SHIMADZU

WP 459

Qualification and Quantitation of Phosphorothioate Oligonucleotides Using a Quadrupole-Time-of-Flight Mass Spectrometer

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1. Overview

The purpose of this study is qualitative analysis and quantitation of a phosphorothioate antisense oligonucleotide (Figure 1) using a LC/Q-TOF-MS. The molecular weight confirmation and the sensitive quantitation are successfully achieved by charge state deconvolution and high-resolution MRM , respectively.

2. Introduction

With the current diversification of variety of oligonucleotide therapeutics, there is an urgent need to improve the efficiency of analytical method development. MS detection is easier way to analyze oligonucleotide in both sample preparation and method development comparing with immune-based detection such as ELISA, and also the only methodology that can distinguish slight difference of modifications and quite resembled impurities based on accurate M.W. information.

Here, we report quantitative and qualitative analysis of modified antisense oligonucleotides in a single system of Q-TOF that combines selectivity with sensitivity.

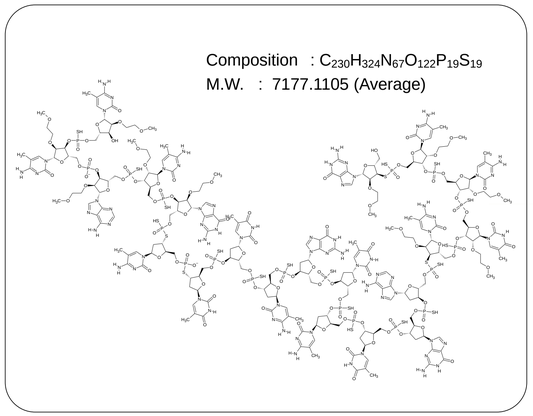

Figure 1 Structure of mipomersen

Table 1 Oligo nucleotide sequence of mipomersen analogues

Name	Oligo nucleotide sequence
Mipomersen	5'-mG-mC*-mC*-mU*-mC*-dA-dG-dT-dC*-dT-dG-dC*-dT-dT-dC*-mG-mC*-mA-mC*-mC*-3'
Mipomersen-2'-deoxy	5'-dG-dC*-dC*-dT-dC*-dA-dG-dT-dC*-dT-dG-dC*-dT-dT-dC*-mG-dC*-dA-dC*-dC*-3'
Mipomersen-OMe	5'-#G-#C-#C-#U-#C-dA-dG-dT-dC*-dT-dG-dC*-dT-dT-dC*-#G-#C-#A-#C-#C-3'
Mipomersen-LNA	5'-G^mC^-mC^-U^-mC*-dA-dG-dT-dC*-dT-dG-dC*-dT-dT-dC*-G^-mC^-mA^-mC^-mC^-3'

m : 2'-O-(2-methoxyethyl) nucleoside
* : Substitution at 5-position of cytosine and uracil base with a methyl group
d : 2'-deoxynucleoside (2'-deoxy)
: 2'-O-Methyl nucleoside (OMe)
^ : Locked nucleic acid (LNA)
All phosphodiester bonds between nucleotides are substituted with phosphorothioate bonds.

3. Methods

Mipomersen and mipomersen analogues were analysed using a quadrupole time of flight mass spectrometer (LCMS-9030; Shimadzu, Japan) coupled with liquid chromatography (Nexera, Shimadzu). Oligo nucleotide sequences of mipomersen, mipomersen-2'-deoxy, mipomersen-OMe, and mipomersen-LNA were shown in Table 1. The LC separation was carried out with binary gradient of 50mM HFIP and 10mM DIPEA aqueous in water and acetonitrile using a C18 column (Shim-pack Scepter C18 2.1x50mm, Shimadzu). The molecular weight confirmation of each oligonucleotide was carried out using an analytical software for charge state deconvolution (Insight Explore CSD, Shimadzu). Quantitative data acquisition was achieved by high-resolution MRM with fragment ion of m/z 94.9358 (PSO2-) as a monitored ion. The calibration range and detection limit of each oligonucleotide were estimated based on correlation coefficient of calibration curve using the peak area.

High-Resolution and Accurate Mass Spectrometer

Resolution power	: > 30,000 FWHM at m/z 1,972 / 1,626
Mass accuracy	: 1 ppm
Maximum acquisition rate	: 100 Hz

Figure 2 LCMS-9030 quadrupole time-of-flight mass spectrometer

Table 2 Analytical condition

[HPLC conditions] (Nexera)		
Column	:	Shim-pack Scepter C18 (2.0 x 75, 1.9μm)
Mobile phases	:	A) 50 mmol/L HFIP*2 and 10 mmol/L DIPEA*3 B) Acetonitrile
Gradient Program	:	B 5% (0-0.5 min) – 15% (0.5-6min)
Flow rate	:	0.2 mL/min
Column Temp.	:	50℃
Injection volume	:	5 μL
[MS conditions] (LCMS-9030)		
Ionization	:	ESI (Negative mode)
Probe Voltage	:	-3 kV
Mode	:	Full scan（m/z 500 - 3000） MRM（803.4626 > 94.9358）
Nebulizing gas flow	:	3.0 L/min
Drying gas flow	:	10.0 L/min
Heating gas flow	:	10.0 L/min
DL Temp.	:	250℃
Heat Block Temp.	:	400℃
Interface Temp.	:	350℃

TEA*1 : Triethylamine
HFIP*2 : 1,1,1,3,3,3-Hexafluoro-2-propanol
DIPEA*3 : N,N-diisopropylethylamine

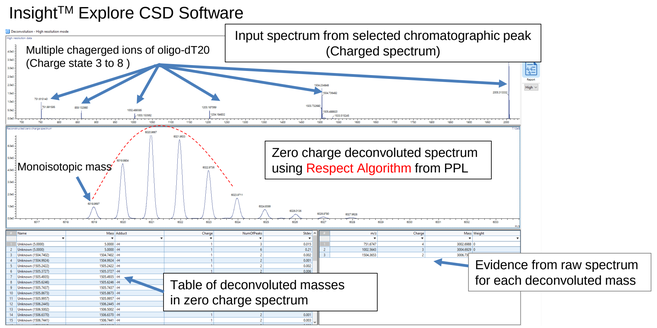

Figure 3 Spectral processing software for charge-state deconvolution
: An analysis example of Oligo-dT20

4. Results

Here we report an example of qualitative analysis and sensitive quantitation using a high-resolution and accurate mass spectrometer (HRMS), a quadrupole time-of-flight mass spectrometer, using modified analogues of a phosphorothioate antisense oligonucleotide.

4-1. Molecular Weight Confirmation by Charge-State Deconvolution

Mass spectra of mipomersen analogues (sample conc.: 50 μg/ml) were acquired in MS full scan. Acquired charged mass spectra were processed with an analytical software for charge state deconvolution (Figure 3). Figure 4 shows an analysis example of mipomerse-2'-deoxy. The charge state distribution was observed in charge state 4 to 8 (Figure 4 *left*). From the results of charge state deconvolution, molecular weight of mipomersen and all its' analogues could be confirmed with high mass accuracy (Table 3).

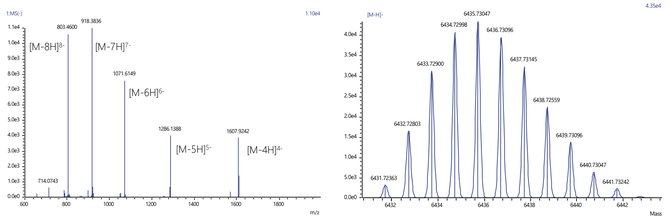

Figure 4 Result of charge state deconvolution: Charged mass spectrum (*left*) and processed uncharged spectrum (*right*) of mipomersen-2'-deoxy.

Table 3 Results of molecular weight confirmation for mipomersen analogues

	Theoretical mass (monoisotopic)	Observed mass (monoisotopic)	Δ ppm
Mipomersen $C_{230}H_{324}N_{67}O_{122}P_{19}S_{19}$	**7172.0918**	**7172.0874**	**-0.607**
Mipomersen-LNA $C_{210}H_{264}N_{67}O_{112}P_{19}S_{19}$	**6711.6731**	**6711.6733**	**0.029**
Mipomersen-OMe $C_{204}H_{272}N_{67}O_{112}P_{19}S_{19}$	**6647.7357**	**6647.7329**	**-0.422**
Mipomersen-2'-deoxy $C_{200}H_{264}N_{67}O_{102}P_{19}S_{19}$	**6431.7240**	**6431.7241**	**0.023**

4-2. Quantitative Analysis by high-resolution MRM

Mipomersen analogues were quantified by MRM acquisition mode using high-resolution mass spectrometer. In this experiment, product ions are detected selectively in the same way as a MRM using triple quadruple mass spectrometer. Consequently, higher sensitivity can be achieved by effectively focusing the m/z range for product ions scan.

All analytical methods for four analogues could be established using a common fragment ion derived from phosphorothioate group (PSO2-, m/z 94.9358) as a monitored ion. Figure 5 shows a typical chromatogram in high-resolution MRM (XIC width: 20 ppm). The calibration ranges of mipomersen-2'-deoxy, mipomersen-OMe, mipomersen-LNA, and mipomersen were 1 ppb to 1 ppm (Figure 5 *right*), 5 ppb to 1 ppm (data not shown), 5 ppb to 1 ppm (data not shown), and 10 ppb to 1 ppm (data not shown), respectively.

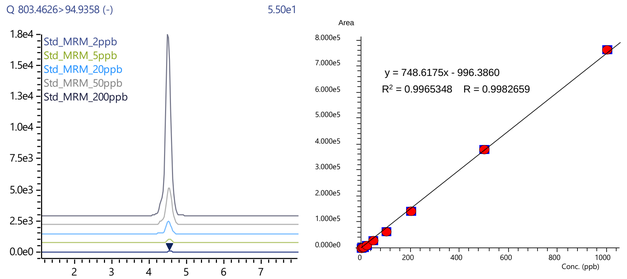

Figure 5 Quantitative result of mipomersen-2'-deoxy
: MRM chromatogram (*left*) and calibration curve (*right*)

5. Conclusions

- The molecular weight confirmations of phosphorothioate oligonucleotides were successfully achieved with a high mass accuracy less than 1 ppm error.
- The quantitative methods with high sensitivity were established by high-resolution MRM using a phosphorothioate fragment