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Application News

No. C219

Liquid Chromatography Mass Spectrometry

Quantitative Analysis and Determination of Molecular Weight of siRNA Type Oligonucleotides by LCMS™-8060

Introduction

Nucleic acid drugs are synthetic oligonucleotides which are designed to bond specifically with target RNA or proteins. Although most nucleic acid drugs approved to date are the antisense type, the aptamer type and siRNA type have also been approved. Many nucleic acid drugs consist of around 20 bases and have molecular weights on the order of 6,000. Precision mass analysis techniques such as MALDI-TOF type and Q-TOF type LC/MS are used in measurements of the molecular weight of drug substances. On the other hand, the triple quadrupole mass spectrometer is generally used in quantitative analysis such as analysis of the fate of drugs in the blood, as this instrument offers high sensitivity in combination with a wide dynamic range.

This article introduces an example of analysis of synthetic double-strand oligonucleotides using a Shimadzu LCMS-8060 triple quadrupole mass spectrometer, assuming analysis of the drug substances of siRNA type nucleic acid drugs. Quantitativity was confirmed by SIM (selected ion monitoring). In preparation of the calibration curve by the SIM mode, linearity was confirmed in the range of 1 fmol to 10 pmol. In addition, the molecular weights were determined by deconvolution of a multivalent ion mass spectrum.

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Samples

Sequence: Sample: double strand oligonucleotide (21 nt):
AS-RNA, 5′-pU CGA AGU AUU CCG CGU ACG dTdT-3′
Mw: 6646.0 (average mass)
SS-RNA, 5′-pC GUA CGC GGA AUA CUU CGA dTdT-3′
Mw: 6669.0 (average mass)

Analysis Conditions

Table 1 shows the HPLC and MS analysis conditions.

Table 1 Analysis Conditions

[HPLC conditions] (Nexera™)		
Column	:	Commercial C18 column (100 mm × 2.1 mm I.D., 1.7 µm)
Mobile phases	:	A) 200 mM HFIP*1 and 7.5 mM TEA*2/water B) Methanol
Gradient program	:	B conc. 4 % (0 min) – 20 % (8.0 min)
Flow rate	:	0.2 mL/min
Column temp.	:	75 °C
Injection volume	:	10 µL
[MS conditions] (LCMS-8060)		
Ionization	:	ESI (negative mode)
Probe voltage	:	-3 kV
Mode	:	Q3 scan (*m/z* 500 - 1800) SIM *m/z* 1666.0 (SS), *m/z* 1659.9 (AS)
Nebulizing gas flow	:	3.0 L/min
Drying gas flow	:	5.0 L/min
Heating gas flow	:	15.0 L/min
DL temp.	:	250 °C
Heat block temp.	:	500 °C
Interface temp.	:	350 °C

*1 1,1,1,3,3,3-hexafluoro-2-propanol
*2 Triethylamine

SIM Chromatogram

Fig. 1 shows the SIM chromatogram of a mixed solution of SS-Oligo and AS-Oligo (100 fmol each). According to ion pair chromatography, elution occurred at 6.88 min and 6.94 min, respectively, and resolution R was 0.3.

Fig. 2 shows the mass spectra of the two components. Ions with valences of 4 to 9 were detected in both SS-Oligo and AS-Oligo. The tetravalent ions were selected in the SIM chromatogram.

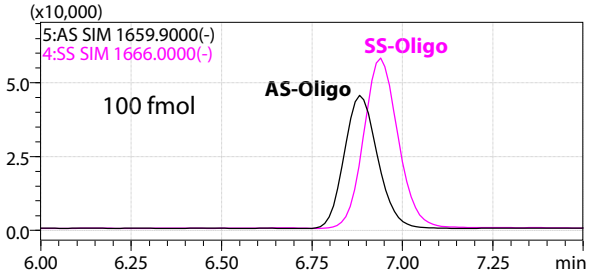


Fig. 1 SIM Chromatogram

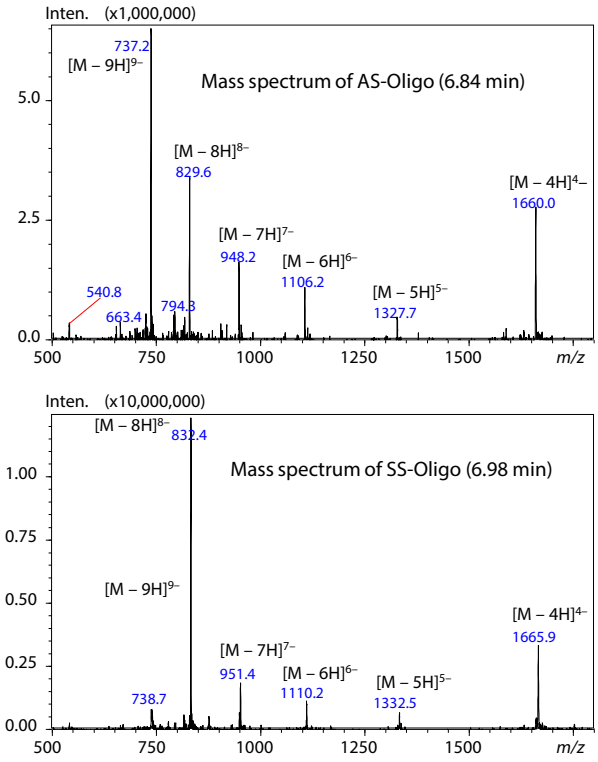


Fig. 2 Mass Spectra of Synthetic Oligonucleotides

Calibration Curves

Calibration curves for AS-Oligo and SS-Oligo were obtained in the range from 1 fmol to 10,000 fmol. Fig. 3 shows the SIM chromatograms for the injection volumes of 1 fmol (limit of detection, LOD) and 5 fmol (limit of quantitation, LOQ). Fig. 4 shows the calibration curves. The coefficient of determination (R^2) was 0.997 for AS-Oligo and 0.995 for SS-Oligo. Although an analysis by MRM (multiple reaction monitoring) was also attempted, it was not possible to obtain results that exceeded the SIM analysis results for LOQ. The 5 fmol calibration point was undetected in the MRM results. As reference, Fig. 5 shows the MRM chromatograms for the injection volume of 50 fmol.

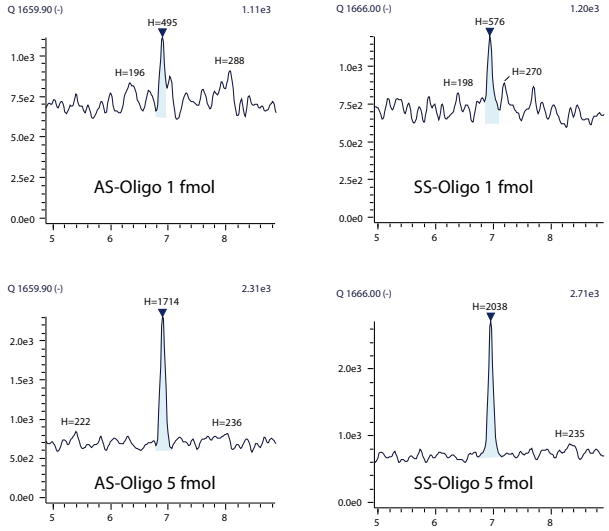


Fig. 3 SIM Chromatograms of Synthetic Oligonucleotides (H : Peak Height)

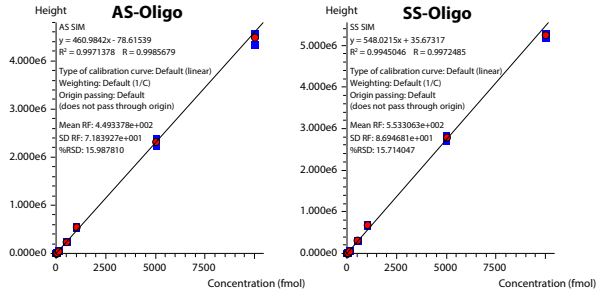


Fig. 4 Calculation Curves Obtained from SIM Chromatograms

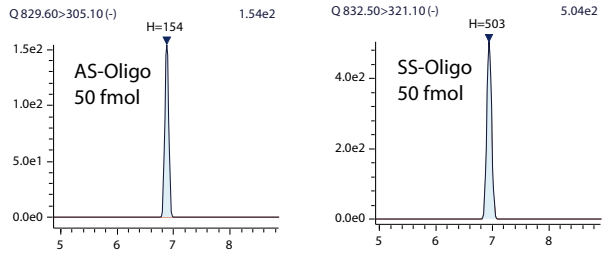
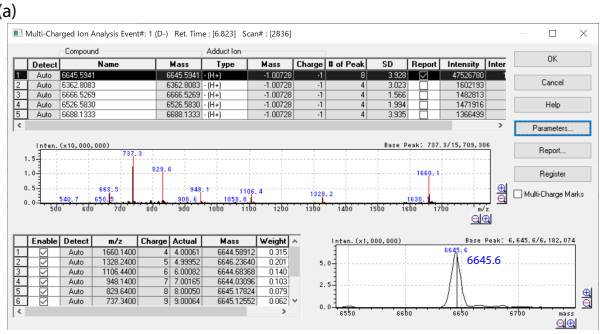


Fig. 5 MRM Chromatograms of Synthetic Oligonucleotides (H : Peak Height)

Deconvolution

Molecular weight calculations were done using the deconvolution function of the Shimadzu software LabSolutions™ (Fig. 6). As shown in the deconvoluted spectra, the estimated molecular weights of AS-Oligo and SS-Oligo are 6,645.6 and 6,667.1, respectively, and the corresponding errors from the average molecular weights were 0.4 Da and 1.9 Da.

(a)



(b)

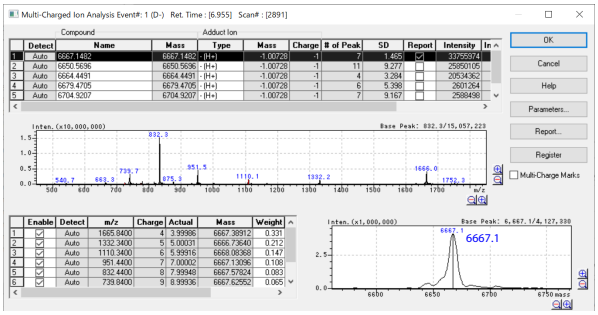


Fig. 6 Deconvoluted Spectra (a) AS-Oligo, (b) SS-Oligo

Conclusion

This article introduced an example of a quantitative analysis of siRNA type oligonucleotides by SIM using an LCMS-8060. It was possible to obtain calibration curves for the range of 1 fmol to 10 pmol. The limit of quantitation was 5 fmol and was approximately 3 ng/mL at the concentration with the injection volume of 10 uL.

With the oligonucleotides used in this experiment, the calibration curves could be prepared to lower concentrations by SIM than by MRM. Although the data are not shown here, when an octavalent ion was used as the precursor, the product ion with the strongest intensity was obtained by MRM. However, the LOQ of MRM did not achieve the same level as SIM. The cause of this difference is thought to be inadequate absolute sensitivity for the product ion.

Although the mass spectra obtained with a triple quadrupole mass spectrometer have low resolution, the molecular weights could be determined within an error range of a few Da by utilizing the deconvolution function.

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