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Analysis of Antibody Drug Conjugate Using High Flow HPLC Coupled to Time-of-Flight Mass Spectrometry

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Introduction

Antibody Drug Conjugates (ADCs) are monoclonal antibodies in which cytotoxic drugs are covalently conjugated (figure 1). The antibody brings specificity to deliver drug to the target by binding to epitopes at the site of action. The drug is then slowly released at the target site. These ADC's are gaining a lot of attention as better cancer treatments. ADCs being protein molecules can undergo number of chemical alterations during drug conjugation, formulation and storage. Thus they demand reliable and sensitive methods for characterization. Liquid chromatography/mass spectrometry (LC/MS) technology provides a good platform to investigate ADCs at intact, subunit level and also to calculate drug antibody ratio (DAR). This study describes an optimized liquid chromatography/mass spectrometry method for analysis of ADC molecules

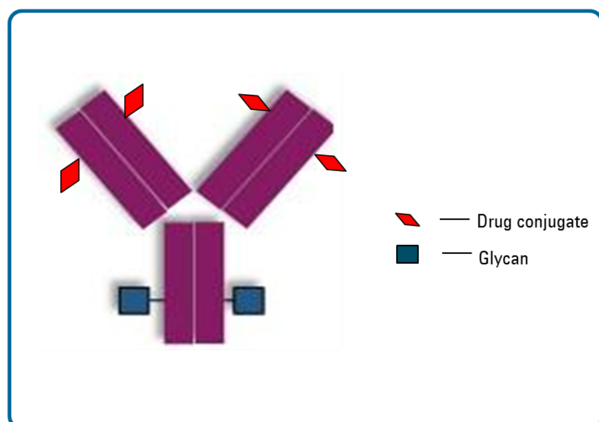

Figure 1. Schematic diagram of Antibody Drug Conjugate

Experimental

To characterize the antibody drug conjugates and its subunits. The workflow as shown in figure 2 was followed. The workflow consists of first preparing ADC for intact, deglycosylated and the reduced subunit forms of light and heavy chains. Then analyzed the samples using the Agilent 1290 Infinity LC coupled with the Agilent 6550 iFunnel Q-TOF LC/MS System. The data obtained from the LCMS was analyzed using BioConfirm software to obtain the masses and also to calculated the drug antibody ratio as discussed in the results section.

Experimental

Sample

Antibody drug conjugate (ADC) were diluted to 1 mg/mL using 0.1% formic acid in water. 5 µL was injected. For reduction of ADC, the protein were reduced using Dithiothreitol (DTT) at 60°C for one hour. Deglycosylation of the mAb was performed using PNGase in 20 mM Tris-HCl buffer pH 8.2 as described by the manufacturer(Sigma-Aldrich)

LC system: Agilent 1290 Infinity LC with Agilent Poroshell 300SB-C8 Column 2.1mmx 75mm was used.

MS systems: Agilent 6550 iFunnel Q-TOF LC/MS System with Agilent JetStream ion source.

LC Parameters	
Mobile phase A	0.1% formic acid in water
Mobile phase B	70% IPA/20% ACN/ 10% water with 0.1% formic acid
Gradient (Segmented)	At 0 min → 15% B At 4 min → 20%B At 5 min → 75%B At 10min → 100%B At 10.1 min → 15%B
Stop time	10.1 min
Post time	4 min
Column Temperature	60°C
Flow rate	0.4mL/min

MS parameters	
Ion mode	Positive ion mode (Profile)
Drying gas temperature	290 °C
Drying gas flow	14 L/min (nitrogen)
Nebulizer	45 psi
Sheath gas temperature	400 °C
Sheath gas flow	12L/min
Capillary voltage	5500 V
Fragmentor voltage	400 V
Nozzle voltage	2000 V
Oct RF Vpp	750 V
Acquisition parameters MS mode	Data were acquired at 1GHz, MS only mode, mass range 2000–5000 m/z.

Data Analysis: The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software. Maximum Entropy deconvolution algorithm was used for obtaining zero-charge spectrum of mAb.

Results and Discussion

Antibody drug conjugate

— Drug conjugate

— Glycan

Deglycoslylated

Intact

Reduced

1290 Infinity HPLC

6550 iFunnel QTOF

Data analysis by BioConfirm

Agilent Jet Stream

Hexabore Capillary

Dual Stage Ion Funnel

Figure 2. Workflow for characterization of Antibody Drug Conjugate

TIC

Mass spectrum

Deconvoluted spectrum

A

Counts vs. Acquisition Time (min)

Counts vs. Mass-to-Charge (m/z)

Counts vs. Deconvoluted Mass (amu)

151099.56

152060.30

153016.19

150145.23

149186.41

153973.69

155091.28

155891.01

TIC

Mass spectrum

Deconvoluted spectrum

B

Counts vs. Acquisition Time (min)

Figure 3. Total ion chromatogram(TIC),Mass spectrum and deconvoluted spectrum for (A) Intact and (B) Deglycosylated ADC.

Results and Discussion

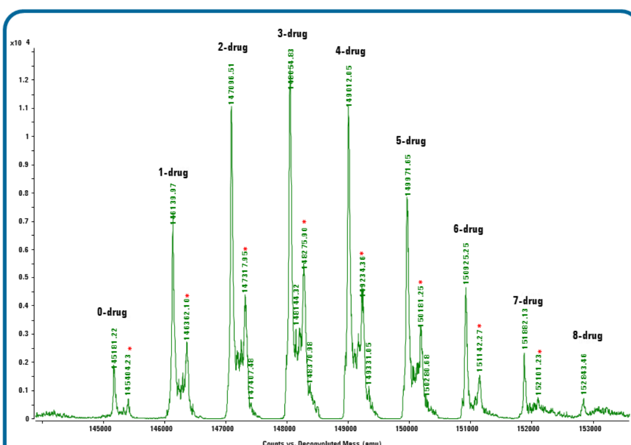

Figure 4. Deconvoluted MS spectra of deglycosylated ADC. The number of drugs attached to ADC is indicated in the figure. Extra linker was observed and labelled with (*). Reference: Characterization of the drug-to-antibody ratio distribution for antibody–drug conjugates in plasma/serum. *Bioanalysis* (2013) 5(9), 1057–1071

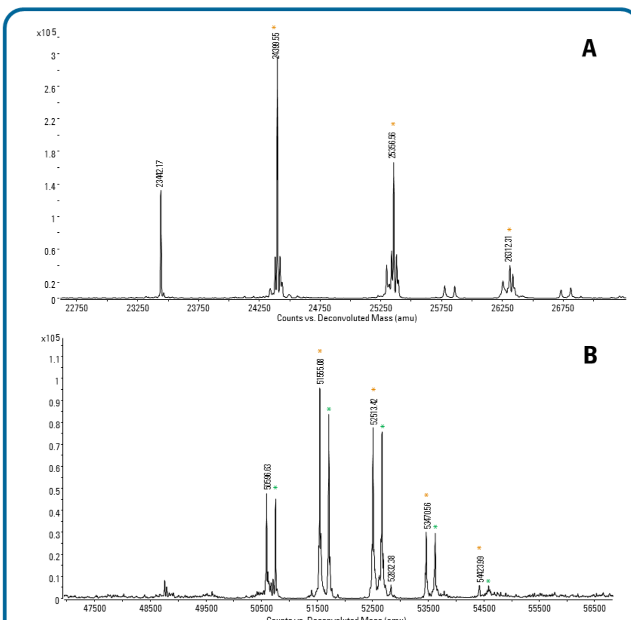

Figure 5. Deconvoluted spectra for light chain (A) and heavy chain(B) of ADC under study. The (*) indicates drug attached drug, while (*) corresponds to glycan attached to the molecules

Table 1:Drug Load Distribution (Peak Area %) and Calculated DAR Values

Drug Load	Peak area (%)	Weighted peak area (%)
0	2.579	0
1	11.34	11.34
2	18.87	37.74
3	23.14	69.42
4	19.42	77.70
5	13.95	69.73
6	6.501	39.01
7	2.738	19.17
8	1.464	11.72
DAR		~3.4

The intact MS results showed that the ADC mass spectrum under study is heterogeneous with different populations of ADC due to different types of glycans and the attached drugs. The deconvoluted spectrum of intact ADC was difficult to interpret due to complex nature of the sample (figure 3A). In order to simplify the interpretation, the ADC was deglycosylated. The deglycosylated mass spectrum showed well resolved charge states which suggested the removal of the glycans from ADC leaving only the covalently attached drug molecules (figure 3B). The deconvoluted spectrum showed 8 major drug attachments (figure 4) and from this the drug antibody ratio was calculated using the formula:

DAR = Σ(relative peak area × number of loaded drugs)/100

DAR values were calculated using the relative peak area (%) of each peak and the corresponding number of drugs loaded from the deconvoluted spectrum. Then multiplying the relative peak area (%) and the corresponding number of loaded drugs gives weighted peak percentage, which measures the contribution of individual drug loaded species to DAR(table 1)

Conclusions

- The analysis of ADC using Agilent 1290 Infinity LC coupled to Agilent 6550 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) has been demonstrated with excellent performance.
- Agilent MassHunter BioConfirm software provided data extraction, deconvolution which helped in calculation of DAR. Calculated DAR value was ~3.4 which is in close agreement with the published data for this ADC