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Application News

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer
Quadrupole Time-of-Flight Liquid Chromatograph Mass Spectrometer

Analysis of Drug-to-Antibody Ratio in Antibody-Drug Conjugates—Multifaceted Evaluation by LC-QTOF and MALDI Analysis—

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User Benefits

- The MALDI-8030 system enables drug/antibody ratios to be evaluated quickly and easily.
- The high mass stability of the LCMS-9030 system enables reliable evaluations of intra-day and inter-day drug-to-antibody ratios of antibody-drug conjugates.
- Using the MALDI-8030 and LCMS-9030 systems together to analyze the same antibody-drug conjugate enables multifaceted evaluations of antibody-drug conjugates.

■ Introduction

Antibody-drug conjugates (ADC) are biopharmaceuticals with a linker that links a monoclonal antibody (mAb) to a cytotoxic drug (payload).

Since ADCs offer both the high cancer specificity of mAbs and the cancer cell lethality of payload drugs, they are attracting attention as a new type of therapeutic drug for fighting cancer. Consequently, pharmaceutical manufacturers around the world have been actively engaged in developing them[1)].

The efficacy of ADCs depends greatly on their drug-to-antibody ratio (DAR), which is expressed by the number of payloads per mAb molecule. Therefore, it is necessary to evaluate their DAR as a parameter for analyzing their ADC properties.

This Application News article describes a multifaceted evaluation of the same ADC sample using a benchtop MALDI-8030 linear matrix-assisted laser desorption/ionization time-of-flight mass spectrometer system and an LCMS-9030 quadrupole time-of-flight mass spectrometer system (Fig. 1). The LCMS-9030 was also used to evaluate the intra-day and inter-day precision of DAR values.

Fig. 1 MALDI-8030 (Left) and Nexera™/LCMS™-9030 (Right) Systems

■ Sample Preparation

Samples were prepared by spiking human blood plasma with brentuximab vedotin, a type of ADC, to a concentration of 100 µg/mL. The samples were deglycosylated using PNGase F, reduced by TCEP, and pretreated by affinity purification. They were then collected as a 10 % aqueous acetonitrile solution that contained 10 mM TCEP and 1 % acetic acid. The final sample solutions contained a 10 µg/mL concentration of brentuximab vedotin that had been reduced by TCEP, with the light and heavy chains separated.

The LC-QTOF analysis with the LCMS-9030 system involved injecting the pretreated sample directly into the LC unit, separating the contaminant components with an analytical column, and then detecting its components.

The analysis using the MALDI system involved air-drying 3.75 µL (in 0.75 µL increments) of the pretreated sample, which had been sandwiched between matrix solutions on a MALDI target plate, and then analyzing the resulting residues in the MALDI-8030 system. For the matrix solution, a 10 mg/mL concentration of sinapinic acid (SA) dissolved in a 50 % aqueous acetonitrile solution containing 0.1 % trifluoroacetic acid (TFA) was used.

■ Analysis Conditions

Simple Analysis Using MALDI-8030 System

Table 1 shows the analysis condition settings for the MALDI-8030 analysis of reduced ADC. With the MALDI-8030 system, simple analysis of samples, from loading analytical samples onto a target plate to analyzing the data, can be accomplished within about 30 minutes.

Since MALDI analysis mainly generates singly-charged ions, it is easy to interpret the mass spectra, which eliminates the process of analyzing multiply-charged ions to determine molecular weight values from *m/z* values. Consequently, DAR values can be calculated without using specialized software.

Table 1 MALDI-8030 Analysis Conditions

System:	MALDI-8030
Laser:	Solid Laser (λ = 355 nm)
Matrix:	10 mg/mL sinapinic acid in 50 % acetonitrile, 0.1 % TFA
Polarity:	Positive
TOF Mode:	Linear
Pulsed Extraction:	*m/z* 65000
Mass Calibration:	External Standard BSA $[M+H]^+$ *m/z* 66431 BSA $[M+2H]^{2+}$ *m/z* 33216 BSA $[M+3H]^{3+}$ *m/z* 22144

Analysis by Nexera and LCMS-9030 System

Table 2 shows the analysis condition settings for Nexera and LCMS-9030 analysis of reduced ADC.

Table 2 Nexera and LCMS-9030 Analysis Conditions

System:	Nexera + LCMS-9030
Mobile Phase:	A: 0.1 % Formic acid – water B: 0.1 % Formic acid – Acetonitrile
Column:	Reversed phase column
Column Oven:	30 °C
Flowrate:	0.2 mL/min
Gradient:	B conc. 5 % (0 min) → 95 % (15.0-20.0 min) → 5 % (20.1 -25.0 min)
Injection Volume:	40 µL
Ionization:	ESI Positive
Interface Voltage:	5.0 kV
Nebulizer Gas Flow:	2.0 L/min
Heating Gas Flow:	10.0 L/min
Drying Gas Flow:	10.0 L/min
Interface Temperature:	250 °C
DL Temperature:	250 °C
Heat Block Temperature:	400 °C
Probe Position:	+3 mm
TOF Range:	*m/z* 701 to 2500
Event Time:	0.400 sec
Threshold:	Low

■ Simple DAR Evaluation of ADC Using MALDI-8030 System

The mass spectrum obtained from the TCEP-reduced ADC sample spiked with human blood plasma using the MALDI-8030 system is shown in Fig. 2. The mass spectrum shows a signal detected from light chains in the *m/z* 23000 to 25000 range and from heavy chains in the *m/z* 48000 to 53000 range.

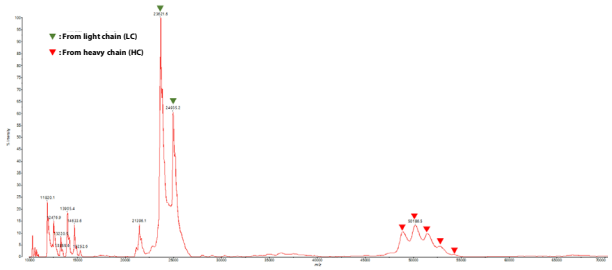

Fig. 2 Mass Spectrum from ADC-spiked Human Blood Plasma

An enlargement of the *m/z* range from 10000 to 37000 is shown in Fig. 3. A signal from a light chain without a linked payload (d0) was detected near *m/z* 23655 and from a light chain with one linked payload (d1) near *m/z* 24971. The shoulder signals near *m/z* 23856 and 25168 were presumably from a d0 or d1 matrix adduct and are commonly observed when using SA matrices.

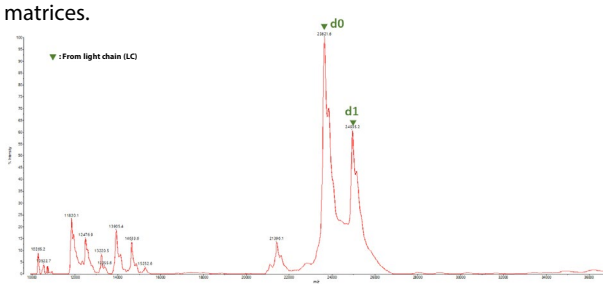

Fig. 3 Mass Spectrum Enlargement near Light Chains

An enlargement of the *m/z* range from 40000 to 62000 is shown in Fig. 4. Signals from heavy chains were detected near *m/z* 48904, 50195, 51493, 52772, and 54400 (corresponding to a heavy chain without a payload linked (D0), with 1 linked payload (D1), with 2 linked payloads (D2), with 3 linked payloads (D3), and with 4 linked payloads (D4), respectively). Based on the *m/z* value, the signal near *m/z* 47500 was presumably from a dimer of d0.

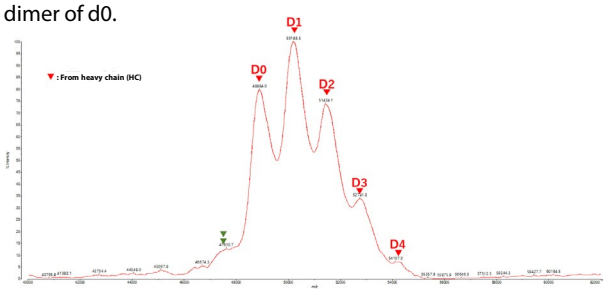

Fig. 4 Mass Spectrum Enlargement near Heavy Chain

The mass spectral peak intensity for the respective molecular species and DAR values determined by equation (1) are indicated in Table 3. The total DAR value was 3.32.

Table 3 Signal Intensity and DAR of Spectrum Obtained Using MALDI-8030 System

Attribution	Signal Intensity (mV)
Light Chain d0	3.99
Light Chain d1	2.42
Heavy Chain D0	4.20×10^{-1}
Heavy Chain D1	5.26×10^{-1}
Heavy Chain D2	3.89×10^{-1}
Heavy Chain D3	1.78×10^{-1}
Heavy Chain D4	3.77×10^{-2}
Total DAR	3.32

■ Evaluation of DAR Inter-Day Precision by Nexera and LCMS-9030 System

Data obtained using the LCMS-9030 system was analyzed using Byos software from Protein Metrics. Fig. 5 shows the total ion current chromatogram obtained from analysis of the ADC-spiked human blood plasma sample. Using Byos to confirm the eluted substance for each peak determined that the ADC eluted within 7.0 to 9.5 minutes.

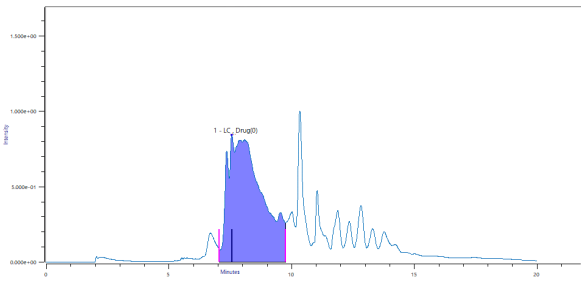

Fig. 5 Total Ion Current Chromatogram from ADC-Spiked Human Blood Plasma Sample

Fig. 6 shows the average mass spectrum from ADC elution peaks (peaks in the highlighted section in Fig. 5). Fig. 7 shows the mass spectrum obtained from multiply-charged ion analysis of the mass spectrum in Fig. 6.

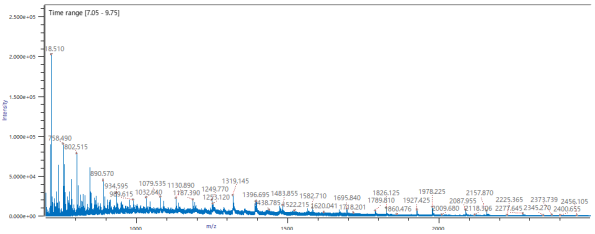

Fig. 6 Mass Spectrum from ADC-Spiked Human Blood Plasma Sample Using LCMS-9030 System

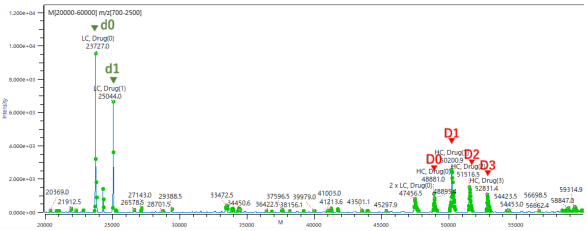

Fig. 7 Spectrum from Multiply-Charged Ion Analysis

The MALDI system detected all fragments from the ADC as singly charged ions ($[M+H]^+$), which resulted in a simple mass spectrum. However, the LCMS-9030 system detected the multiply-charged ions from the ADC as multiply-charged ions with 11 to 75 charges, resulting in an extremely complex mass spectrum (Fig. 6). Therefore, the software plotted the mass spectrum horizontal axis values from multiply-charged ion analysis converted to molecular weight values. Fig. 7 shows a signal from d0 detected near a molecular weight of 23727, a signal from d1 near 50201, a signal from D0 near 48881, a signal from D1 near 50201, a signal from D2 near 51517, and a signal from D3 near 52831. It also shows a signal near a molecular weight of 47457, presumably from a dimer of d0, which was also identified by the MALDI system.

$$Total\ DAR = \left(\frac{\sum_{i=0}^{1} i \times di\ peak\ hight}{\sum_{i=0}^{1} di\ peak\ hight} + \frac{\sum_{i=0}^{4} i \times Di\ peak\ hight}{\sum_{i=0}^{4} Di\ peak\ hight}\right) \times 2 \cdots (1)$$

■ Evaluation of DAR Inter-Day Precision by Nexera and LCMS-9030 System

The precision of intra-day and inter-day DAR values was determined to confirm the consistency of the DAR values obtained by the LCMS-9030 system.

The intra-day precision was calculated by preparing 3 identically pretreated ADC-spiked human blood plasma samples and then successively analyzing a sample from each of the 3 vials on the same day (Table 4).

The inter-day precision was calculated by preparing 3 identically pretreated ADC-spiked human blood plasma samples and then analyzing each of the samples once a day for 3 days (Table 5).

Table 4 Intra-Day Precision of DAR Values Using LCMS-9030 System

	Signal Intensity (counts per sec)		
Attribution	First	Second	Third
Light Chain d0	8.61×10^3	7.98×10^3	7.86×10^3
Light Chain d1	5.69×10^3	5.43×10^3	5.20×10^3
Heavy Chain D0	8.30×10^2	7.37×10^2	7.34×10^2
Heavy Chain D1	2.09×10^3	1.84×10^3	1.82×10^3
Heavy Chain D2	1.22×10^3	9.91×10^2	1.01×10^3
Heavy Chain D3	7.46×10^2	5.16×10^2	5.54×10^2
Heavy Chain D4	N.D.	N.D.	N.D.
Total DAR	3.57	3.44	3.47
Average DAR		3.49	
Relative Standard Deviation of DAR Values (%RSD)		1.90	

Table 5 Inter-Day Precision of DAR Values Using LCMS-9030 System

	Signal Intensity (counts per sec)		
Attribution	Day 1	Day 2	Day 3
Light Chain d0	9.56×10^3	5.20×10^3	3.75×10^3
Light Chain d1	6.66×10^3	3.23×10^3	2.21×10^3
Heavy Chain D0	1.15×10^3	2.66×10^2	2.20×10^2
Heavy Chain D1	2.62×10^3	9.45×10^2	8.35×10^2
Heavy Chain D2	1.53×10^3	5.60×10^2	5.20×10^2
Heavy Chain D3	1.11×10^3	2.63×10^2	1.68×10^2
Heavy Chain D4	N.D.	N.D.	N.D.
Total DAR	3.63	3.57	3.47
Average DAR		3.56	
Relative Standard Deviation of DAR Values (%RSD)		2.29	

Table 4 shows the DAR intra-day precision and Table 5 shows the inter-day precision. The results show that the DAR values from the samples tended to decrease over time (Table 5). A more detailed look found that the D3 ratio decreased, whereas the D1 and D2 ratios increased. That might have occurred due to payload detachment from the target ADC or other problems, rather than remaining stable within the sample solution.

The DAR relative standard deviation values obtained from repeatedly analyzing the samples 3 times within the same day were 2 % or less. When the samples were repeatedly analyzed each day for 3 days, the DAR values were 3 % or less. This confirms that the LCMS-9030 can be used to reliably evaluate DAR values. For samples with highly stable ADCs, the precision values would probably be even higher.

■ Conclusion

This Application News article describes using MALDI and LC-QTOF systems for multifaceted evaluations of the drug-to-antibody ratio (DAR) values in pretreated samples of human blood plasma spiked with an antibody-drug conjugate (ADC).

The MALDI system enables simple measurements because components are generally not separated by LC, so DAR values can be calculated without using specialized software. Although the resolution of MALDI-TOF MS is limited, it enables direct and quick evaluations.

The LC-QTOF system can provide highly accurate MS spectra with high resolution. In combination with dedicated Byos software, it enables detailed evaluations of DAR values. It also offers superior mass consistency and extremely consistent results with DAR intra-day relative standard deviation values of 2 % or less and inter-day relative standard deviation values of 3 % or less.

In conclusion, using multiple instruments for multifaceted evaluations enables more reliable ADC analysis.

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