Automated determination and profiling of therapeutic oligonucleotide impurities and metabolites in LC-HRMS data

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ABSTRACT

This presentation highlights several aspects of a typical metabolite and impurity data processing pipeline, that need refinement to support analysis of therapeutic nucleic acids modalities ranging in size from 5 000 to 10000. Entries in a customizable library of oligonucleotides defined in terms of their 5 building blocks, were used to describe the parent molecule and predict its metabolic and impurity products. Isotope pattern fidelity in collected HR MS was instrumental in compound confirmation and the signal from series of isotope peaks was utilized in profiling workflows. Automated data processing of series of samples was enabled through the Molecule Profiler App integrated in the SCIEX OS software.

INTRODUCTION

Nucleic acid modalities, such as siRNA, anti-sense oligonucleotides and aptamers, have become emerging therapeutic candidates in biopharmaceutical development. To be able to succeed in the development and clinical pipelines and meet the regulatory requirements, the biophysical properties and purity of these candidate molecules must be well understood. Recent advancements in the chromatographic separations coupled to high resolution high sensitivity accurate mass spectrometry systems facilitate workflows that are capable of confident identification and relative quantitation of oligonucleotides, as well as their process-related impurities and metabolic or degradation products. To provide timely accurate qualitative and quantitative results, dedicated data reduction tools focused on the mass spectrometry of nucleic acids, need to be deployed in the analytical pipeline.

MATERIALS AND METHODS

Sample Preparation:
representative set of oligonucleotides (11- to 34-mer)
a pure oligonucleotide solution spiked with shortmers at low concentrations was investigated.

Instrumental Analysis:

LC-HRMS System:
Agilent 1290 Infinity LC and SCIEX ZenoTOF 7600 system or TripleTOF 6600+ system.
LC column: Waters ACQUITY UPLC Oligonucleotide BEH C18, 2.1mmx50mm 1.7u 130 Å.

LC/MS Acquisition Method:
The column was maintained at 60 ºC, with a mobile phase consisting of Solvent A: 100 mM hexafluoro-2-propanol [HFIP] and 10 mM triethylamine (TEA), and Solvent B: methanol.

After HPLC separation, the samples were analysed with quadrupole-time-of-flight (QTOF) tandem mass spectroscopy (MS/MS) on a TripleTOF 6600 system (SCIEX) equipped with a DuoSpray ion source.

DISCUSSION

Oligonucleotide chemical space

Name	type	# nucleotides	Molar mass g-mol−1	monoisotopic to base peak	Index of base peak
Fomivirsen	DNA, phosphorothioate	1*21	6682.35	15.0%	5
Mipomersen	2-methoxyethyl DNA mix, phosphorothioate	1*20	7594.76	11.7%	5
Inotersen	2-methoxyethyl DNA mix, phosphorothioate	1*20	7183.08	11.6%	5
Eteplirsen	Phosphorodiamidate morpholino	1*30	10305.89	4.3%	6
Golodirsen	Phosphorodiamidate morpholino	1*25	8647.401	8.9%	5
Nusinersen	2-methoxyethyl	1*18	7500.86	12.2%	5
Patisiran	Double stranded RNA with 2'-O-methyl	2 * 22	13424.39	2.2%	7
Givosiran	Double stranded 2' O-methyl and 2' fluoro	21+23	16300	0.5%	9
Pegaptanib	5' 40kDaPEG single stranded and double stranded mix	27	~50 000	<0.1%	
Defibrotide	single stranded and double stranded mix	variable		<0.1%	

Table 1. List of 10 FDA approved drugs to 2020[1], their chemical make-up and characteristics to be considered in LC-MS data analysis.

The last two drugs do not have the isotope pattern resolved in TOF MS at R=40000, and thus they were not considered in our project.

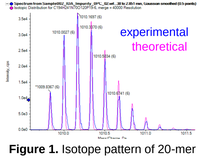


Figure 1. Isotope pattern of 20-mer ssDNA (M=6065.04)
CGG CTA CCT TGT TAG CAC AT

Oligonucleotide structure details in hypothesis-driven data mining

Many expected metabolic or degradation products are results of hydrolysis, cleaving a portion of the molecule, or modifications associated with a specific part of oligonucleotide. For efficient data analysis, studied oligonucleotide molecule is described as a chain of residues. Each residue has up to 5 building blocks that describe the base, sugar, linker (phosphate) core and 2 linker atoms that connect the linker core from 3' and 5' sides to a sugar. Some of the parts can be missing for special cases of residues, such as 3' and 5' termini and some linker cores (such as morpholino). Residue symbols have special characters and abbreviated information on modifications to base, sugar and linker core, for example, "*" indicates phosphorothioate linker.

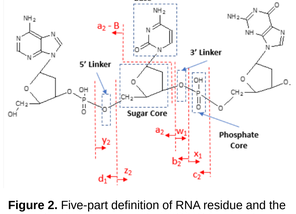


Figure 2. Five-part definition of RNA residue and the respective in-silico generated MS/MS fragments.

Five -part oligonucleotide structure representation enables:
- prediction of hydrolytic cleavages
- in-silico generation of accurate MS/MS fragments
- ability to predict synthetic impurities, including internal omission and addition impurities, and assess compatibility with a putative degradation reaction

Transforming LC/HRMS oligonucleotide data into qualitative workflow results

Challenge	*Solution*
The TOF MS signal of an oligo is distributed across isotope patterns for range of charge states and, optionally, it has different H-alkali metal exchanges	To capture the total biomolecule response, series of accurate TOF MS XICs are summed or, the peak areas in accurate XICs are summed.
Isotope patterns have many peaks and monoisotopic peak for low abundant species may be immersed in noise	Average mass supported with a score for isotope pattern fit and where possible monoisotopic mass confirm compound mass.
Single precursor isotope peak not transmitted through Q1 and results in complex MS/MS spectrum with fragment isotope signal	Fragment charge state and fragment isotope pattern provide more confident fragment assignment
Impurities and metabolites are not fully chromatographically separated	HRMS enables the orthogonal separation of closely eluting peaks
Ionization efficiencies of parent and impurities may not be the same	Parallel UV detection and processing

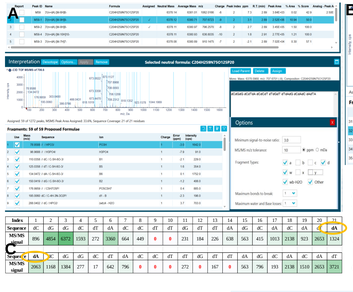
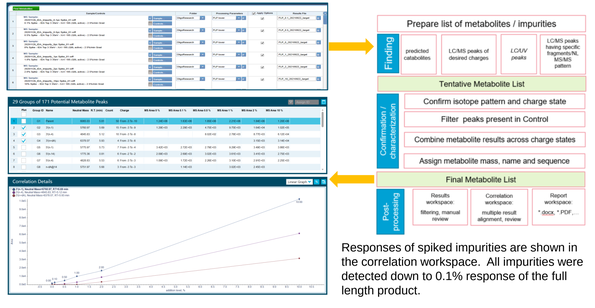


Figure 2 illustrates steps in sequence characterization of an "n+dA" spiked impurity. Terminal MS/MS fragment assignments lead to unambiguous confirmation of sequence that has adenine attached at 3' terminus (B). Isotope patterns of fragments aid in sequence confirmation (C).

Figure 3. Role of Isotope Score in metabolite assignment.

As large biomolecules have complex isotope patterns, two putative molecules of similar size are expected to have common peaks in their isotope pattern series. Isotope Score reflects how well each peak in respective isotope series fits the theoretical biomolecule isotope distribution, and thus it is instrumental in resolving competing identification assignments.

Figure 6. Processing steps in a workflow for relative quantification and structural confirmation of metabolites integrated in the Molecule Profiler App.



Responses of spiked impurities are shown in the correlation workspace. All impurities were detected down to 0.1% response of the full length product.

CONCLUSIONS

- Negative polarity LC HRMS with the information dependent acquisition gave isotopically resolved accurate mass data for series of tested sequences of oligonucleotides having molar masses up to 10000. The high-resolution MS/MS data allowed both quantitative and qualitative analysis to be performed in a single run.
- Expanded LC-HRMS data processing workflow for metabolite and impurity profiling streamlined data analysis in support of these new modalities.
- Five-part nucleotide residue description was developed to cover the breadth of emerging chemistries in the oligonucleotide drug design.
- The new tool, integrated into SCIEX OS software as a Molecule Profiler App, was instrumental in detecting, resolving and annotating expected and unexpected cleavage impurities as well as constructing semi-quantitative impurity profiles.

REFERENCES

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