An Integrated Workflow for the Comprehensive Analysis of Antibody-Drug Conjugates

Using Microfluidic HPLC-Chip/MS

Application Note

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Abstract

A highly automated and integrated method for determining intact mass, deglycosylated intact mass, drug-to-antibody ratios, and glycan composition of antibody drug conjugates has been developed using the Agilent HPLC-Chip solution and time-of-flight (TOF) mass spectrometry.

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Introduction

Antibody Drug Conjugates (ADCs) are a new generation of targeted biopharmaceuticals created by attaching cytotoxic agents through a linker to monoclonal antibodies (mAbs) targeting specific cells. There are different types of conjugation chemistries, including cysteine-linked, lysine-linked, and site-specific conjugation. Depending on the type of conjugation chemistry, different methodologies are used for the characterization.

The production and characterization of potent, highly selective, and low toxicity ADCs are more complicated than traditional mABs. Rigorous quality control is required to ensure that ADCs have a consistent glycan composition and drug-to-antibody ratio (DAR). Achieving the proper DAR is crucial to optimize the efficacy and minimize the toxicity of the ADC. The DAR is frequently determined using liquid chromatography/mass spectrometry (LC/MS), as is the glycan composition. Deglycosylation is often done offline before analysis to reduce the complexity of the ADC LC/MS results used for the DAR calculation.

This application note describes a novel and rapid integrated workflow for the determination of intact mass, DAR, and glycan composition of a lysine-linked ADC using the Agilent HPLC-Chip solution and time-of-flight (TOF) MS. For intact ADC analysis, separation of the glycosylated and drug-conjugated species was performed on a standard protein ID chip with a reversed-phase column. A mAb-ProtID-Chip equipped with an online reactor containing immobilized PNGase F enzyme as well as reversed-phase enrichment and analytical columns was used to determine the deglycosylated mass and DARs of the ADC. An alternate chip with the same deglycosylation reactor and porous graphitized carbon enrichment and analytical columns was used to characterize the released glycans. Nanogram amounts on-column can be analyzed, and each analysis can be accomplished in 15 minutes. In addition, online deglycosylation provides a solution that can be implemented in any clinical research laboratory with multiple users.

Experimental

Instruments

This workflow was developed using an Agilent 1260 capillary and nanoflow HPLC interfaced with the HPLC-Chip Cube and an Agilent 6230 Accurate-Mass TOF LC/MS. Different microfluidic chips were used for the characterization of the ADC. For standard intact ADC analysis, a reversed-phase chip was used. The mAb-ProtID-Chip with an enzyme reactor containing immobilized PNGase F and a PLRP-S 300Å, 5-µm column was used for intact deglycosylated ADC analysis and DAR calculations. The mAb-Glyco-Chip with immobilized PNGase F and porous graphitized carbon (PGC) as the stationary phase was used for characterization of the released N-glycans from the intact ADC. Triplicate injections were performed for all analyses. Table 1 lists the instrument conditions.

Data analysis

Data analysis was performed using Agilent MassHunter Qualitative Analysis with BioConfirm Software. Deconvolutions were performed using BioConfirm software's Maximum Entropy algorithm. The DAR calculation was performed using the Agilent DAR Calculator.

Table 1. LC and TOF MS conditions.

	Intact ADC analysis	DAR analysis, deglycosylated ADC	N-glycan analysis
LC Run conditions			
HPLC-chip	ProtID-Chip-150 (II) 150 mm × 300 Å C18 chip with 40 nL trap column	mAb-ProtID-Chip, PNGase F enzyme reactor, PLRP-S reversed-phase enrichment column and analysis column (p/n G4240-63001 SPQ 494)	mAb-Glyco-Chip, PNGase F enzyme reactor, graphitized carbon enrichment column and analysis column (p/n G4240-64021)
Injection volume	2 µL	2 µL	2 µL
Mobile phase	A) 0.1 % formic in H_20 B) 0.1 % formic in acetonitrile	A) 0.1 % formic in H_20 B) 0.1 % formic in acetonitrile	A) 0.1 % formic in H_20 B) 0.1 % formic in acetonitrile
Flow rate	0.5 µL/min	0.5 µL/min	0.5 µL/min
Linear gradient	Time (Min) %B 0.00 2 1.00 2 3.00 60 8.00 90 12.00 95	Time (Min) %B 0.00 2 6.00 2 7.50 32 8.00 85 9.00 85 9.01 2	Time (Min) %B 0.00 2 6.00 2 7.50 32 8.00 85 9.00 85 9.01 2
TOF MS conditions			
Ion mode	Positive ESI	Positive ESI	Positive ESI
Drying gas	N_2, 13 L/min, 250 °C	N_2, 13 L/min, 250 °C	N_2, 13 L/min, 250 °C
Fragmentor voltage	400 V	400 V	125 V
Skimmer 1 voltage	65 V	65 V	65 V
Capillary voltage	1,850 V	1,850 V	1,850 V
OctopoleRF peak	750 V	750 V	750 V
Scan rate	1 spectra/sec	1 spectra/sec	1 spectra/sec
Min range	500 *m/z*	500 *m/z*	200 *m/z*
Max range	7,000 *m/z*	7,000 *m/z*	1,700 *m/z*

Results and Discussion

Drug-to-antibody determinations

Using a standard protein ID chip, intact ADCs with the glycoforms were observed. Figure 1 shows a magnified view of the DAR 1 species. Heterogeneity of the glycan (G0, G1, and so forth) was characterized and annotated using BioConfirm in MassHunter Qualitative Analysis. The glycoform delta mass errors are indicated in parts per million (ppm) above each peak. The unlabeled peaks in Figure 1 correspond to an extra unconjugated linker (no drug attached) present on the ADC. The extra unconjugated linkers were observed for all DAR values in both intact and deglycosylated ADC runs.

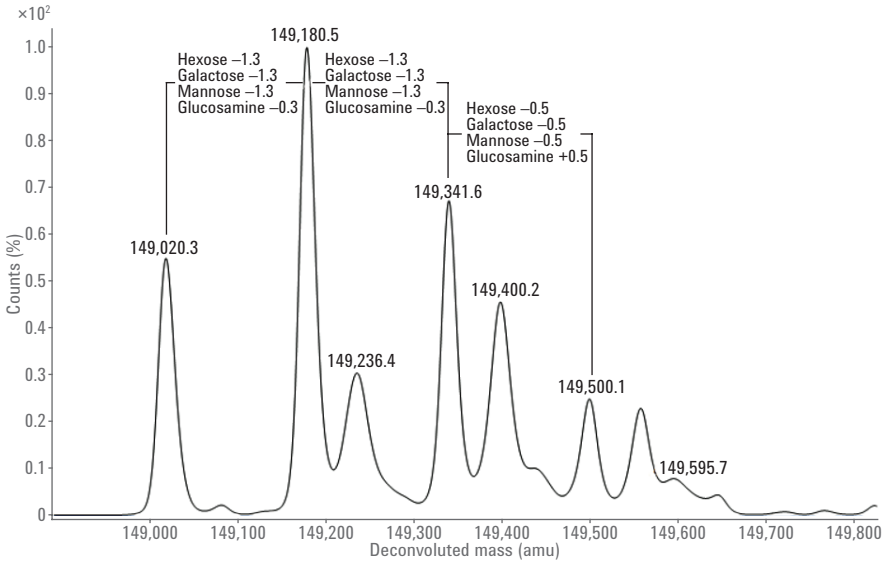

Figure 1. Deconvoluted spectrum of the intact ADC DAR 1 species with glycosylation and drug linker attached. Glycan heterogeneity generates multiple peaks (G0, G1, and so forth), and the mass errors ppm for each component of the possible core glycan are displayed above each peak. Peaks labeled only with a mass value are ADC glycan species with an additional unconjugated linker.

Using the mAb-ProtID-Chip for online deglycosylation, the DARs were calculated. The measured masses of the ADCs were within 10 ppm of the expected masses. Figure 2 shows the triplicate overlay of the deconvoluted spectra of the ADC after online deglycosylation on the chip. A distribution from DAR 0 to DAR 7 was observed. The DAR ratios were reproducibly calculated using the Agilent DAR Calculator, with relative standard deviations (RSDs) less than 5 %.

An average DAR value of 3.0 for the ADC was determined using the Agilent DAR Calculator (Figure 3). The DAR Calculator provides the flexibility for the user to select which peaks to integrate for the DAR calculation. As show in Figure 3, the first peak in each DAR species was used, and the resulting DAR value was consistent with the DAR value calculated when all peaks were integrated.

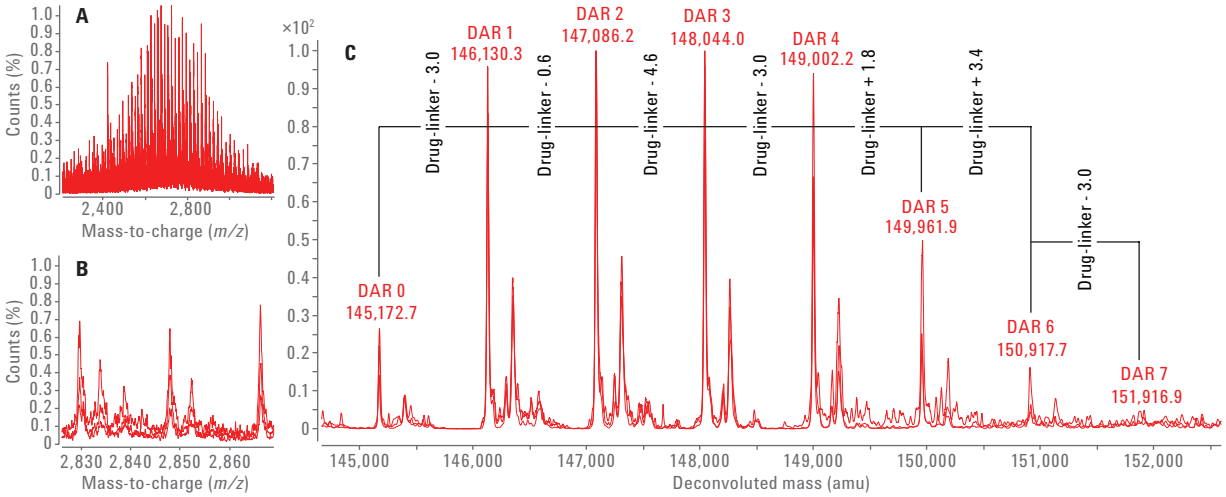

Figure 2. Triplicate injections of 1 µg of online deglycosylated lysine-conjugated ADC on the mAb-ProtID-Chip, using the Maximum Entropy algorithm for deconvolution. The triplicate overlay of the mass spectrum is shown in A, with the magnified view in B. The delta ppm mass error for the drug-linker complexes is shown for each DAR 1 to 7 in C.

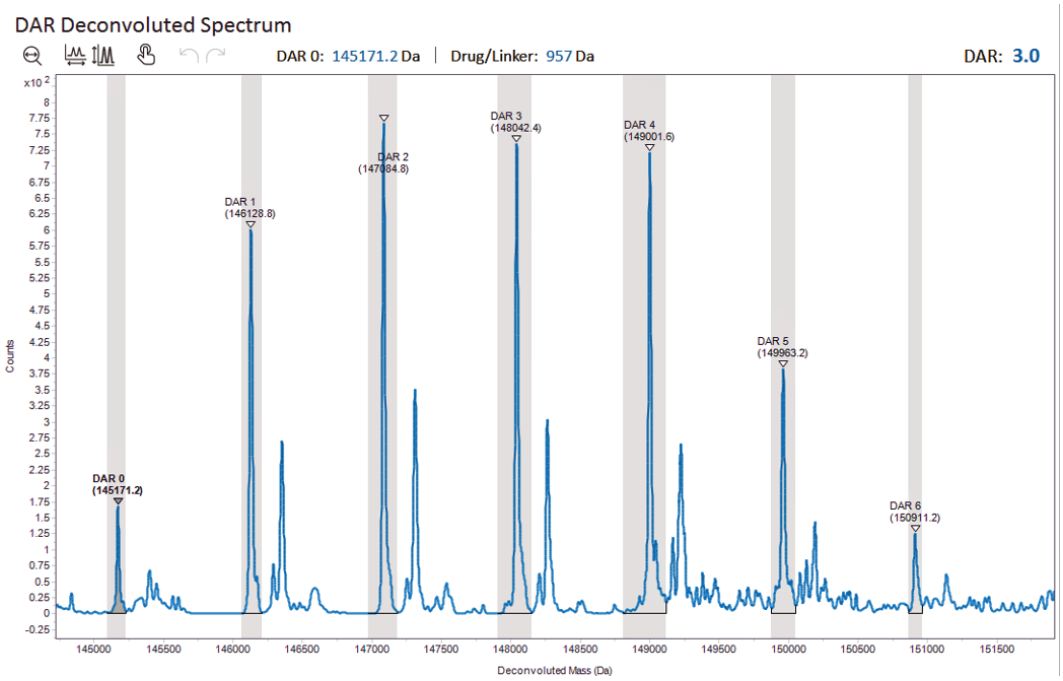

Figure 3. Agilent DAR Calculator software interface with selected peaks for DAR value calculation. The accurate mass for each chosen peak is also shown in parentheses. The average DAR value was calculated to be 3.0. The additional peaks for each DAR have additional unconjugated linkers attached.

Glycan identification

The mAb-Glyco-Chip with online deglycosylation was used to determine the glycan composition of the ADC (Figure 4). The N-glycans were reproducibly identified using Find by Formula in Agilent MassHunter Qualitative Analysis and matched against the mAb-Glyco-Chip Personal Compound Database Library (PCDL) provided with the chip.

Conclusions

An automated and integrated microfluidic ADC characterization workflow was developed using the HPLC-Chip. For intact ADC analysis, separation of the drug conjugated species was performed on a standard protein ID chip with a reversed-phase column. Online deglycosylation of the ADC was performed on chips with immobilized PNGase F enzyme, followed by separation of the deglycosylated ADC species on reversed-phase as the enrichment and analytical columns. For glycan analysis, online deglycosylation was followed by separation of the N-glycans on porous graphitized carbon as the enrichment and analytical columns. Using this novel microfludic nanoLC/MS workflow, accurate mass measurements were reproducibly observed for ADCs (glycosylated and deglycosylated) within 10 ppm of the expected masses. Reproducible DAR ratios were determined with RSDs less than 5 % using the Agilent DAR Calculator. An analysis time of 15 minutes was needed for each ADC or glycan analysis. Using this integrated microfluidic nanoLC/MS workflow, only nanogram amounts of ADC are needed and analysis times are shortened by using an immobilized enzyme reactor for the online deglycosylation of the ADC with automated analysis.

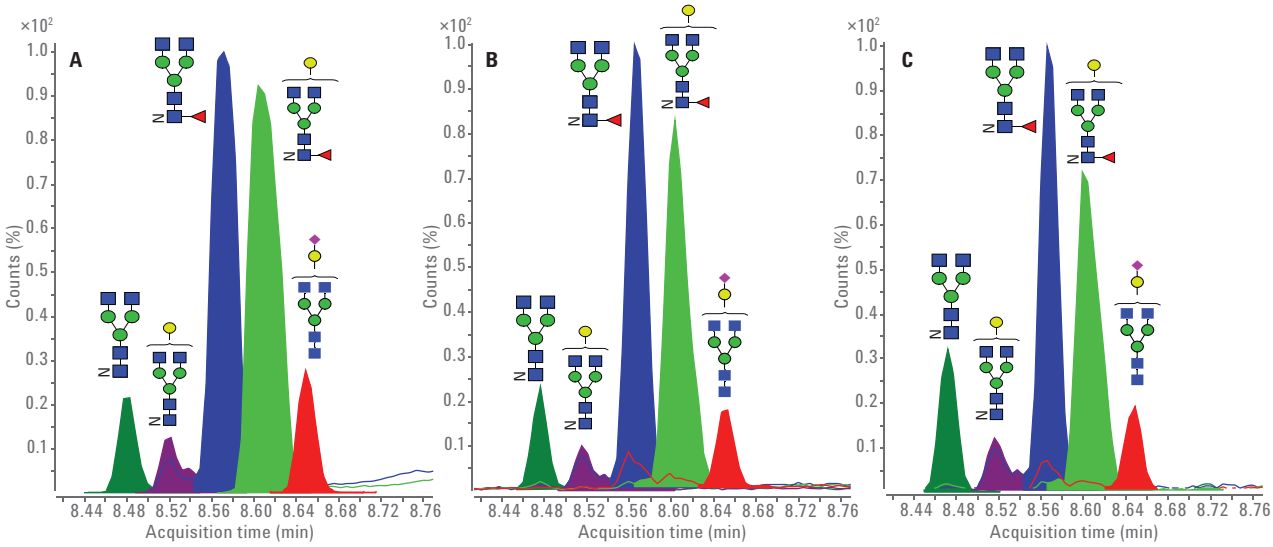

Figure 4. Triplicate analysis of the major N-glycans identified from the ADC using the mAb-Glyco-Chip with immobilized PNGase F in the enzyme reactor and porous graphitized carbon as the stationary phase in the analytical column for the characterization of the released N-glycans.

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