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

Ken Cook1, Ulrik Mistarz1, Alexander Schwahn2, Fiona Rupprecht3 1 Thermo Fisher Scientific, Hemel Hempstead, United Kingdom, 2Thermo Fisher Scientific, Reinach, Switzerland, 3 Thermo Fisher Scientific, Dreieich, Germany

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-sCdsAd-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)	%В	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 insource 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

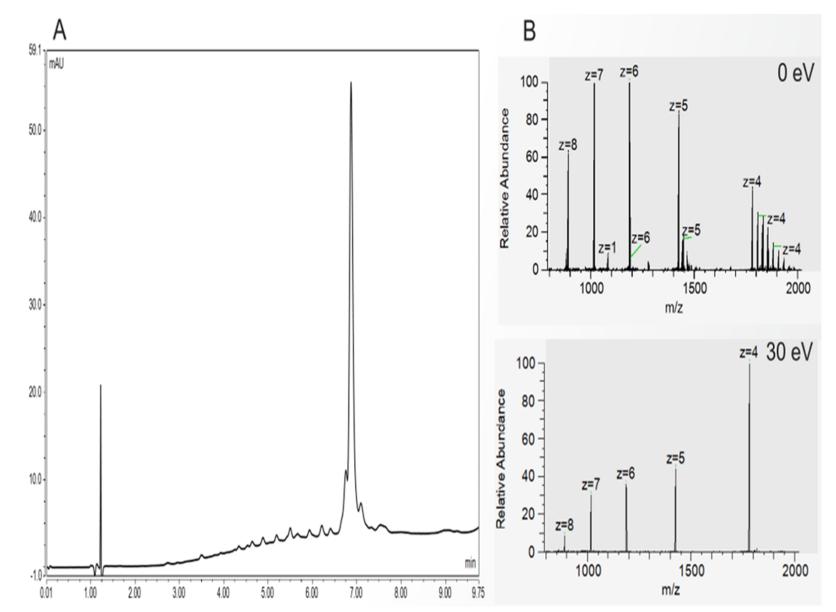
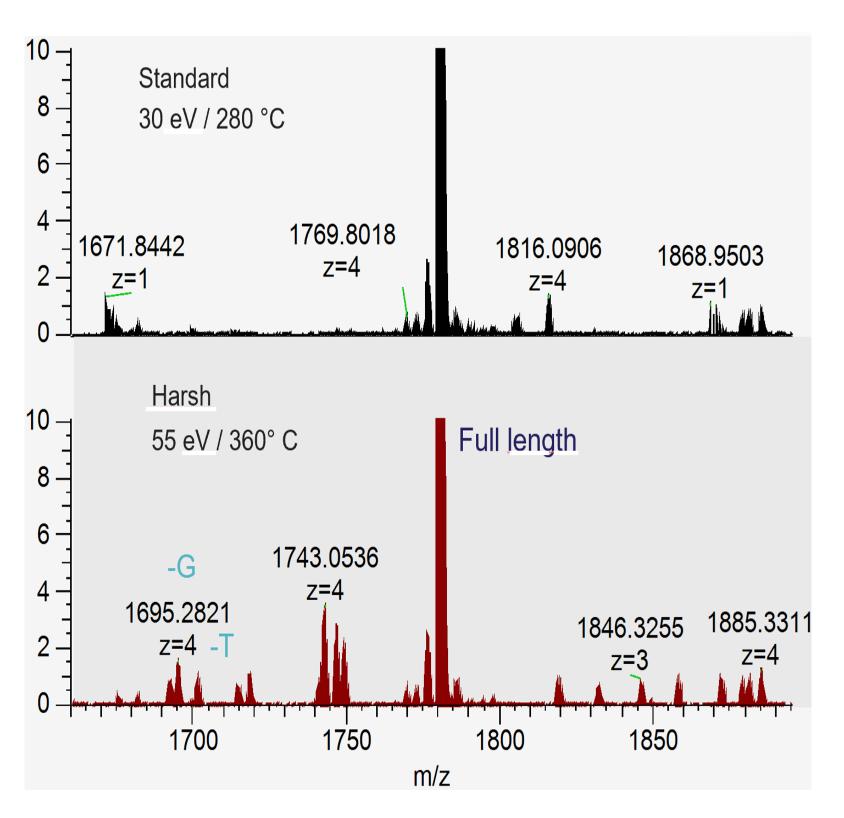


Figure 1 shows the chromatography resolution achieved with the HA/HFIP eluent system from an unpurified 20nt long, fully thiolated, synthetic ASO DNA sample. Panel B shows the removal of the HA adducts from the charge -4 and -5 states using in-source collision energy of 30eV. The data quality is extremely 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 adducts¹. The optimized MS source conditions used with this eluent system does not produce insource mpurity 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 insource 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 analysis². 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.

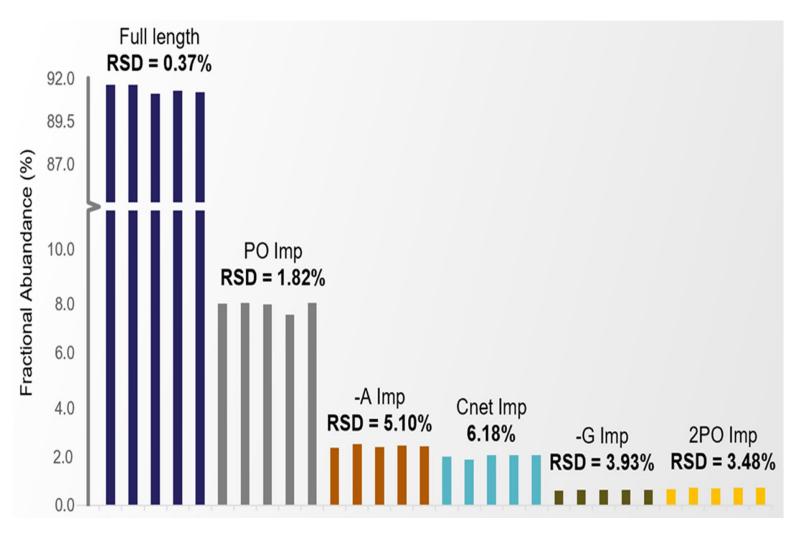


Figure 3. relative standard deviation of the deconvoluted impurity data

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

_	Imp	ourity ta	ab									Sequ	lence	list							
lm Da	purity delta mass	impurity name impurity tolerance Range min Max # TIC > Name			*ExpetedMass [[Position	sition Volume Instrument Method					ocessing Meth	od Stat	IUS						
1 -32	29.035	-A BASE 1 -330.035 -328.035 1		ev	6402.62	32 Unkn	nown	Y:E1		1.00 120k	FullScan PA	30eV ITT 275 V	T 250 M	- IS Quantitative	Fin	nished					
	05.023	-C BASE -G BASE	1	-306.023	3 -304.023 -344.03	2		FullThio 20ev		6402 62	32 Unkn	nown	Y:E1				20eV_ITT_275_V		IS Quantitative	Fin	ished
	45.03 20.023	-G BASE -T BASE	1	-346.03	044.00			FullThio 30ev		6402 62											nished
5 -30	04.49	-U BASE	1	-305.49	-303.49	<u> </u>	2	-									30eV_ITT_275_V	-	IS Quantitative		
	35.054	Adenine loss	1	-136.054	-104.004	4 <u> </u>	2	FullThio_5%N+1		6402.62			Y:E7		1.00 120k	FullScan_PA	30eV_ITT_275_V	T_250 M	IS Quantitative	Fin	nished
	11.043 51.049	Cytosine loss Guanine loss	1	-112.043		;	2	FullThio_10%N+1		6402.62	32 Unkn	nown	Y:E7		1.00 120k	FullScan_PA_	30eV_ITT_275_V	T_250 M	IS Quantitative	Fin	nished
2	26.043	Thymine loss	1	-127.043		;	2	FullThio 10%N+1 20		6402.62	32 Unkn	nown	Y:E7		1.00 120k	FullScan PA	20eV_ITT_275_V	T 250 M	IS Quantitative	Fin	nished
	17.044	A Depurination	1	-118.044		7		FullThio 5%		6402 62	32 Unkn	Yown	Y·E7				20eV ITT 275 V		IS Quantitative	Fin	ished
	33.039 .027	G Depurination Cnet	1	-134.039	9 -132.039 54.027		1	1011110_0		0702.02		NWIII	1.57		1.00 1200		2007_111_273_7	-200 1		11.00	Janou
	5.977	PO	1	-16.977	-14.977		•										11		<u>с п</u>		
	2.011	Acetyl	1	41.011	43.011	• •	Repo	ort co	ompa	res ex	bec	cted r	nass	and	the	Impl	iritv m	ass	to th	е	
	.995	Oxidation	1	14.995	16.995						P • •									•	
	.16	Pentylamine LAC	1	86.16	88.16	~	vno	rimo	ntal i	sotopi	o m	nace t	o ido	ntif	com	non	onto				
S874	8.21 9.5	Pentylamine + AC N-2 T+A	1 1	127.21 648.5	129.21 650.5	e	syhe	IIIIIE	IIIdi I	Solopi		1922 1	U IUE	iiuiy	COLL	ιροπ	ents.				
19 -31		P02	1	-32.9	-30.9		•														
Result (Component	Monoisotopic Mass	Sum Intensit	v	Relative Abundance	Frac		Number of Stat		Charge State Distribution		Number of cted Interval	. Delta	Mass	Scan R	ange	Start Time (mir	n) Stop	Time (min)	Ape	ex RT
-								Juli			Deter					-					
+ Result C	Component 1	6402.6268	33 1.13E	+09	100.00		82.06		64	. 9		1	4	0.00000	1519 - 164	4	13.2	254	14.35	3	13
+ Result C	Component 2	6386.6520)7 1.82E	+08	16.19		13.29		64	.9		1	4 -	15.97477	1496 - 159	В	13.0)53	13.95	2	13
+ Result C	Component 3	6370.6763	30 1.45E	+07	1.29		1.06		64	9			9 -	31.95053	1484 - 156	3	12.9	948	13.64	6	13
+ Result C	Component 4	6082.6070)8 8.57E	+06	0.76		0.63		44.	.8			9 -3	20.01975	1496 - 156	3	13.0)53	13.64	6	13
+ Result C	Component 5	6073.5954	14 7.18E	+06	0.64	1	0.52		54.	. 8			8 -3	29.03139	1507 - 156	9	13.1	149	13.69	9	13
+ Result C	Component 6	6097.6007	79 5.90E	+06	0.52		0.43		3 4	- 6			7 -3	05.02604	1513 - 156	9	13.2	201	13.69	9	13
+ Result C	Component 7	5753.5804	18 <u>5</u> .08E	+06	0.45	1	0.37		54	. 8		1	-6	49.04636	1444 - 151	B	12.5	599	13.25	4	12
+ Result C	Component 8	6057.6041	19 3.54E	+06	0.31		0.26		4 4	. 7			9 -3	45.02265	1490 - 155	B	13.0	001	13.60	3	13
		Component 1		Component 2		Component 3			Compon		mponent 4	nent 4		Component 5							
ExpetedM	Matches IPE		A CONTRACTOR OF	Name	Fractional	Isotopic	Delta	Name	Fractiona		Delta	Name	Fractional	Isotopio		Name	Fractional	Isotopic		Name	Frac
ass	Component	? Mass	Mass		Abundance	Mass	Mass		Abundanc	e Mass	Mass		Abundance	Mass	Mass		Abundance	Mass	Mass		Abu
Da)	Da	Da		%	Da	Da		%	Da	Da		%	Da	Da		%	Da	Da		
6402.6232	Yes, Most Abund			Target	82.37	6386.7	-15.97	PO	13.33	6370.7	-31.95	PO2	0.63	6082.6	-320.02	-T BASE	0.63	6073.6	-329.03	-G BASE	
6402.6232	Yes, Most Abune			Target	82.63	6386.7	-15.97	PO	13.12	6370.7	-31.94	PO2	0.64	6082.6	-320.01	-T BASE	0.64	6073.6	-329.02	-G BASE	
6402.6232	Yes, Most Abun			Target	82.44	6386.7	-15.97	PO	13.01	6370.7	-31.94	PO2	0.61	6082.6	-320.01	-T BASE	0.61	6073.6	-329.02	-G BASE	
6402.6232	Yes, Most Abune	114 C		Target	82.62	6386.7	-15.96	PO PO	12.67	6370.7	-31.94	PO2 PO2	0.62	6082.6	-320.01	-T BASE Oxidation	0.62	6073.6	-329.02	-G BASE -T BASE	L.
6402.6232	Yes, Most Abun			Target	82.71	6386.7	-15.96	PO	12.67	6370.7		PO2 PO2	0.76	6417.6	15.02	-T BASE	0.76	6082.6	-320.00	-T BASE	l
6402.6232 6402.6232	Yes, Most Abune Yes, Most Abune			Target Target	83.18 82.85	6386.7 6386.7	-15.96 -15.96	PO	12.60 12.75	6370.7 6370.7	-31.93	PO2 PO2	0.62 0.63	6082.6 6082.6	-320.00		0.62	6073.6 6073.6	-329.01 -329.01	-G BASE	l
0402.0232	Tes, MOST ADUN	Jan 0402.0	0.02	larger	02.00	0300.1	-10.90	PU	12.15	0310.1	-21.93	PUZ	0.05	0002.0	-320.00	-I DASE	0.05	0013.0	-329.01	-O DAGE	L L

Thermo Fisher S C I E N T I F I C

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

- 1. Christina J. Vanhinsbergh, Angela Criscuolo, Jennifer N. Sutton, Keeley Murphy, Andrew J. K. Williamson, Ken Cook, and Mark J. Dickman. Characterization and Sequence Mapping of Large RNA and mRNA Therapeutics Using Mass Spectrometry. Analytical Chemistry. doi.org/10.1021/acs.analchem.2c00765
- 2. Claus Rentel, Hans Gaus, Kym Bradley, Nhuy Luu, Kimmy Kolkey, Bao Mai, Mark Madsen, Megan Pearce, Brandon Bock, and Daniel Capaldi. Assay, Purity, and Impurity Profile of Phosphorothioate Oligonucleotide Therapeutics by Ion Pair–High-Performance Liquid Chromatography–Mass Spectrometry, Nucleic acid therapeutics. DOI: 10.1089/nat.2021.0056.

Acknowledgements

We would like to thank Professor Mark Dickman from the University of Sheffield for advice and supplying additional purified samples to further authenticate these results.

Trademarks/licensing

© 2024 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others. PO177-2024-EN