11) metabolite (bottom) was observed. Dotted yellow line highlights the monoisotopic peak with identified charge state.

82 Groups of 187 Potential Metabolite Peaks

Name	Neutral Mass	ppm	% Area	Count
Parent	7172.05	-5.2	35.35	7
5'(n-9)	3866.59	-4.4	7.81	5
5'(n-10)	3546.57	-4.9	6.67	5
5'(n-7)	4505.65	-4.4	5.17	7
5'(n-6)	4850.68	-5.3	3.94	6
5'(n-3)	5966.84	-5.0	3.89	8
Desulfurization	7156.07	-8.2	3.56	4
5'(n-1)	6752.98	-5.7	3.10	6
5'(n-2)	6359.91	-4.9	3.00	7
3'(n-11)	3227.53	-4.1	2.77	4
3'(n-12)	2908.50	-4.3	2.67	3
3'(n-6)	4851.68	-1.5	2.35	2
Loss of A	7036.99	-8.2	1.54	2
5'(n-10)+Desulfurization	3530.60	-4.6	1.51	5
Phosphorylation + Depyrimidination of U	7158.08	5.6	1.39	2

Figure 6. Top 15 of 82 identified potential impurities. Sorted by %area after grouping charge states for an injection of the ASO-20PS2MOE-FLP spiked with 3% (of total weight) of each of the metabolites. "Count" shows the number of charge states of the impurities that were found. When multiple potential metabolites were listed for a specific peak, the lowest scoring metabolite was removed. All 9 spiked-in metabolites were identified.

Although in-source formation of this product is possible, a difference in retention time for the FLP and desulfurization product was observed, ruling out in-source fragmentation as the cause for the observed desulfurization product.

Table 4 shows a summary of 3 replicate analyses of the spiked-in metabolite sample. Reproducibility of the relative quantification was excellent, with the RSD for each metabolite <10%. The reported relative areas for the metabolites were in-line with the spike-in, except for B, C and E analytes which were reported to be 1.7 to 2.5x higher as the calculated spike-in. Metabolite E was present in a significant amount in the control sample (FLP without spike-in), which can partly explain the higher relative amount reported for this metabolite. In addition, it is possible that (some of) the other analytes used for spike-in contained B, C and/or E analytes, derived from the serial synthesis approach of the ordered analytes.

Confirmation of structures using MS/MS data

For the FLP and spiked-in metabolites MS/MS data were acquired using an MRM^{HR} method. The Molecule Profiler software was used to annotate the peaks in the spectra and to generate a sequence coverage map. Figure 7 shows part of the raw MS/MS spectrum of the -6-charge state of the 5'(n-9) metabolite and the corresponding, deisotoped spectrum in the Molecule Profiler software. The doubly charged γ 4 ion was automatically assigned by the Molecule Profiler software. Figure 7 also shows that using MS/MS data derived from the -6 charged precursor only, full sequence coverage with evidence for the position of each nucleotide was achieved. A minimum S/N setting of 50 was used for the assignments and only *a*, *c*, *d*, *w* and γ -ions were considered with the possible loss of 1 H₂O molecule.

Figure 8 shows a summary of the sequence coverage found based on the MS/MS spectra for the FLP and all spiked-in metabolites. Except for the 5'(n-6) metabolite, sequence coverage was 100% for the metabolites. To enable easy review of information, the software combines assigned fragments from the MS/MS spectra from all charged states for a given metabolite. This can improve sequence coverage significantly, as some fragments might only be generated from specific charge states.

Conclusions

- Confident identification of metabolites spiked-in at the 3% (*w/w*) in a plasma extract sample was achieved with excellent TOF MS data quality and mass accuracy of the X500B QTOF system
- Relative quantification using Molecule Profiler software showed good correlation of the reported relative amount with the spike-in percentage with excellent reproducibility (RSD <10%)
- The confirmation of the structure of oligonucleotide-based drugs and impurities is greatly accelerated by automatic annotation of TOF MS/MS spectra with the most commonly found fragment ions based on a proposed structures in Molecule Profile software

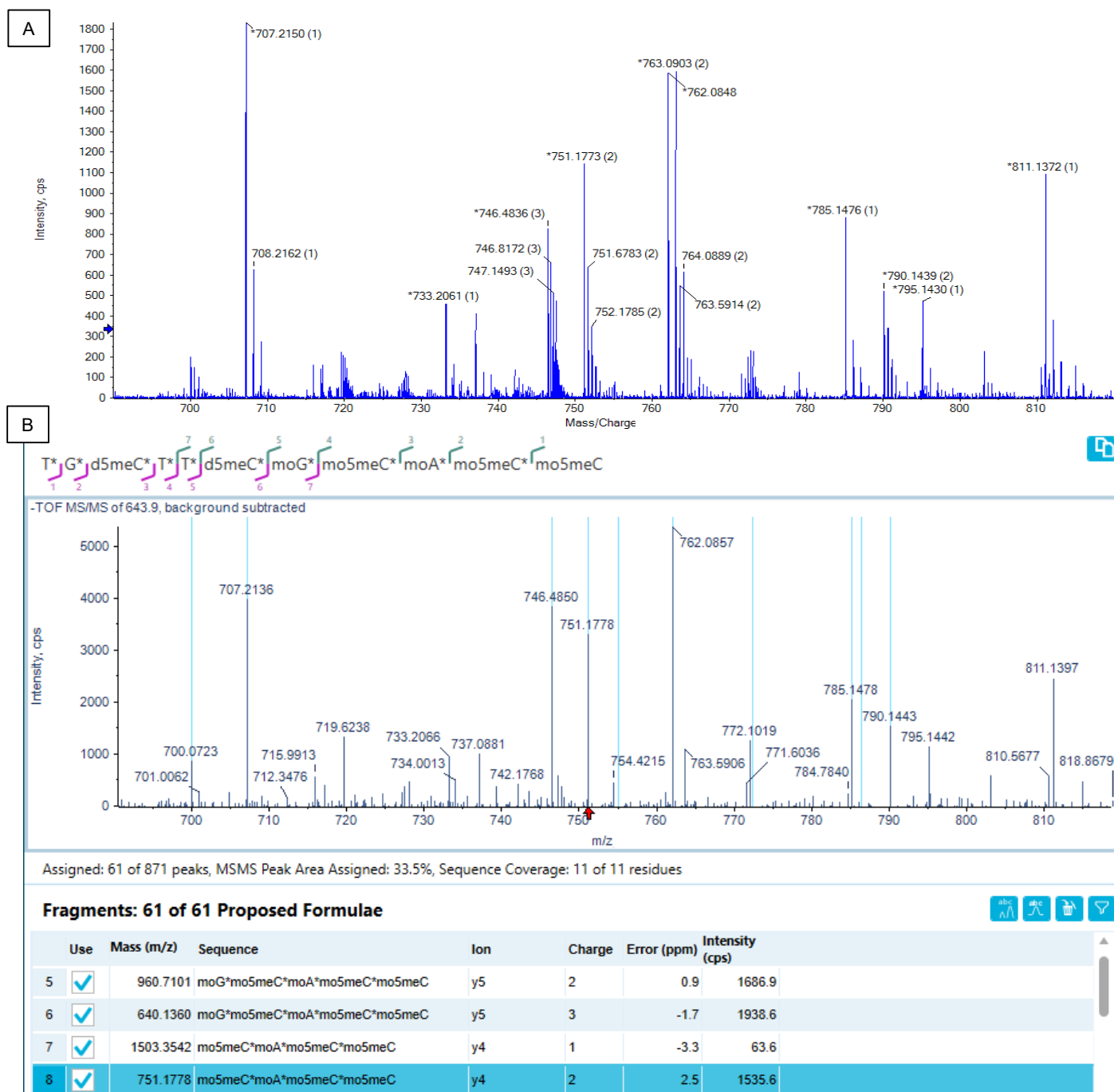


Figure 7. Confirmation of the sequence of the 5'(*n*-9) metabolite using MS/MS of the [M-6H]6- charge state. A zoom-in of the raw MS/MS spectrum is shown on top (A). To enable easier data review, the MS/MS spectrum was de-isotoped in the Molecule Profiler software (B). Using a, c, d, w and y terminal ions and allowing for 1 water or base loss, the full sequence could be confirmed considering fragments with a minimal S/N ratio of 50.



Figure 8. Summary of sequence coverage for the FLP (Parent) and all spiked-in metabolites. Sequence coverage is determined using fragments assigned in all identified charge states of a metabolite that MS/MS spectra were acquired for. A, c, d, w and y terminal ions were considered and 1 water or base loss was allowed. A minimum S/N ratio of 50 was used.

References

- Richard S. Geary, Brenda F. Baker, Stanley T. Crooke.
Clinical and Preclinical Pharmacokinetics and
Pharmacodynamics of Mipomersen (Kynamro®): A Second-
Generation Antisense Oligonucleotide Inhibitor of
Apolipoprotein B. [Clin Pharmacokinet \(2015\) 54:133–146](#)

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