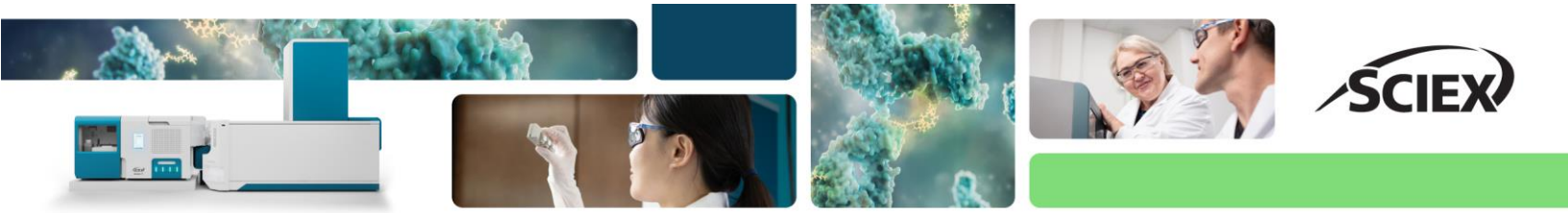

Charge variant analysis of antibody-drug conjugates using an icIEF-UV/MS workflow

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This technical note demonstrates a novel integrated workflow using the Intabio ZT system for charge variant analysis of antibody-drug conjugates (ADCs). This innovative system offers direct chip-based integration of icIEF with mass spectrometry (MS), achieving separation and confident identification of proteoforms with different payloads, reliable quantitation of charge variants and determination of drug-to-antibody ratios (DARs). Further, the streamlined icIEF-UV/MS platform significantly reduces acquisition and analysis time compared to ion exchange chromatography (IEX) with fraction collection, eliminating multiple weeks of effort.[1]

The icIEF-UV/MS data were interpreted using an Intabio intact icIEF-UV/MS workflow within Biologics Explorer software for automated protein deconvolution and accurate DAR calculation. With a single injection, 8 different trastuzumab emtansine (T-DM1) payloads were electrophoretically separated and an average DAR of 3.6 was measured.

ADCs are cutting-edge biotherapeutics that selectively target specific cells by linking potent cytotoxic drugs to monoclonal antibodies (mAbs).[2] The drug and mAb are connected through a cysteine-based, lysine-based or site-specific linker. Variations in the number of drug molecules attached to the linkers result in a complex mass spectrum. Additionally, the types of linkers used significantly affects the charge heterogeneity of ADCs.[3] Depending on the linker type, the DAR of an ADC can reach as high as 8. Additionally, post-translational modifications (PTMs), such as glycosylation, glycation and deamidation can contribute to the heterogeneity of an ADC product.[4] Therefore, performing charge-based separation prior to MS analysis simplifies the complexity of the spectrum and improves accurate DAR measurements (Figure1).

Key benefits of intact ADC analysis using the icIEF-UV/MS platform method

- **Single-injection** icIEF-UV/MS analysis **of intact ADCs:** This streamlined workflow provides charge-based separation of intact ADCs using icIEF, confident charge-variant identification through high-resolution MS and accurate DAR measurement in a single injection
- **Superior separation of** charge variants**:** The Intabio ZT system offers powerful electrophoretic separation for each of the DM1 payload
- **Accurate DAR measurement**: Biologics Explorer software offers automated protein deconvolution, confident peak annotation and DAR calculation
- **Streamlined workflow:** The icIEF-UV/MS workflow is streamlined from data acquisition using the Intabio ZT system and ZenoTOF 7600 system to automated data analysis by intuitive Biologics Explorer software
- **Routine ADC assessment:** The icIEF-UV/MS workflow can be readily incorporated into standard procedures to assess and monitor product quality during ADC development and manufacturing

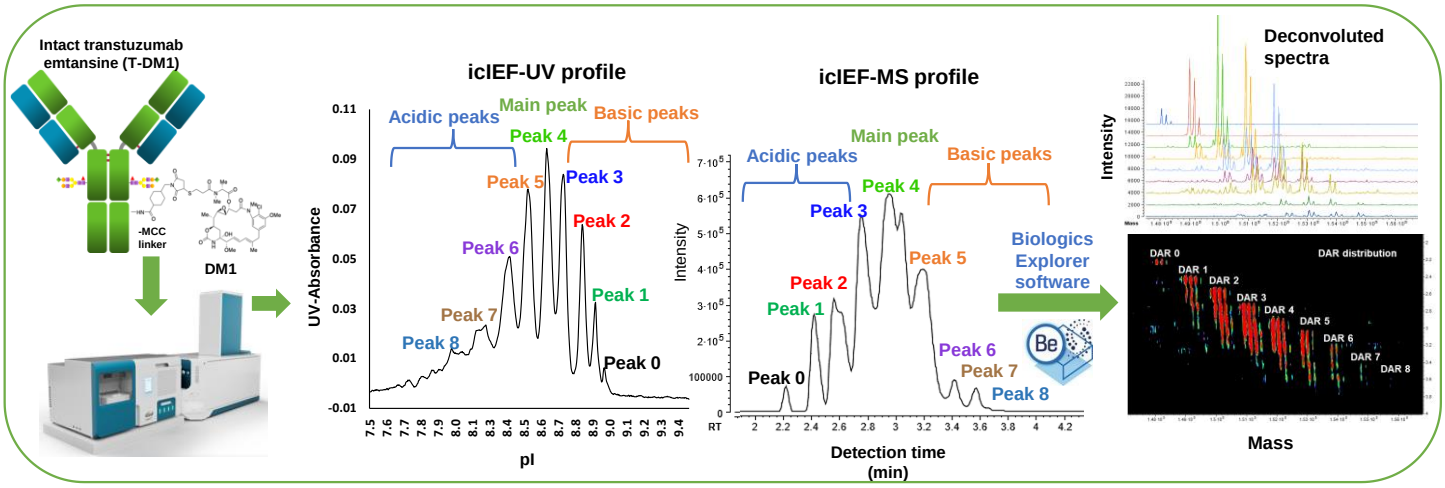

Figure 1. A streamlined intact protein analysis workflow for determining the average DAR of intact T-DM1 using the Intabio ZT system coupled to the ZenoTOF 7600 system. Intact MS data of T-DM1 were analyzed by Biologics Explorer software using Intabio intact icIEF-UV/MS workflow for automated DAR calculation. An average DAR of 3.6 was measured for the intact T-DM1.

Introduction

ADCs exhibit high complexity and structural heterogeneity from various product-related species, such as those containing different numbers of the payload and/or PTMs.[5] The charge heterogeneity arises from cellular processes, chemical degradation and manufacturing conditions.[6] This heterogeneity underscores the need for accurate DAR measurement and comprehensive PTM characterization to achieve high-quality ADC products.[6] Charge-based separation prior to intact MS analysis enables the confident identification of charge variants and accurate DAR measurement. This approach may be valuable for the development and quality control of ADCs to ensure their safety, efficacy and potency.[7] In this technical note, a streamlined icIEF-UV/MS workflow was leveraged to obtain a high-resolution charge variant separation of T-DM1 and accurate DAR measurement.

Methods

Equipment: The Intabio ZT system (SCIEX) and Intabio ZT cartridge (SCIEX, P/N: 5088248) were used to separate T-DM1 and its charge variants. MS detection was performed on the ZenoTOF 7600 system equipped with components of the OptiFlow interface (SCIEX, P/N: 5084645).

Chemicals and reagents: The Intabio system–Electrolytes and Mobilizer kit (P/N: 5088205) consists of the anolyte, catholyte and mobilizer solutions. The stock solutions of the anolyte and catholyte are 1% formic acid and 1% diethylamine, respectively. The mobilizer is composed of 25% acetic acid, 25% acetonitrile and 50% water. The anolyte and mobilizer were used without further dilution. The stock catholyte solution of 1% diethylamine was diluted to 0.25% diethylamine prior to use in the reagent drawer. A 500mM cathodic spacer solution containing free base L-arginine (Arg) (purity ≥ 98.5%, Sigma-Aldrich, P/N: A8094-25G) was prepared by dissolving 870 mg of Arg powder into 10 mL of Milli-Q water. The peptide pI markers (CanPeptide) were individually dissolved in Milli-Q water to a final concentration of 5 mg/mL.

Sample preparation: Prior to icIEF-UV/MS analysis, lyophilized T-DM1 was reconstituted in deionized (DI) water to a final concentration of 5 mg/mL. For the intact T-DM1 analysis, 400 µg of T-DM1 was mixed with the master mix solution, which contains 15mM Arg, 3% Pharmalyte, narrow range 8–10.5 (Cytiva, P/N: 17045501), 3% Pharmalyte, narrow range 5–8 (Cytiva P/N: 17045301) and 6.25 µg/mL peptide pI markers. The mixture was vortexed and then degassed by centrifugation at 3900 cf.

icIEF-UV/MS analysis: A 1 mg/mL T-DM1 solution was prepared as described above and then analyzed using the Intabio ZT cartridges. The icIEF separation parameters used are provided in Table 1. UV absorbance measurements were collected at 1 Hz throughout the focusing and mobilization steps. The samples were introduced to the ZenoTOF 7600 system with a controlled flow of mobilizer solution at 3 µL/min. The TOF MS data were acquired using the parameters listed in Table 2.

Table 1. Key parameters for icIEF separation.

Hold time (s)	Anode voltage (V)	Cathode setting	Mobilization setting	Step
60	*1500*	*0 V*	*0 A*	*Focusing*
60	*3000*	*0 V*	*0 A*	*Focusing*
300	*4500*	*0 V*	*0 A*	*Focusing*
600	*8500*	*0 A*	*5500 V*	*Mobilization*

Table 2. TOF MS parameters.

Parameter	Value
Curtain gas	*25 psi*
Spray voltage	*5500 V*
TOF start mass	*2000 m/z*
TOF stop mass	*6000 m/z*
Accumulation time	*0.5 s*
Source temperature	*100°C*
Declustering potential	*190 V*
Collision energy	*55 V*
Time bins to sum	*150*

***Data processing*:** The icIEF-UV profiles and mass spectra from the icIEF-UV/MS analysis of T-DM1 were analyzed using Biologics Explorer software. Each peak in the icIEF-UV profile was integrated to determine peak area and percentage composition. Intact masses of the main peak and charge variants from the icIEF- MS profile were determined using a deconvolution algorithm. The average DAR of T-DM1 was automatically calculated by the DAR calculator integrated into the software.

Intact T-DM1 charge variant characterization using the icIEF-UV/MS workflow

To determine the charge heterogeneity profile and measure the DAR, T-DM1 was analyzed using the Intabio ZT system coupled to the ZenoTOF 7600 system. Figure 2A shows the icIEF-UV profile of T-DM1 that demonstrates a clear separation of 4 acidic peaks (peaks 5–8), 4 basic peaks (peaks 0–3) and the main peak (peak 4). The peaks separated at lower pI values correspond to the species with higher DARs. Table 3 provides the relative area of each peak that corresponds to a different DAR.

Similar separation profiles were obtained from icIEF-UV (Figure 2A) and icIEF-MS (Figure 2B) measurements. Basic peaks on the icIEF-UV profile that have high pI values are introduced first into the MS system for analysis and therefore appear at earlier detection time points (left side of the icIEF-MS profile in Figure 2B). All the peaks observed in the icIEF-UV profile were also detected in the icIEF-MS profile with similar separation resolution. In addition, the 2 profiles showed similar relative peak areas of the acidic variants (peak 5–8), basic variants (peak 0–3) and main species (peak 4).

Table 3. Relative peak areas of different charge variant peaks in T-DM1 containing different DARs ranging from 0–8 separated by icIEF-UV.

Peaks	Area (%)
Basic 1	*2.6*
Basic 2	*5.1*
Basic 3	*10.3*
Basic 4	*16.7*
Main	*20.2*
Acidic 1	*15.9*
Acidic 2	*15.1*
Acidic 3	*10.3*
Acidic 4	*3.8*

The variation in the number of DM1 payloads linked to the antibody led to different charge isoforms. Figure 2C shows the stacked view of intact protein mass spectra results from Biologics Explorer software. A complex icIEF-MS profile of intact T-DM1 with DARs ranging from 0–8 was observed (Figure 2C). The major peaks identified in the deconvoluted spectrum (Figure 2D) were G0F and G1F glycoforms of T-DM1 carrying 2-6 payloads. A mass difference of +958 Da measured between the adjacent major peaks (Figure 2C) corresponds to the DM1 payload conjugated with the MCC linker.

In addition, a series of low-abundant peaks with a mass difference of +221 Da was observed for each G0F+G1F glycoform (Figure 2D). These species can be attributed to the ADC conjugated with 1 free linker but not the DM1 payload.[7] The MS ion map (Figure 3A) helps visualize the addition of the linker, as the acidic shift shown is larger than the acidic shift by increasing the mass induced by the addition of a linker and a payload.

Integrated DAR measurement workflow for T-DM1

The analysis of intact T-DM1 data was streamlined using an Intabio intact icIEF-UV/MS workflow template in Biologics Explorer software. This intuitive software provides powerful data visualizations, as shown in Figure 3. A representative ion map of intact T-DM1 shows the DAR distribution of T-DM1 (Figure 3A) with the detection time shown on the y axis, demonstrating the separation achieved. The high-resolution 3D display (Figure 3B) shows the 360° view of separation, DAR distribution and peak abundances. Enhanced data visualization and more precise data interpretation are achieved by accurately identifying each glycoform and different payloads, including the low-abundant species with a mass difference of +221 Da from the G0F+G0F major glycoforms. Biologics Explorer software provides automated calculation of the average DAR of an ADC. In this study, an average DAR value of 3.6 was measured for the T-DM1 (Figure 3C), consistent with the values reported for T-DM1 in the literature.[7,8,9]

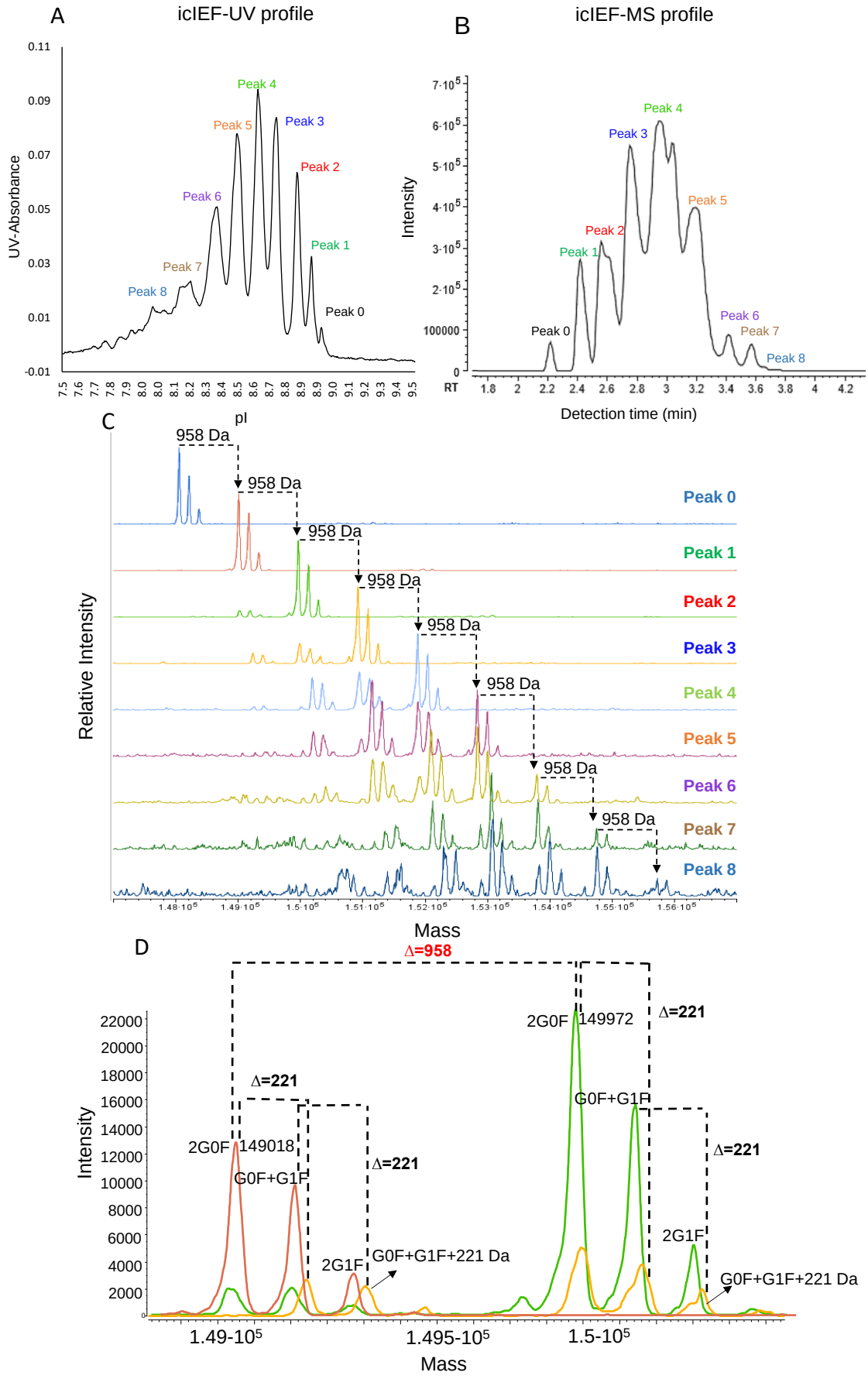

Figure 2. Intact protein separation, detection and deconvolution of T-DM1 using the icIEF-UV/MS workflow. (A) An icIEF-UV profile demonstrating charge variant separation of 9 major species of T-DM1. (B) icIEF-MS profile (intact MS base peak electropherogram (BPE)) of T-DM1 charge variants detected by the ZenoTOF 7600 system. (C) Stacked view of deconvoluted MS spectra of intact T-DM1 with various numbers of the payload separated by icIEF. A mass difference of +958 Da was observed for the adjacent peaks. (D) Zoomed-in view of the deconvoluted spectrum showing 3 major glycoforms with DARs of 1–2. A mass difference of +221 Da was observed for these major glycoforms. The +221 Da species correspond to the antibody conjugated with the linker but no coupled payload.

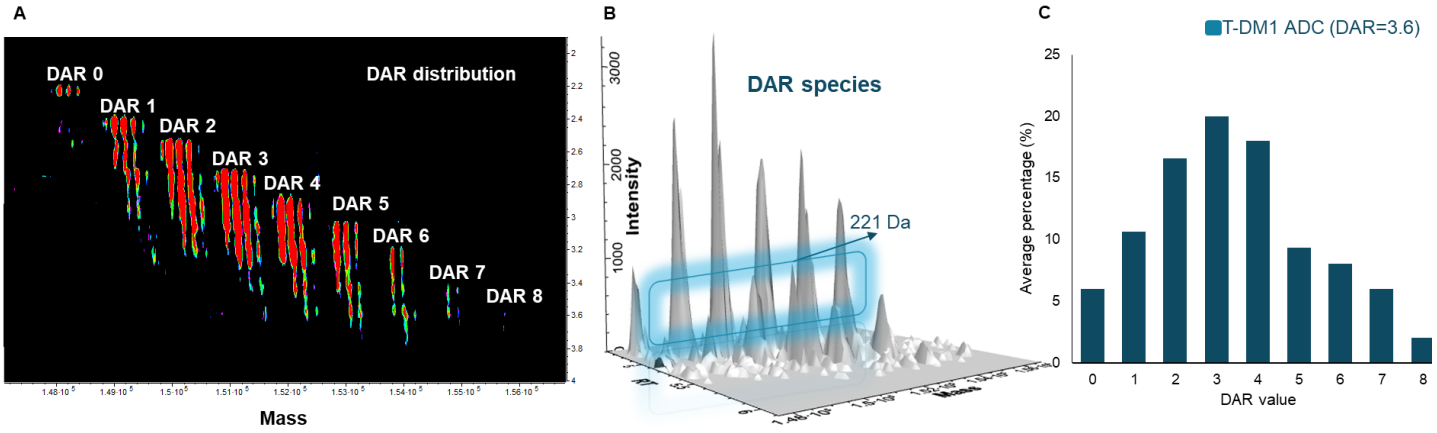

Figure 3. Intuitive data visualization of intact deconvolution and DAR measurement of T-DM1 using Biologics Explorer software. (A) MS ion map view of the identified major peaks of T-DM1 with DAR distribution. (B) 3D view of the identified different DM1 payloads providing confident information about the detected payload, DAR distribution along with lower intensity peaks (+221 Da mass shift) identification. (C) An average DAR value of 3.6 was automatically determined by Biologics Explorer software for the intact T-DM1.

Conclusions

- The streamlined icIEF-UV/MS workflow leverages the power of charge variant separation by icIEF-UV on the Intabio ZT system with high-resolution MS detection offered by the ZenoTOF 7600 system
- The icIEF-UV separation efficiency and resolution of T-DM1 were preserved after mobilization, enabling confident detection and identification of the main peak and its charge variants, including the low-abundant species
- Accurate mass measurements led to high-confidence annotation of T-DM1 glycoforms with up to 8 drug payloads with an average DAR value of 3.6
- Biologics Explorer software offers intuitive workflows, powerful tools and automated data interpretation for accurate intact mass measurements, confident protein deconvolution, automated DAR measurements and rapid results review

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