Oligonucleotide analysis

Green ion pair and HFIP free method for ASO RNA analysis with GLP compliant automated data handling

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Abstract

The progress made with delivery mechanisms for oligonucleotides has increased the development of therapeutic oligonucleotides in recent years. Oligonucleotides such as mRNA and small synthetic therapeutic RNA have quickly become a promising new market on the biopharmaceutical horizon for the treatment of numerous diseases. An ever-growing number of oligonucleotide modifications, such as thiolation of the phosphodiester bonds, incorporation of locked nucleic acids, or methylation of bases and sugars aid efficacy and protect the oligonucleotide drug from nuclease attack. This creates an analytical need to monitor and characterize these new modalities. Method development for characterization of new oligonucleotide therapeutics has never been an easy process, The new modalities arriving make it even more difficult. There is also a move to stop using HFIP (hexafluoroisopropanol) due to the fluoride content and laboratories increasingly shy way for the use of the amine ion pairing agents. In addition, there has never been a GLP compliant methodology approved by the regulatory bodies.

It is difficult to impossible to separate chromatographically all the known impurities present in the oligonucleotide sample. Mass spectrometry has been accepted as a second-dimension detector capable of filling these gaps. Here we will describe a simple workflow to rapidly develop reliable methods for new modalities without using HFIP or ion pairing chromatography that is easy to perform and GLP compliant.

Introduction

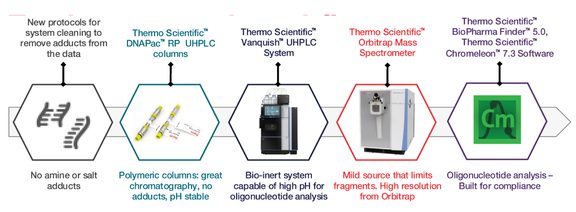
Here we demonstrate how therapeutic oligo-nucleotides such as antisense oligonucleotides (ASOs) can be analyzed rapidly, comprehensively and in compliance with regulatory requirements using reversed-phase (RP) liquid chromatography coupled to a high-resolution Thermo Scientific™ Orbitrap Exploris™ MS. Thermo Scientific™ Chromeleon™ (CM) chromatography control and data handling software provides the compliance. Deconvolution and targeted XIC's were used in the data analysis. We have analyzed pharmaceutically-relevant ASO's at the intact level that feature various modifications such as backbone phosphor-thiolation, incorporation of locked nucleic acids, and O-methoxyethyl modifications. Here, we describe the procedure for analysis and method development using a commercial ASO, Nusinersen. Particular care was given to the avoidance of adduct formation and source induced impurities.

Materials and methods

Sample

Synthetic antisense oligonucleotide (ASO)

- Length: 18 nts Nusinersen (Spinraza)
- Backbone Modifications: Phosphorothioate, 2'-O-2-methoxyethyl



LC-MS Measurement

UHPLC : RP separations were performed with a Thermo Scientific™ DNAPac™ RP column (4 µm, 2.1 ×100 mm) using a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. The ammonium acetate eluent system was developed to allow good separation, low adducts and high sensitivity.

Mass Spectrometry: ASO purity analysis was performed at high resolution on an Orbitrap Exploris MS. Controlled by Chromeleon software.

Data Analysis: Thermo Scientific™ Chromeleon™ 7.3.2 software was used for identification and relative quantitation of the oligonucleotide full-length product (FLP) and their impurities. Characterization used deconvolution, with the impurities found transferred within the software to a component table for quantitation by XIC of the found impurities which were determined to be real. A report was generated with flexible impurity annotation. Quantitation was validated with isotopic sliding windows deconvolution and extracted ion chromatograph signals.

Workflow

- Ion pair free separation with reversed phase at high pH and deconvolution in CM
- Use of CM reporting engine 2.0 – faster, more functions, 64 bit, Thermo Scientific™ Ardia™ platform ready
- Automatic annotation of the full-length product (FLP) and filtered data to show true impurities not adducts or in-source generated impurities
- Internal transfer within CM of identified components to an XIC component table. CM allows input of additional target XIC's
- Automatic reporting, including results tables, deconvoluted spectra, abundance values, XIC's, bar graphs
- Comparison of deconvoluted and targeted XIC results.

Results

Optimisation of source conditions

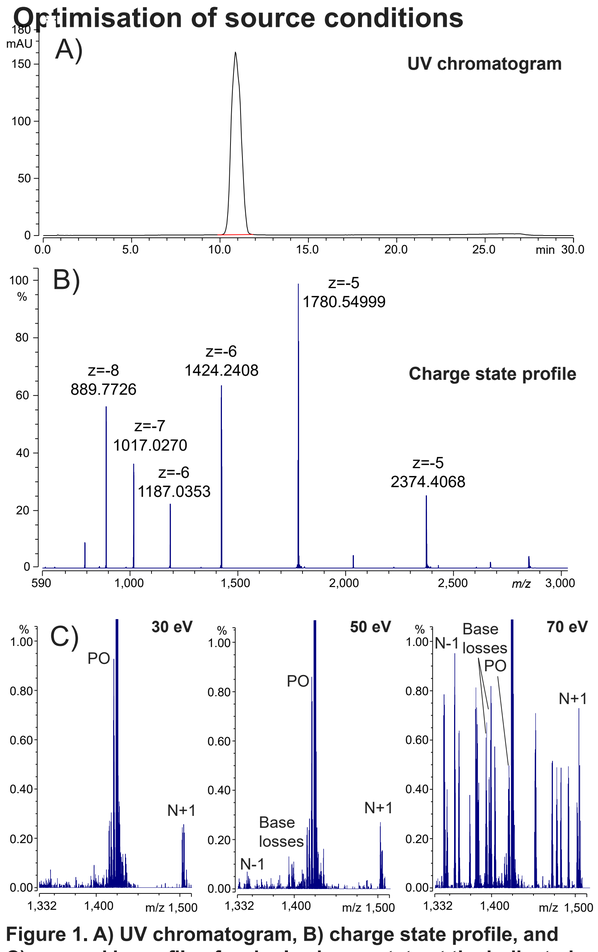


Figure 1. A) UV chromatogram, B) charge state profile, and C) zoomed in profile of a single charge state at the indicated in-source collision energies.

Optimisation of the source conditions is critical to remove adducts without producing in-source fragments that can be mistaken for real impurities. As we have not used any amine ion pairing in the method, the source conditions can be kept low as there will be no amine adducts to remove. **Figure 1C** shows the effect on in-source impurity generation with increasingly harsher source conditions. Nusinersen is quite stable and shows little indication of breaking down in the source until 70 eV is applied. At this voltage base loss appears as well as N-1 impurities that are not present in the original sample.

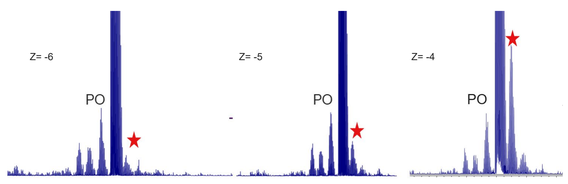
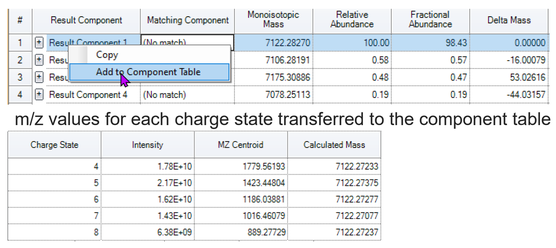


Figure 2. Zoom of different charge states showing differences in the impurities present.

Figure 1B shows there are multiple charge states present in the HRMS profile. This can be used to indicate which impurities are real and require monitoring by XIC. **Figure 2** shows a zoom of the charge states -6, -5, and -4. The PO impurity remains constant in all charge states where the impurity shown with a red star reduces considerably in the higher charge states, indicating it is not real. Most adducts appear preferentially on the lower charge states and could potentially interfere with quantitation.



#	Result Component	Matching Component	Monoisotopic Mass	Relative Abundance	Fractional Abundance	Delta Mass
1	Result Component 1	(No match)	7122.28270	100.00	98.43	0.00000
2	Resu		7106.28191	0.58	0.57	-16.00079
3	Resu		7175.30886	0.48	0.47	53.02616
4	Result Component 4	(No match)	7078.25113	0.19	0.19	-44.03157

m/z values for each charge state transferred to the component table

Charge State	Intensity	MZ Centroid	Calculated Mass
4	1.78E+10	1779.56193	7122.27233
5	2.17E+10	1423.44804	7122.27375
6	1.62E+10	1186.03881	7122.27277
7	1.43E+10	1016.46079	7122.27077
8	6.38E+09	889.27729	7122.27237

Figure 3. Transfer of the XIC values of selected deconvoluted components to the quantitation component table in Chromeleon software.

Figure 3 and **Figure 4** show the steps to internally transfer XIC values for different charge states from the deconvolution result table to the component table for quantitation, all inside Chromeleon. software, Additional components can still be added to the component table manually if required. Once the selected components are in the quantitation table the charges states to be used in the calculations can be selected, the mode of quantitation and the integration parameters can then be optimised.

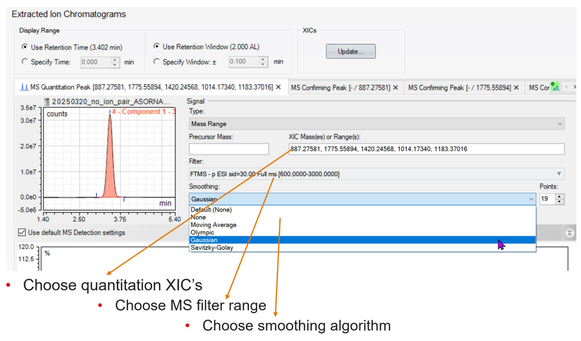


Figure 4. Example of the MS quantitation parameter settings within Chromeleon software.

Quantitation in MS is most often done using the XIC signals of the targeted components, which is simple with small molecules that have only one charge state. Oligonucleotides have multiple charges states that could potentially be used. In this workflow, the quantitation by deconvolution is presented alongside the results using the targeted XIC signals. This gives an extra layer of confidence in the results and aids in choosing the best charge states to use, as shown in **Figure 2**.

Chromeleon software can use the UV as well as the XIC MS signals for quantitation in a GLP environment. This is a market need that has not been previously fulfilled.

Component	Deconvolution	XIC component table
FLP	97.88	97.47
PO	1.22	1.30
CNET	0.30	0.35
-44	0.22	0.25
N+1	0.15	0.12

Table 1. Component quantitation: Comparable results between deconvolution and XIC

The quantitative results shown in Table 1 reveal comparable results obtained by XIC and deconvolution.
Figure 5 shows the annotated impurities found at low level in the Nusinersen sample. The data is very clean and easy to interpret due to the removal of adducts and source induced impurities.
Similar results (not shown) have been obtained using a triple quadrupole MS with the same source and LC conditions.

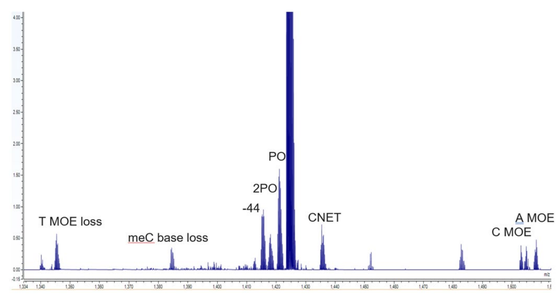


Figure 5. Zoom of the impurity isotopic profiles around the full-length product (FLP) with identifications.

Conclusions

- RP-LC HRAM MS is a powerful technology to analyze the purity of ASOs
- Removing ion pairs allows softer source conditions to prevent in-source impurity generation
- Utilization of deconvolution or an intelligent selection of target XICs and charge states provides comparable results.
- Removing ion pairing agents and HFIP is a goal for many companies
- Chromeleon enables the fast and reliable identification and relative quantification of ASO and their impurities with deconvolution, XIC and UV detection channels.
- ASO purity analysis in CM can be fully automated including reporting
- CM is built for compliance

Acknowledgements

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