# Complete Characterization of Trastuzumab Deruxtecan, a Cysteine-linked antibody drug conjugate, using high resolution accurate mass (HRAM) Mass Spectrometry

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## Abstract

**Purpose:** To demonstrate the capability of comprehensive characterization of cysteine-linked ADC trastuzumab deruxtecan on HRAM mass spectrometry.

**Methods:** A Thermo Scientific™ Vanquish™ Flex UHPLC coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer equipped with BioPharma option was used for all experiments.

**Results:** Complete characterization of trastuzumab deruxtecan at native intact, reduced chains, subunit and peptide level.


## Introduction

Over the last decade, antibody-drug conjugates (ADC) have evolved into promising and efficient therapeutic agents for targeted chemotherapy in cancers. As of August 2023, a total of 16 ADCs have been approved globally for hematological malignancies and solid tumors, and over 100 ADC candidates are undergoing clinical trials[1]. ADCs are generated through the conjugation of monoclonal antibodies (mAbs) targeting specifically the tumor-associated antigens (TAAs) of the tumor cell with highly potent cytotoxic drug payloads via a cleavable or non-cleavable chemical linker. Here we demonstrated the comprehensive characterization of Enhertu® (trastuzumab deruxtecan, T-DXd), developed by AstraZeneca and Daiichi Sankyo, a latest-generation homogenous cysteine conjugated-ADC with a high DAR, using a Vanquish Flex UHPLC coupled to a Orbitrap Exploris 240 mass spectrometer equipped with BioPharma Option.

## Materials and methods

### Sample Preparation

Native Intact Mass Analysis: Commercially available T-DXd was dissolved in $ddH_2O$ and desalted using 10k cut-off filter, final concentration is 2mg/mL in $ddH_2O$.

Reduced chains analysis: T-DXd was diluted to 1mg/mL using denaturing buffer (7M guanidine hydrochloride, 50mM Tris-HCl, pH=8.3) followed by DTT reduction.

Subunit analysis: T-DXd was diluted to 0.5mg/mL using 50mM Tris-HCl (pH=7.9), followed by IdeS digestion then DTT reduction.

Peptide mapping: T-DXd was diluted to 1mg/mL using denaturing buffer, then reduced, alkylated and digested with trypsin after buffer exchanging to 50mM Tris-HCl.

### UHPLC Separation

Thermo Scientific™ MAbPac™ SEC-1 (P/N 088790) was used for native intact MS separation, Thermo Scientific™ MAbPac™ RP (P/N 088648) was used for reduced chains and subunit analysis and Thermo Scientific™ Acclaim™ VANQUISH™ C18 UHPLC (P/N 071399-V) column was used for peptide mapping.

### Mass Spectrometry:

An Orbitrap Exploris 240 mass spectrometer with Biopharma option was used for all analysis.

### Data Analysis

Data analysis was performed using Thermo Scientific™ BioPharma Finder™ software 5.2.

**Figure 1. The Schematic of T-DXd.**

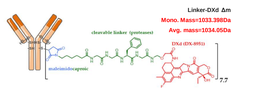



**Figure 2. Comprehensive LC-MS characterization workflow for T-DXd.**

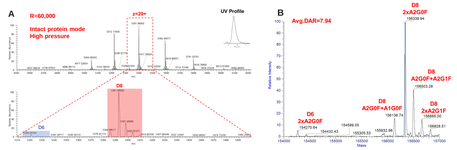

## Results

### Native intact MS analysis and DAR measurement

In this study, comprehensive characterization of T-DXd at multiple levels were achieved using HRAM mass spectrometry. The schematic of T-DXd is displayed in Figure1[2]. Figure2 illustrates the whole LC-MS based workflow.

During T-DXd conjugation, the interchain disulfide bonds of the mAb were reduced, resulted in non-covalently bonding of the light and heavy chains (LC and HCs). Therefore, intact MS under native condition is essential for accurate measurement of molecular weight and DAR at intact ADC level. In this experiment, 20μg sample was loaded for analysis. As shown in Figure3, the main peak corresponds to mAb bearing 8 conjugated drugs (expected mass of 156,339 Da for 2*A2G0F). Low level of intact ADC contains 6 payloads (~5%) was also detected. The average DAR is 7.94, which is calculated by BioPharma Finder software automatically, in agreement to previous publication[2].

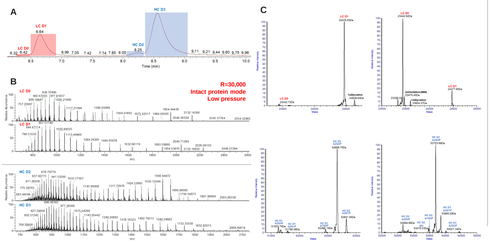
**Figure 3. Native intact MS of T-DXd.**
**A, full MS raw spectra . B, deconvolution result.**




### Reduced chains and subunit analysis under denaturing condition

To further understand the heterogeneity of this biomolecule, analyses of reduced chains and subunits under denaturing conditions were conducted. 5μg reduced sample was loaded for analysis. After reduction, the remaining interchain disulfide bonds were broken, resulting in a mixture of LC/HC isoforms. These isoforms can be separated on RPLC (Figure 4A). Full MS spectra of each LC/HC isoform are showed in Figure4B, and the molecular weight(MW) is presented in the deconvolution results (Figure 4C), where LC D1 and HC D3 were the main payloads, which agrees with native intact MS results. Minor LC D0 and HC D1/D2 were also detected. Other modifications, like oxidation and glycation on LC were also observed. Both payload and glycosylation distributions of the HC were identified.

**Figure 4. Reduced chains analysis of T-DXd under denaturing condition.**
**A,RPLC separation profile. B, full MS spectra. C, deconvolution results.**




For subunit analysis, the sample was digested with IdeS enzyme followed by DTT reduction. 5μg sample was loaded for analysis. Subunits carrying different payloads (LC D0 and D1, Fd' D2 to D3) and N-glycosylation (Fc) were chromatographically separated and successfully identified (Figure 5A-B). The relative abundance of LC D0 and Fd' D2 are very low(<5%), which is consistent with native intact MS and reduced chains analysis results. 240,000 resolution and intact protein mode (low pressure) were applied for subunit analysis data acquisition, provided baseline separation of isotopic peaks and highly sensitivity. Figure 5C displays the deconvolution results of subunits with different payloads. In addition to N-glycoforms distribution in Fc region and payload distribution in light chain and Fd' region, more PTMs, such as succinimidation, oxidation and glycation were detected.

Table 1 and 2 show lists of calculated weighted averages for the DAR of reduced chains and subunits, respectively. The proportion of each peak area was calculated and the sum of the peak area proportions totaled 100%. The weighted average of DAR = 2 X (Σ weighted peak area of each chain(or subunit) )/ 100, the weighted average of DAR for T-DXd was calculated to be 7.77(reduced chains)or 7.74(subunit).

**Figure 5. Subunit analysis of T-DXd under denaturing condition. A,RPLC separation profile. B, full MS spectra. C, deconvolution results.**

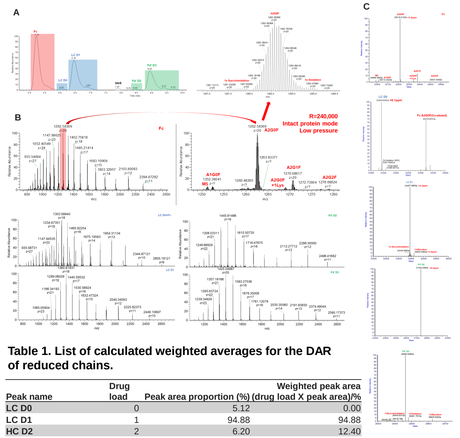



**Table 1. List of calculated weighted averages for the DAR of reduced chains.**

| Peak name | Drug load | Peak area proportion (%) | Weighted peak area (drug load X peak area)/% |
|---|---|---|---|
| LC D0 | 0 | 5.12 | 0.00 |
| LC D1 | 1 | 94.88 | 94.88 |
| HC D2 | 2 | 6.20 | 12.40 |
| HC D3 | 3 | 93.80 | 281.40 |
| Weighted average DAR | 7.77 | | |

**Table 2. List of calculated weighted averages for the DAR of subunits.**

| Peak name | Drug load | Peak area proportion (%) | Weighted peak area (drug load X peak area)/% |
|---|---|---|---|
| Fc | 0 | 100.00 | 0.00 |
| LC D0 | 0 | 4.99 | 0.00 |
| LC D1 | 1 | 95.01 | 95.01 |
| Fd' D2 | 2 | 8.06 | 16.12 |
| Fd' D3 | 3 | 91.94 | 275.81 |
| Weighted average DAR | 7.74 | | |

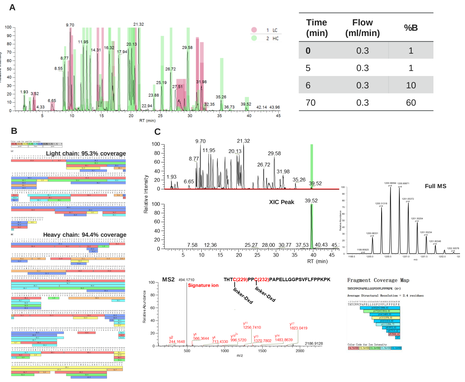
### Peptide mapping and conjugation peptides identification

Peptide mapping is a widely used analytical method to characterize the biopharmaceuticals, providing detailed information about the molecule, including sequence coverage, PTM identification, localization and relative quantification, sequence variants, and disulfide bonds. In this study, 10μg trypsin digested T-DXd was loaded, and the sequence coverage of this ADC was 94.4% (HC) and 95.3% (LC) with MS/MS identification(Figure 6A-B). The incomplete coverage was attributed to the generation of short peptides, only two or three amino acids in length, which were too short for MS/MS identification but can be detected by MS1.

Four potential conjugation sites were identified in this molecule: LC C214, and HC C223, C229, and C232. The payload conjugation increases the hydrophobicity of drug-loaded peptides and can diminish MS signals, as observed. However, the exceptional sensitivity of the Orbitrap analyzer allowed for the collection of sample information on these peptides. We identified all theoretical conjugation sites and calculated the site occupancy (Figure6C and Table3). Since this homogenous Cys-ADC with a high DAR, light chain C214, heavy chain C223, C229 and C232 were highly occupied (>95%).

Other common modifications in biotherapeutic products, such as deamidation, succinimidation, aspartic acid isomerization, glycation and oxidation were identified and relatively quantified (Figure6D and Table4). It is worth noting that deamidation% of light chain N30 is 10.63% and 1.09% of heavy chain N55. The most abundant N-glycoform is A2G0F (77.86%).

**Figure 6. Peptide mapping results and conjugation peptides identification. A, base peak chromatograms using 65 min LC-gradients. B, sequence coverage map. C, XIC peak, full MS spectrum, MS2 spectrum and fragment coverage map of hinge region peptide *THTC(229)PPC(232)PAPELLGGPSVFLFPPKPK*. D, relative abundances of other common PTMs.**




| Time (min) | Flow (ml/min) | %B |
|---|---|---|
| 0 | 0.3 | 1 |
| 5 | 0.3 | 1 |
| 6 | 0.3 | 10 |
| 70 | 0.3 | 60 |

**Table 3. conjugation sites occupancy, calculated based on peptide XIC area.**

| Modification | Abundance inj.01 | Abundance inj.02 | Abundance inj.03 | Abundance Avg. | Abundance Avg. CV |
|---|---|---|---|---|---|
| Dxd (HC C229 and C232) | 95.60% | 95.48% | 95.12% | 95.40% | 0.26% |
| Carboxymethylation (HC C229 and C232) | 4.40% | 4.52% | 4.88% | 4.60% | 5.48% |
| Dxd(LC C214) | 99.85% | 99.83% | 99.84% | 99.84% | 0.01% |
| Carboxymethylation (LC C214) | 0.15% | 0.17% | 0.16% | 0.16% | 5.13% |
| Dxd(HC C223) | 100.00% | 100.00% | 100.00% | 100.00% | 0.00% |

D

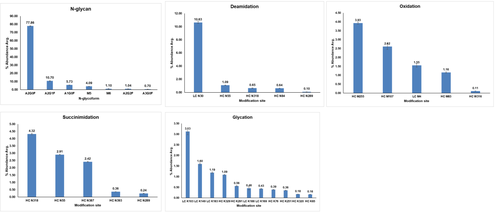



**Table 4. relative abundances of HC D283 isomerization and C terminal Lysine truncation, calculated based on peptide XIC area.**

| Modification | Peptide Sequence | Abundance inj.01 | Abundance inj.02 | Abundance inj.03 | Abundance Avg. | Abundance Avg. CV |
|---|---|---|---|---|---|---|
| HC D283 Isomerization | FNWYVDGVEVHNAK | 0.13% | 0.11% | 0.11% | 0.12% | 7.60% |
| HC K Loss | SLSLSPGK | 86.45% | 86.34% | 86.40% | 86.39% | 0.06% |

## Conclusions

- In this work we demonstrated outstanding performance of HRAM Orbitrap mass spectrometry for comprehensive characterization of Trastuzumab deruxtecan, a latest-generation cysteine-linked ADC using HRAM mass spectrometry.
- High resolution and sensitivity benefits all applications, allowing for DAR measurement at native intact level, N-glycoforms and PTMs identification at subunit level, confident detection and relative quantification of very low abundant PTMs and nonconjugated peptides at peptide level.

## References


1. Antibody-drug conjugates come of age in oncology. Nat Rev Drug Discov 2023; 22: 641–61.
2. Pharmaceuticals 2020, 13, 245; doi:10.3390/ph13090245.


## Trademarks/licensing