Method considerations for therapeutic ASO RNA analysis. Adducts and insource impurity generation

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Abstract

Development of a new high resolution mass spectrometry (HRMS) method to simplify the impurity analysis of ASO RNA and siRNA therapeutic oligonucleotides. A new chromatography method which maintains chromatographic resolution yet allows amine, HFIP and metal ion adducts to be essentially eliminated in the HRMS data. A Thermo Scientific™ Orbitrap Exploris™ 240 and an MX Mass Spectrometer was used for sensitivity, with optimised source conditions to remove adducts without generating in-source impurities. Thermo Scientific™ BioPharma Finder™ V 5.2 Software was used for identification and relative abundance using sliding windows Xtract algorithm with isotopically resolved data. Thermo Scientific™ Chromeleon™ 7.3.2 software was used for GLP compliance and reporting with the same deconvolution engine.

The finalized method is fully automated, GLP compliant and can be applied to multiple RNA drug products with little effort.

Introduction

Oligonucleotide analysis has gained considerable interest over the last few years with the successful introduction of mRNA vaccines and the synthetic short interfering RNA therapeutics. Synthetic RNA drug products extend the need for new analytics to accurately determine impurities generated during manufacture. The highly charged, linear chain structure already provides analytical challenges. The introduction of sulphur onto an asymmetric phosphate group for stability causes additional chromatography related problems. Amine ion pair and metal adducts create a quantitation problem due to multiple split signals. In-source generated impurities compound this problem. A successful routine method with optimized HRMS conditions has been developed for synthetic oligonucleotide impurity analysis which resolves all of these problems.

Materials and methods

Sample preparation: Synthetic modified RNA and DNA oligonucleotides were purchased from Thermo Fisher Scientific as a full-length product [FLP] and with integrated PO, N-1 and N+1 impurities. These were diluted in water to 1mg/ml.

Td-sAd-sCd-sAd-sGd-sCd-sAd-sTd-sCd-sGd-sGd-sCd-sCd-sTd-sGd-sGd-sAd-sCd-sAd-sTd

UHPLC Separation: IPRP 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 eluent system was developed to allow elimination of amine and metal adducts while maintaining high chromatographic resolution. Condition are in Table 1.

Mass Spectrometry: Characterization assays were performed on an Orbitrap Exploris 240 and MX mass spectrometer. Settings are listed in Table 2.

Data Analysis: Chromeleon software was used for compliant relative quantification of the oligonucleotide FLP and their impurities. A report was generated with flexible impurity annotation. Quantitation was validated with isotopic sliding windows deconvolution and extracted ion chromatograph signals.

Time(min)	Flow (ml/min)	%B	Curve	Temperature
0.0	0.25	20.0	5	50
7.5	0.25	67.0	3	50
7.6	0.25	100	5	50

Buffer A – 15 mM Hexylamine, 60mM HFIP in water
Buffer B – 15 mM Hexylamine, 60 mM HFIP in 40% acetonitrile

Table 2. HRMS Conditions

Full MS – HMR mode, trapping gas pressure setting=1.0	
Sheath gas: 35Arb	In-source CID: 30eV
Aux gas: 10 Arb	RF Lens (%): 75
Spray voltage: Negative Ion 2.5kV	Microscans: 1
Ion Transfer Tube Temp.: 320°C	Resolution: 120,000
Vaporizer temp.: 200 °C	Scan range: 500-2500

Results

Synthetic oligonucleotides are relatively new therapeutics which have proven difficult to characterize. Traditional single quadrupole methods utilize a large hydrophobic amine to suppress stereoisomer separation. The single quadrupole MS systems are limited in sensitivity and have problems with adducts and in-source impurity generation. The smaller amine used here in conjunction with HFIP produces the same suppression of stereoisomer separation but is easier to remove as an adduct with low in-source collision energy which also prevents in-source generation of impurities.

Figure 1. Chromatography and mass spectrum of ASO RNA

UV chromatogram panel A, full mass spectrum panel B, of the synthetic ASO RNA using no in-source energy and the optimized method

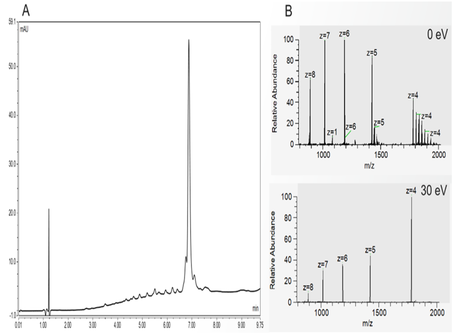
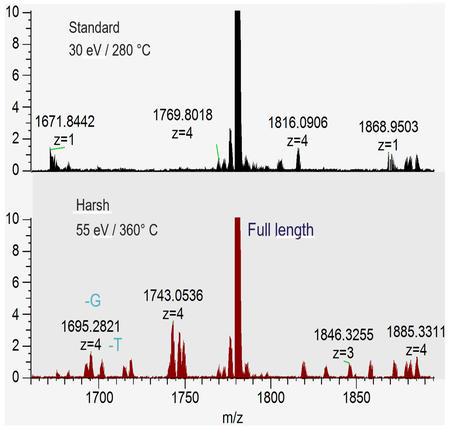


Figure 1 shows the chromatography resolution achieved with the HA/HFIP eluent system from an unpurified 20nt long, fully thiolated, synthetic ASO DNA sample. Panel B shows the removal of the HA adducts from the charge -4 and -5 states using in-source collision energy of 30eV. The data quality is extremely clean which makes quantitation much simpler with the added advantage of high-resolution chromatographic peaks devoid of MS adducts. The data presented here resolves stereoisomer issues with chromatography conditions that also partially separate PO, N-1 and N+1 impurities

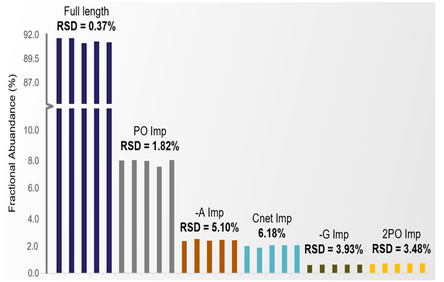
Metal adducts have become accepted as inevitable with oligonucleotide analysis. Using novel cleaning techniques for the UHPLC, the use of high-quality reagents, and avoidance of any silicate glass, results in no detectable metal adducts[1]. The optimized MS source conditions used with this eluent system does not produce in-source impurity artifacts or adducts making quantitation simple.
MS source optimization showed specific in-source impurity increases are observed with gradually harsher source conditions. This creates additional impurity signals that are not present in the original sample and make accurate quantitation difficult. A balance can be achieved where the amine adducts are removed with no in-source impurities generated.

Figure 2. Source created impurities using harsh conditions



Increasingly harsh conditions in the source have been shown to fragment oligonucleotides and create impurities during the analysis[2]. Multiple source conditions were examined and two of these are shown in Figure 3. The standard conditions reported in table 2 removed amine adducts but did not form additional impurities. Increasing the in-source collision energy and the temperature of the source was found to produce increasing amounts of fragmented impurities before and after the FLP m/z. The most common is single base loss. This is an easy and fast way to ensure the impurity peaks found are real and not generated during the analysis.
Isotopic sliding windows deconvolution of the data provides additional retention time information from XIC traces generated from the deconvolution data to help further verify the authenticity of the impurity identification.
Figure 3 shows that using deconvoluted data the relative abundance RSD values of the FLP are below 0.4% and that even the low-level impurities are 6% and below. XIC data from specific m/z values in the same experiment confirmed the results calculated with isotopic deconvolution. Calibration curves from serial dilutions in water and in FLP confirm linearity and the lack of suppression for impurities under the main peak.

Figure 3. relative standard deviation of the deconvoluted impurity data



BioPharma Finder (BPF) software is used with the input of the ASO RNA sequence to rapidly identify the impurities present in the sample and give the relative quantitation. For GLP compliance, Chromeleon software is used. This controls the UHPLC and the HRMS, it also has the same deconvolution engine as BPF. The parameters from BPF can be seamlessly transferred into the intact deconvolution inside CM via a workbook generated in BPF.
A report depicted in Figure 4 has been developed for the ASO RNA analysis which uses custom variable columns in the Chromeleon sequence editor to input the expected mass of the ASO RNA sample. This value is used in the report to compare with the isotopic experimental mass value found for the FLP. A column confirms the identification of the target compound. The delta mass values found for the impurities in the sample are compared to the impurity mass values which have been input in the custom impurity list. The report will generate a list of the impurities found giving the relative abundance in the sample.

Figure 4. Compliant report for ASO RNA analysis

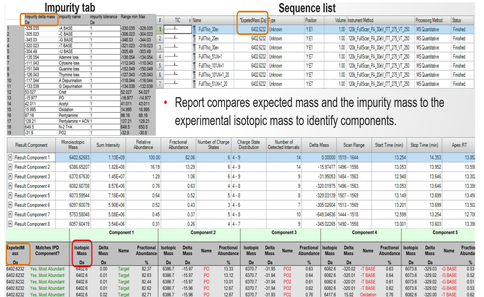


Figure 4 shows the different simple stages of utilizing a specific report in Chromeleon to annotate and quantitate different ASO RNA drugs. Creation and use of custom defined columns to input the expected mass into the Chromeleon sequence. The impurity Tab in the report is used to input expected impurities and their mass. The final depictions show the deconvoluted isotopic mass result in Chromeleon, with the report generated from comparison of the expected mass and the experimental isotopic mass found in the analysis. Annotation of expected impurities are shown in the report. Unlike targeted SIM analysis, unknown or unexpected impurities will still be seen and quantified.
The new LC/HRMS workflow is robust, simple and shows considerable advantages to previous methods using low resolution MS systems.

Conclusions

High resolution mass spectroscopy using an Exploris 240, or MX system gives the sensitivity and resolution required to use simple isotopic deconvolution to accurately identify and quantitate impurities in ASO RNA samples. A QC friendly, mass only, Exploris MX system is designed to give identical results to the Exploris 240 used in this study. The optimized new method using HRMS is robust, simple, fast and fully automatable. This shows considerable advantages to methods using low resolution MS systems. The use of Chromeleon software including the analytical report provides a full GLP compliant workflow for the QC and R&D environment.

References

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