Antibody Drug Conjugate Analysis using automated affinity purification and sensitive intact protein based LC/Q-TOF analysis

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Introduction

Quantitation of proteins in biological systems is traditionally performed either by ligand binding assays (LBA) or multiple reaction monitoring (MRM). LBAs can be highly sensitive but do not provide information about the physical state of the biomolecules (i.e. mass) and can be affected by non-specific binding. MRM assays are sensitive, but surrogate peptides represent only a portion of the total protein. Both LBA and MRM assays may miss unexpected changes to biomolecules that can alter the efficacy and immunogenicity. We developed a workflow using automated affinity purification of antibody-drug conjugates (ADCs) from serum with streptavidin cartridges, followed by UHPLC separation coupled to a newly developed 6545XT AdvanceBio LC/Q-TOF providing a reproducible, sensitive and accurate quantitation method for bioanalytical analysis of intact proteins.

Experimental

Immobilization

- Her2 extracellular domain (ECD) was biotinylated using EZ-Link™ Sulfo-NHS-LC biotin kit.
- 2 μg of biotinylated Her2 ECD was immobilized on each streptavidin (SA-W) cartridge using AssayMAP Bravo.

Affinity Purification and On-Cartridge Deglycosylation using AssayMAP Bravo

- Commercially obtained ADC (T-DM1) was diluted with water from 10 mg/mL to 1 mg/mL.
- Rat serum was centrifuged at > 14,000g for 5 min. The supernatant was diluted 1:1 with HEPES buffer (10 mM HEPES, 150 mM NaCl, pH-7.4). ADC was spiked into diluted rat serum to make 10, 3.2, 1, 0.32, 0.1 ng/μL samples.
- 100 μL ADC samples (n=6) were loaded on SA-W cartridge at 3 μL/min, followed by 150 μL HEPES buffer wash, and 50 μL deglycosylation buffer (20 mM Tris, pH-8.0) wash at 10 μL/min.
- 6 μL of Rapid PNGase F at 37 °C or a buffer control was aspirated onto each cartridge containing ADC and heated for 30 minutes. 10 μL of deglycosylation buffer was aspirated through the cartridge to remove the released glycan and enzyme after the reaction.
- Each cartridge was washed with 50 μL 1 M NaCl in HEPES buffer, and 50 μL 0.003% formic acid at 10 μL/min.
- The purified ADC was eluted with 15 μL of 1% formic acid into sample plate containing 15 μL 0.5% ammonium hydroxide to neutralize the purified ADC samples.

LC/MS Analysis

- Samples were analyzed using Agilent 1290 Infinity II UHPLC system with a PLRP-S column (PL1912-3802) coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF with a Dual Agilent Jet Stream ESI source.

Deconvolution and DAR calculation

- Spectra were extracted, averaged and deconvoluted using MassHunter BioConfirm. Drug-to-antibody (DAR) of ADC were determined using the MassHunter DAR Calculator. Peak area integrated in DAR calculator was used to generate the quantitation curve.
**DAR Determination of T-DM1 Purified from Serum**

![Graphs showing deconvoluted spectra and DAR values](image)

Figure 2-7. Deconvoluted spectra and DAR of (a) glycosylated and (b) deglycosylated T-DM1 purified from rat serum ADC samples in serum were simultaneously processed on the AssayMAP Bravo system. The steps taken include: 1) all ADC samples were purified with streptavidin affinity purification cartridge. 2) Half of the sample were treated with buffer control and half were treated with on-cartridge PNGase F digestion. 6 μL of final processed ADC samples were injected into LC/Q-TOF in triplicate. The injection amounts were 200, 64, 20, 6.4, 2 and 0.64 ng. DAR values were compared between (a) glycosylated and (b) deglycosylated ADCs at each injection amount (Fig. 2-7). The lowest injection amount where DAR values can be reliably determined were 6.4 ng for glycosylated T-DM1 and 2 ng for deglycosylated T-DM1.

![Graph showing relative abundance of DAR](image)

Figure 8. Relative abundance of different DAR species at different levels

The relative abundance of different DAR species (D0-D7) were compared for deglycosylated T-DM1 across different samples. (Fig. 8). The relative abundance (represented by the size of the bubbles) for each DAR species was consistent with D3, D4 being the most abundant and D0, D7 being the least abundant. The relative abundance of D0 and D7 increased when at very low sample amounts potentially due to interference of the background.
The peaks in deconvoluted spectra were integrated using the DAR calculator. The total peak area was calculated from D0 to D8. The average of the total peak area were calculated from triplicate injections and plotted against T-DM1 on-column injection amount. As shown in Figure 9, for deglycosylated T-DM1, a linear response from the detection limit of 2ng up to an injection of 200ng was achieved.

**Conclusions**

A complete workflow for ADC quantitation was implemented that integrates automated affinity purification, on-cartridge deglycosylation, LC/MS analysis, deconvolution and quantitation with DAR calculation.

- AssayMAP Bravo enables wide range of automated protein sample preparation applications, such as affinity purification and on-cartridge deglycosylation.
- 6545XT AdvanceBio Q-TOF provides high resolution and high analytical sensitivity for ADC sample analysis with 2 ng on-column limit of detection.
- MassHunter DAR calculator delivers fast and reliable peak integration and DAR values even at low detection levels.

**References**


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