A simple robust method for synthetic therapeutic RNA. New chemistry, new quantitation

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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 Mass Spectrometer was used for sensitivity, with optimised source conditions to remove adducts without generating in-source impurities. Thermo Scientific™ BioPharma Finder™ Software was used for identification and relative abundance using sliding windows Xtract algorithm with isotopically resolved data. Thermo Scientific™ Chromeleon™ software was used for GLP compliance with the same deconvolution engine.

INTRODUCTION

Oligonucleotide analysis has gained considerable interest over the last few years with the successful introduction of mRNA vaccines using a lipid nano particle transport system. This has led to new analytical methods for mRNA including sequencing by HRMS[1].Synthetic short interfering therapeutic RNA extends the need for new analytics in this area even further. 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. The split signals produced from multiple adducts also reduces sensitivity. A successful routine method has been developed for short synthetic oligonucleotide impurity analysis which resolves all of these problems.

MATERIALS AND METHODS

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

Ur-sAr-sCr-sAr-sGr-sCr-sAr-sUr-sCr-sGr-sGr-sCr-sCr-sUr-sGr-sGr-sAr-sCr-sAr-sUr

UHPLC Separation: IPRP separations were performed with a 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 amine and metal adducts while maintaining high resolution. Condition are in Table 1.

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

Data Analysis: Biopharma Finder was used for identification with Chromeleon software 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.

Table 1. HPLC gradient conditions

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

Method optimization

Synthetic oligonucleotides are relatively new therapeutics which have proven difficult to characterize. Chromatography conditions are required that eliminate adducts and suppress stereoisomer separation. Large amines give good chromatography and a single charge state for quantitation[2]. However, all the adducts reside on that charge state and the large amine adducts are difficult to remove with in-source collision energy. A smaller amine used in conjunction with HFIP produces the same suppression of stereoisomer separation by fully coating the hydrophobic resin surface. The Hexylamine used here is much easier to remove as an adduct with in-source collision energy. MS source optimization also prevents in-source generation of impurities. Metal adducts have become accepted as inevitable with oligonucleotide analysis. With novel cleaning techniques for the UHPLC, the use of high-quality reagents, and avoidance of any silicate glass, results in no detectable metal adducts. The optimized MS source conditions used with this eluent system does not produce impurity artifacts or adducts making quantitation simple.

Figure 1. Chromatography and mass spectrum of ASO RNA

UV chromatogram panel A, full mass spectrum panel B, of the synthetic ASO RNA using the optimized method

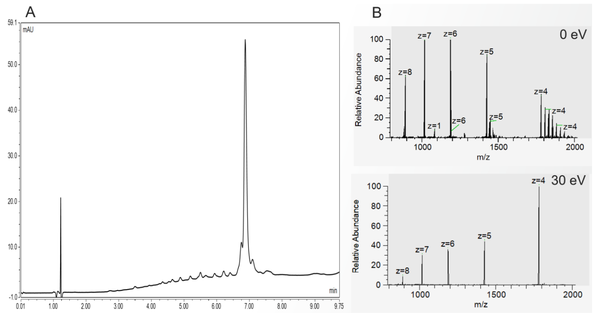
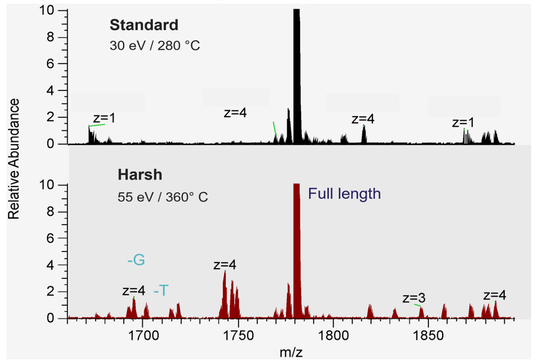


Figure 1 shows the chromatography resolution achieved with the HA/HFIP eluent system form an unpurified 20nt long, fully thiolated, synthetic ASO RNA sample. Panel B shows the removal of the HA adducts from the charge -4 and -5 states using in-source collision energy of 30eV. The data quality is extremely clean which makes quantitation much simpler with the added advantage of high-resolution chromatographic peaks devoid of MS adducts.

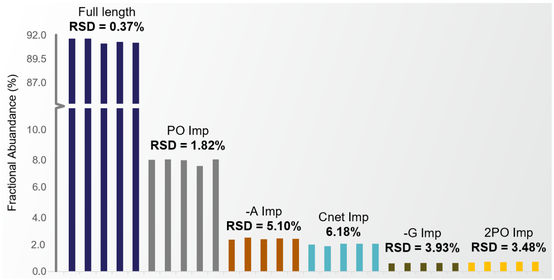
The source conditions used here were examined to ensure that there were no impurities being generated in the source that did not originate from the sample. Increasingly harsh conditions in the source have been shown to fragment oligonucleotides and create impurities during the analysis[2]. Multiple source 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. This is an easy and fast way to ensure the impurity peaks found are real and not generated during the analysis.

Figure 3. Source created impurities using harsh conditions



Isotopic sliding windows deconvolution of the data provides additional retention time information to help further verify the authenticity of the impurity identification. Figure 2 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 the same experiment confirmed the values calculated with isotopic deconvolution. Calibration curves from serial dilutions in water and in FLP matrix confirm linearity and the lack of suppression for impurities under the main peak.

Figure 2. relative standard deviation of the deconvoluted impurity data



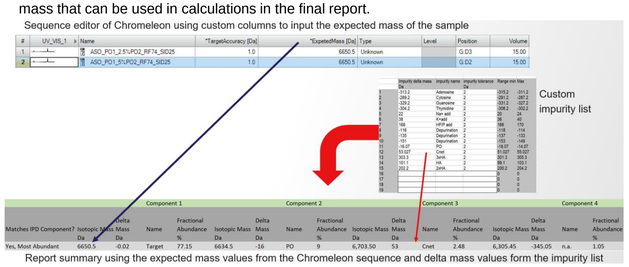
BioPharma Finder(BPF) software is used with the input of the ASO RNA sequence to rapidly identify the impurities present in the sample and give the relative quantitation. For GLP compliance, Chromeleon software is used. This controls the UHPLC and the HRMS, it also has the same deconvolution engine as BPF. The parameters from BPF can be seamlessly transferred into the intact deconvolution inside CM.

A report depicted in Figure 4 has been developed for the ASO RNA analysis which uses custom variable columns in the 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 and impurities. 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 into the custom impurity list in the expected impurity sheet. 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

The use of custom columns in the Chromeleon sequence to add values for expected mass that can be used in calculations in the final report.

Sequence editor of Chromeleon using custom columns to input the expected mass of the sample



Report summary using the expected mass values from the Chromeleon sequence and delta mass values form the impurity list

CONCLUSIONS

High resolution mass spectroscopy using an Exploris 240 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 and 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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TRADEMARKS/LICENSING

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