**IN-DEPTH CHARACTERIZATION OF LYSINE-CONJUGATED ANTIBODY-DRUG CONJUGATES (ADCs) BY LC/MS QUALITATIVE AND QUANTITATIVE ANALYSIS**

Lixiu Chen, Henry Shiyong Ying Qing Yu, Weibin Chen  
Waters Corporation, Milford, MA 01757, USA

**INTRODUCTION**

Antibody drug conjugates (ADCs) is a sub-class of biopharmaceuticals which consists of monoclonal antibodies (mAbs) and cytotoxic drugs linked to mAbs by chemical linkers. The reactivity of primary amines and their compartmental availability in monoclonal antibodies (mAbs) make them a popular target for chemical conjugation in antibody-drug conjugates (ADCs). However, conjugation reactions of lysine residues result in highly heterogeneous mixtures with drugs in many combinations at different lysine sites on the mAb. Hence, the structural complexity and intrinsic heterogeneity of lysine-conjugated ADCs impose a prominent analytical challenge for current characterization methods. Quantification of conjugated peptides and site occupancy ratio determination was traditionally done by UV methods. The drawbacks for UV quantification include low sensitivity, insufficient selectivity and relative long analysis time. MS based quantification can provide higher selectivity and sensitivity compared to UV based methods. The biopharmaceutical industry lacks a complete workflow that enables efficient identification and quantification of ADC peptides, therefore facing a great challenge to make direct comparison on the site occupancy of ADCs from multiple sources. In this study, we present an integrated approach that combines multiplexed MS/MS acquisition for the in-depth primary structure characterization of lysine-conjugated ADCs. Both data-independent acquisition and data-dependent acquisition (DDA) methods were used to identify the lysine-conjugated peptides, confirm the conjugation sites, determine the relative site occupancy ratio and compare conjugated peptide levels across samples.

**METHODS**

Sample Preparation:
Peptide mapping analysis of Trastuzumab (Tmab) and trastuzumab emtansine (Tmab-DM1) were dematured, alkylated and digested by trypsin and Asp-N endoproteinase, respectively. Lysine enrichment (LSEnR) was added to each sample at final concentration of 50 fmol/ul as an internal standard. Enzyme digests of Tmab and Tmab -DM1 were analyzed in triplicate injections.

All samples were treated with PNGase F overnight to remove the N linked carbohydrates. Enzyme digests of Tmab and Tmab-DM1 were analyzed in triplicate injections.

**Intact Mass Analysis**

The distribution of the drug load is determined by LC/MS. The distribution profile was obtained by quantification of the difference between adjacent peaks, which is in agreement to the mass of covalently linked DM1 drug with one MCC linker. In both the innovator and candidate biosimilar ADC, a MCC peak corresponding to drug (D + 1, drug, etc.) is the least abundant peak relative to the unreacted linkage, which is attributed to the unlinked linker that modified the antibody but did not react with DM1.

**Peptide Mapping—Site Identification**

Peptide mapping workflows were used to identify the conjugated peptides. The signature fragment ion corresponds to the covalent bond within the drug conjugation. The CID fragmentation of the drug linkage site (conjugated pep. peak) generates a signature fragment ion (drug charge +1), commonly for all drug-conjugated peptides. The signature fragment ion corresponds to a partial drug fragment.

**RESULTS AND DISCUSSION**

**Peptide Mapping—Site Quantification**

For Tmab-DM1, 80 out of 92 conjugation sites were observed. UNIFI provided automated workflow for:  
- In-depth primary structure characterization of lysine-conjugated ADC 
- Site specific localization of ADC conjugation (Peptide Mapping workflows)  
- Quantification of relative site occupancy (Accurate Mass Screen-Depth workflow)  

While this presentation has focused on lysine-conjugated ADCs, these UNIFI workflows are directly applicable to other classes of ADC biopharmaceuticals.

**CONCLUSIONS**

**Figure 5. LC/MS chromatogram (BPI) of tryptic peptide mapping analysis for Tmab vs Tmab-DM1 in UNIFI comparison mode.**

**Figure 6. LC/MS chromatogram (BPI) of AspN peptide mapping analysis for Tmab vs Tmab-DM1 in UNIFI comparison mode.**

**Figure 7. MS/MS spectra to confirm conjugation sites for positionomers for Asp-N peptide.**

**Figure 8. Relative site occupancy determined using Asp-N digestion.**

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