Application Note

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Intact- and Peptide-Level Characterization of Trastuzumab Emtansine In Vitro Transformation

Using the Agilent AssayMAP Bravo and 6545XT AdvanceBio LC/Q-TOF

Suitable for Agilent
1290 Infinity III LC

Author

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Abstract

Antibody-drug conjugates (ADCs) are designed to facilitate the targeted delivery of cytotoxic drugs, improving antitumor effects while minimizing toxicity. Loss of the conjugated drug from the ADC leads to compromised efficacy and safety. In this application note, we demonstrate a highly specific, sensitive, and flexible workflow that combines ligand-binding affinity capture with high-resolution mass spectrometry (MS). Using this workflow, we successfully characterized the transformation of trastuzumab emtansine (T-DM1) after a four-day incubation in phosphate-buffered saline (PBS) and plasma at both intact and peptide levels.

Introduction

Trastuzumab emtansine (T-DM1) is an ADC for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer.[1] Monoclonal antibody trastuzumab serves as antibody backbone of T-DM1. The lysine amines of trastuzumab are conjugated to the cytotoxic agent emtansine (DM1). The drug-to-antibody ratio (DAR) ranges from 0 to 8, with an average of approximately 3.5.[2] However, the conjugated drug may become detached from the ADC due to factors such as storage condition or stress over time. Drug loss leads to ADC compositional change and can affect its efficacy and safety. Therefore, characterization of the ADC transformation under certain conditions is crucial to elucidate the metabolic mechanism of the ADC.

In this application note, a ligand binding LC/MS (LB-LC/MS) hybrid assay was developed to evaluate the changes of T-DM1 at both intact and peptide levels after incubation in buffer and rat plasma for four days. The LB-LC/MS hybrid assay has advantages in specificity, sensitivity, and flexibility compared to traditional ELISA-based ligand binding assays. This hybrid assay consists of the Agilent AssayMAP Bravo protein sample prep platform, the Agilent 1290 Infinity II bio LC, and the Agilent 6545XT AdvanceBio LC/Q-TOF system. Data analysis was performed with Agilent MassHunter BioConfirm software, version 12.1. The workflow components are illustrated in Figure 1.

Experimental

Materials

Immunoaffinity and deglycosylation: Recombinant human HER2 extracellular domain (ECD) was purchased from ACROBiosystems (Newark, DE, U.S.). EZ-Link Sulfo-NHS-LC-Biotin and Zeba Spin Desalting columns, 7K MWCO, 0.5 mL, were from Thermo Fisher Scientific (Pittsburgh, PA, U.S.). Rat serum was from InVivos (Singapore). Rapid PNGase F was from New England Biolabs (Ipswich, MA, U.S.). AssayMAP Streptavidin cartridges (SA-W) were from Agilent. Trizma base, HEPES, ammonium hydroxide, and NaCl were from MilliporeSigma (Burlington, MA, U.S.).

Trypsin digestion: Urea, Trizma base, dithiothreitol (DTT), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma.

Generic: LC/MS-grade formic acid (FA) was purchased from Fisher Scientific (Pittsburgh, PA, U.S.).

The T-DM1 sample was purchased from Alliance Pharm (Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system.

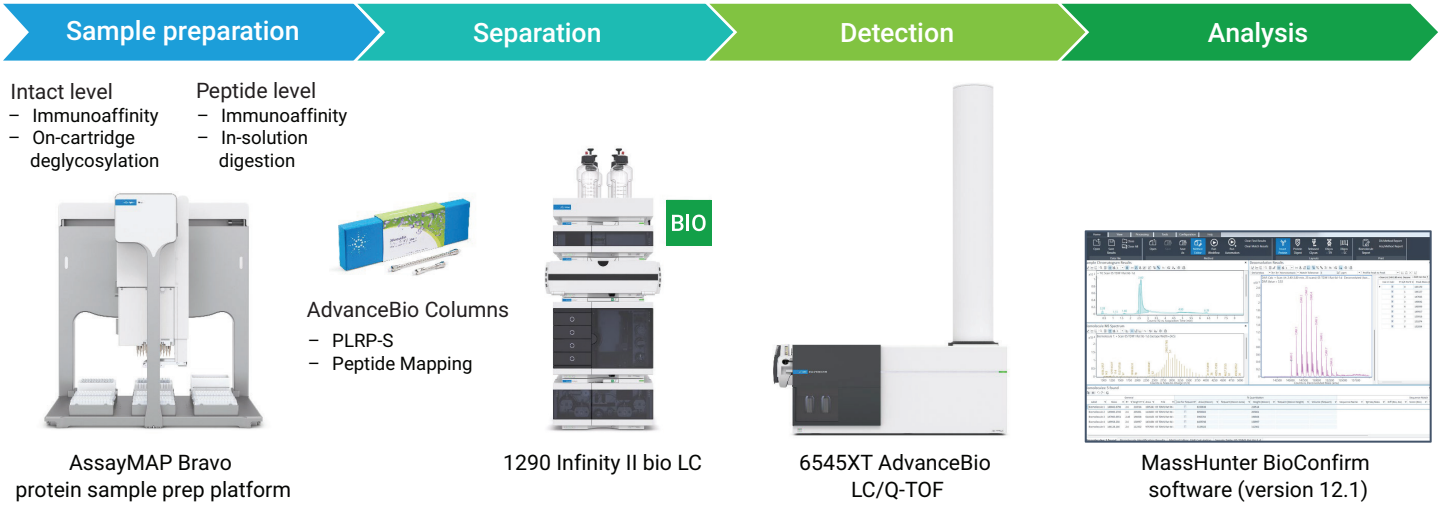

Figure 1. Agilent LB-LC/MS workflow components.

Sample preparation

Incubation: T-DM1 was spiked into rat plasma and PBS buffer separately at the concentration of 100 µg/mL. The spiked samples were incubated at 37 °C for four days. After the four-day incubation was completed, the control sample was prepared by spiking T-DM1 into plasma at 100 µg/mL concentration. All three samples were then subject to other procedures together.

Immunoaffinity purification: For this step, 0.2 mg HER2 ECD was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin followed by clean up with the Zeba Spin Desalting column, as per the manufacturer's instructions. The SA-W cartridges were conditioned with 1% FA. Then, the biotinylated HER2 ECD was immobilized onto the cartridges using the Immobilization app on the AssayMAP Bravo protein sample prep platform (Figure 2A). The cartridges were primed and equilibrated with 100 µL binding buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) at the indicated flow rates in Figure 2B. The biotinylated HER2 ECD, in 100 µL binding buffer, was loaded onto the SA-W cartridges at 5 µL/min, then washed with binding buffer at 10 µL/min. Next, 50 µL sample was 1:1 diluted with binding buffer and loaded onto the ECD-SA-W cartridge at speed of 5 µL/min. After the sample was loaded, the cartridge was washed sequentially with 50 µL high-salt buffer (10 mM HEPES, 1 M NaCl, pH 7.4) then 50 µL of binding buffer, as shown in Figure 2B. For intact-level analysis, the cartridge was ready for deglycosylation.

On-cartridge deglycosylation: The T-DM1-bound cartridge was washed with 50 µL of 20 mM Tris (pH 8.0) and then reacted with heated PNGase F enzyme. The temperature of the Peltier deck was set at 45 °C to maintain an on-cartridge reaction temperature of approximately 37 °C. After 30 minutes, the reaction was completed. The cartridge was washed with 50 µL high-salt HEPES buffer and then with 0.003% FA. The deglycosylated T-DM1 was eluted with 15 µL of 1% FA into an elution plate containing 15 µL of 0.5% ammonium hydroxide. The application settings are shown in Figure 3A. A detailed description of each step is available in a previous application note.[3]

In-solution trypsin digestion: For peptide-level analysis, additional steps in affinity purification were carried out, including the stringent syringe wash and elution (Figure 2B). T-DM1 was eluted with 20 µL of 0.25% FA in 10% acetonitrile into 10 µL of 1 M ammonium bicarbonate. Next, using a single-plate in-solution digestion application (Figure 3B), DTT was added at 10 mM final concentration to the T-DM1 eluted from the affinity purification step. The plate was incubated, off deck, at 60 °C for 1 hour. After cooling down, IAA was added at 20 mM final concentration and the reaction took place, off deck, in darkness, for 30 minutes at room temperature. Additional DTT was added to neutralize excessive IAA. Lastly, 0.5 µg trypsin was added to each well and incubated at 37 °C overnight. Digestion was quenched with 10 µL of 10% TFA. The sample was subject to LC/MS analysis.

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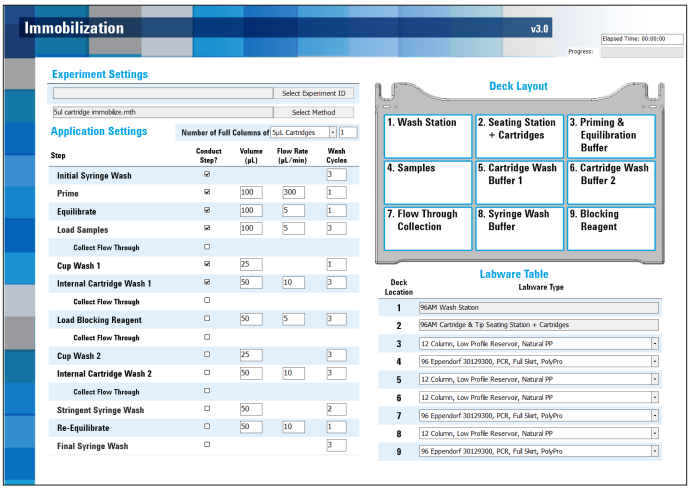

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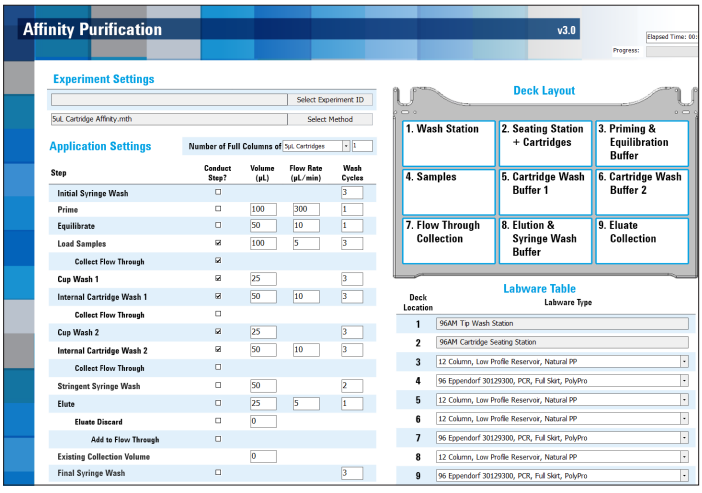

Figure 2. Agilent AssayMAP Bravo protein sample prep platform application settings for (A) immobilization and (B) affinity purification.

Instrumentation

Automated sample preparation was performed using the Agilent AssayMAP Bravo protein sample prep platform (G5571AA).

For separation, the Agilent 1290 Infinity II bio LC system was used, including:

- Agilent 1290 Infinity II bio high-speed pumps (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A) with Agilent Infinity II sample cooler (option #101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bioinert Quick Connect heat exchanger, standard flow (option #065)

Samples were analyzed on the Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with the Agilent Dual Jet Stream ESI source.

Software

The following software was used in this study:

- Agilent VWorks Automation Control software, version 14.2
- Agilent AssayMAP Protein Sample Prep Workbench software, version 4.0
- Agilent MassHunter acquisition (TOF/Q-TOF) software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1

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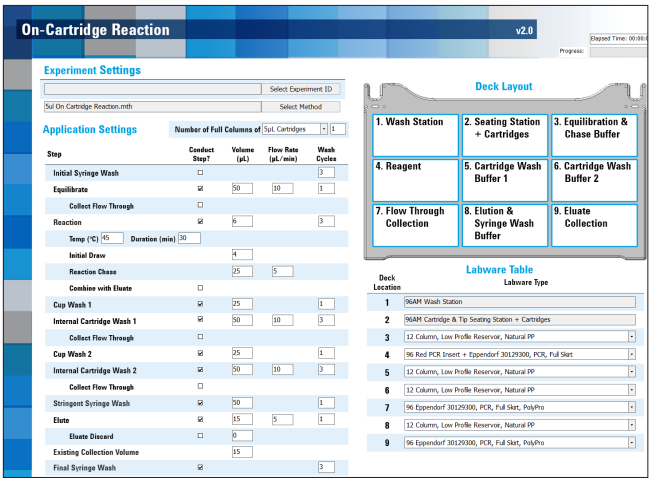

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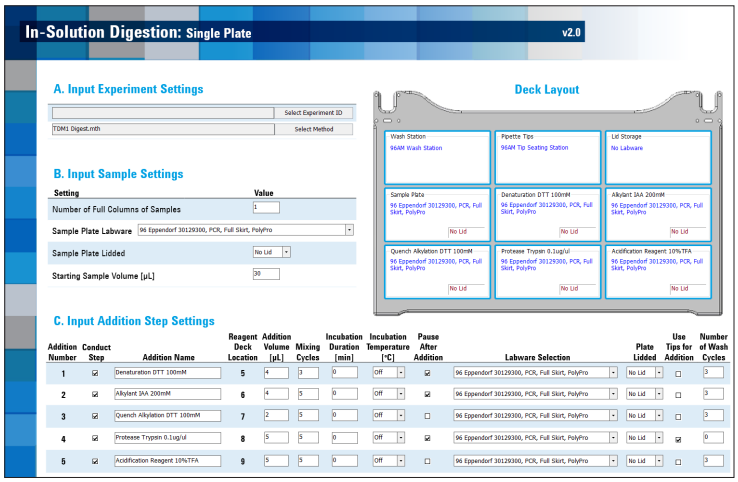

Figure 3. Agilent AssayMAP Bravo protein sample prep application settings for (A) on-cartridge deglycosylation and (B) in-solution digestion.

LC/MS analysis

Tables 1 and 2 list the acquisition parameters for LC and MS. Table 3 displays the DAR calculation parameters in MassHunter BioConfirm software.

Table 1. LC parameters.

	Intact	Peptide
Column	Agilent PLRP-S 1000 Å column, 2.1 × 50 mm, 5 µm (p/n PL1912-1502)	Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm, 120 Å (p/n 653750-902)
Thermostat	8 °C	8 °C
Solvent A	0.1% FA in H_2O	0.1% FA in H_2O
Solvent B	0.1% FA in acetonitrile	0.1% FA in 90% acetonitrile
Flow Rate	0.4 mL/min	0.4 mL/min
Gradient	Time (min) %B 0.0 10 1.0 10 2.0 37 4.0 37 4.5 50 5.5 50 6.0 10	Time (min) %B 0.0 3 1.0 3 70.0 45 71.0 90 73.0 90
Post-Time	2.5 min	5 min
Injection Volume	20 µL	20 µL
Column Temperature	60 °C	60 °C

Table 2. MS data acquisition parameters.

	Intact	Peptide
Source	Agilent Dual Jet Stream ESI	Agilent Dual Jet Stream ESI
Polarity	Positive	Positive
Drying Gas Temperature	350 °C	325 °C
Drying Gas Flow	12 L/min	13 L/min
Nebulizer	60 psi	35 psi
Sheath Gas Temperature	400 °C	275 °C
Sheath Gas Flow	11 L/min	12 L/min
Capillary Voltage	5,500 V	4,000 V
Nozzle Voltage	2,000 V	0 V
Fragmentor	380 V	175 V
Acquisition Mode	High mass range (30,000 *m/z*) Extended dynamic range (2 GHz)	Low mass range (1,700 *m/z*) Extended dynamic range (2 GHz)
Mass Range	*m/z* 900 to 5,000	*m/z* 300 to 1,700
Acquisition Rate	1 spectra/s	2 spectra/s
Reference Mass	*m/z* 922.0097, 1,821.9523	*m/z* 322.0481, 922.0097

Table 3. Agilent MassHunter BioConfirm software DAR calculation parameters.

	Parameters
DAR 0 Mass(es)	145,162
Linker Type	Lysine-linked (all)
Drug + Linker Mass (Da)	957.00
Peak Area	Use most abundant peak height
Max DAR Peak Number	8

Results and discussion

Intact-level T-DM1 characterization

An automated sample preparation workflow was developed on the AssayMAP Bravo platform to address the challenging sample matrix of plasma. The workflow included immobilization of target antigen on the SA-W cartridge, immunoaffinity purification of T-DM1 from matrix, and deglycosylation of purified T-DM1 on-cartridge.

The deglycosylated T-DM1 was analyzed in high-mass-range mode on the LC/Q-TOF system. Figure 4 is the compilation of total ion chromatograms (A to C), raw spectra (D to F), and deconvoluted spectra (G to I) of the samples. All samples eluted within 2.4 to 3.4 minutes, with a mass envelope ranging from *m/z* 2,000 to 4,000, corresponding to charge states between +37 to +68. The clean total ion chromatogram and high-resolution raw spectra indicate the success of the automated sample preparation workflow in immunocapturing and deglycosylation of T-DM1 from matrix.

Nine drug-conjugated T-DM1 species were detected in the control and PBS-4d samples with mass matching DAR0 to DAR8; whereas, in the plasma-4d sample, only eight species were detected, missing DAR8. A profile shift toward lower DAR species was observed in both four-day samples, indicating the loss of drug in T-DM1 over the incubation period. The most abundant species shifted from DAR3 in the control to DAR2 in the incubated samples. The abundance of high DAR species, DAR7 and DAR8, either decreased significantly or totally disappeared in the incubated samples. All these changes attributed to the average DAR value decrease from original 3.5 to 2.7 in the incubated samples.

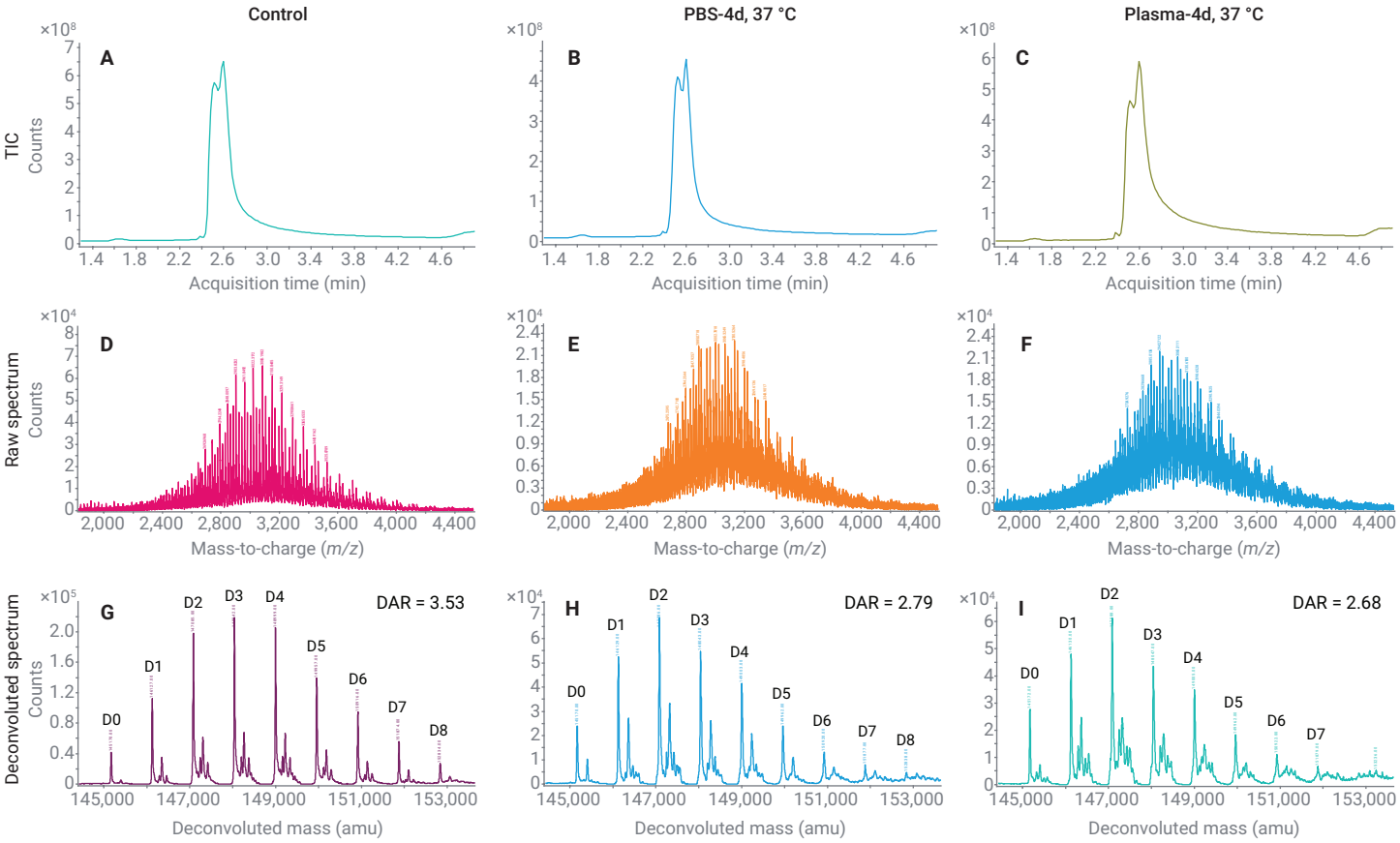

Figure 4. Total ion chromatograms, raw spectra, and deconvoluted spectra of control samples, and PBS-4d, and plasma-4d samples incubated at 37 °C.

In addition to the major drug conjugated species (DAR0 to DAR8), there were also minor species detected and well resolved from main DAR peaks (Figure 5) thanks to the high resolution of the 6545XT AdvanceBio LC/Q-TOF system. Based on the chemical structure of T-DM1 (Figure 6) and the observed delta mass from major DAR species, those minor peaks were assigned to the best of our knowledge and listed in Table 4.

Figure 5 displays the zoomed-in view between DAR2 and DAR3 of the samples. It is a representation of the spectra profiles between other DARs. Peaks a, b, and c are present across all three samples. They represent glycation of antibody, DAR2 addition of free MCC linker[4], and DAR3 with loss of DM1 and addition of a cysteine residue.[5] Peaks d and e are only present in PBS-4d and plasma-4d samples.

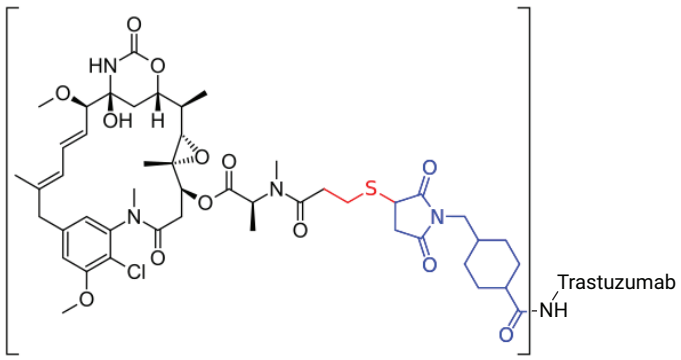

Figure 6. Chemical structure of T-DM1.

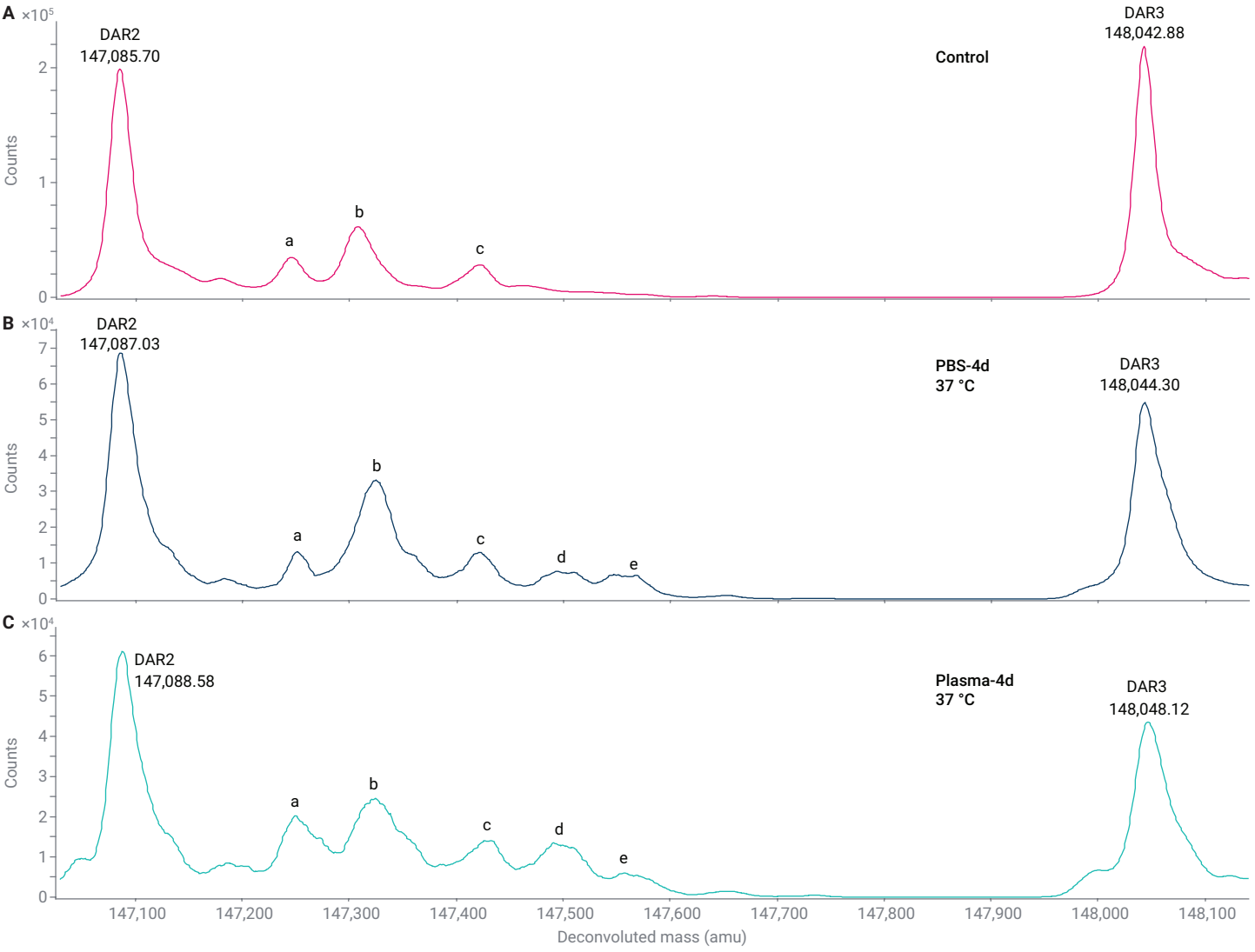

Figure 5. Zoomed-in view of deconvoluted spectra between DAR2 and DAR3 of (A) control, (B) PBS-4d, and (C) plasma-4d.

Table 4. Assignment of minor peaks between DAR2 and DAR3.

Peak	Present In	Delta Mass from DAR2 (Da)	Delta Mass from DAR3 (Da)	Species
a	All	+161	–	Glycation of Trastuzumab
b	All	+220	–	DAR2+MCC
c	All	–	−622	DAR3-DM1+Cys
d	PBS-4d plasma-4d	–	−547	DAR3-maytansinol+OH
e	PBS-4d plasma-4d	–	−490	Unknown degradant

Peak d is DAR3 loss of maytansinol and addition of OH via ester hydrolysis. Peak e, with a delta mass of 490 Da from DAR3, is defined as an unknown species. However, it is certain that peaks d and e, absent from the control, were induced from incubation at 37 °C. Thus, they are related to T-DM1 degradation.

In addition to the above findings, a mass increase of each DAR was observed in the incubated samples compared to the control. For example, in the plasma-4d sample, the 3 and 6 Da mass increase on DAR2 and DAR3 respectively is displayed in Figure 5. The mass increase could be attributed to multiple site hydrolysis of MCC-DM1 over the course of incubation.[5]

The LB-LC/MS workflow, encompassing high-specificity immunoaffinity purification, a high-resolution and accurate mass LC/Q-TOF system, and a reliable deconvolution algorithm, enables the thorough characterization of T-DM1 at intact level.

Peptide-level T-DM1 characterization

In a previous application note[6], the drug-conjugated peptides were identified in neat T-DM1 using an in-solution digestion peptide mapping workflow. Our study further involved the immunoaffinity purification step to clean up T-DM1 from matrices before trypsin digestion. The resulting peptides were then analyzed using LC/Q-TOF in MS-only mode. To access the peptide-level difference between the samples, the precursor mass of those conjugated peptides was extracted and integrated in extracted ion chromatogram (EIC).

Three representative peptides are shown as examples in Figure 7. Generally, the abundance of the conjugated peptides decreases in both incubated samples compared to the control, indicating drug loss over the course of incubation. This observation aligns with the intact-level DAR value decrease in the incubated samples.

Comparing individual peptide of PBS-4d and plasma-4d reveals that the sites of drug loss varied between PBS and plasma condition. For K*VEPK and ICNVNHK*PSNTK peptides, they are either very scarce or completely undetected in plasma-4d. However, it is the reverse situation for ADYEK*HK peptide that the abundance of it in plasma-4d surpasses that in PBS-4d. These findings may suggest that the linked drug at these conjugation sites may be susceptible to loss in the matrices.

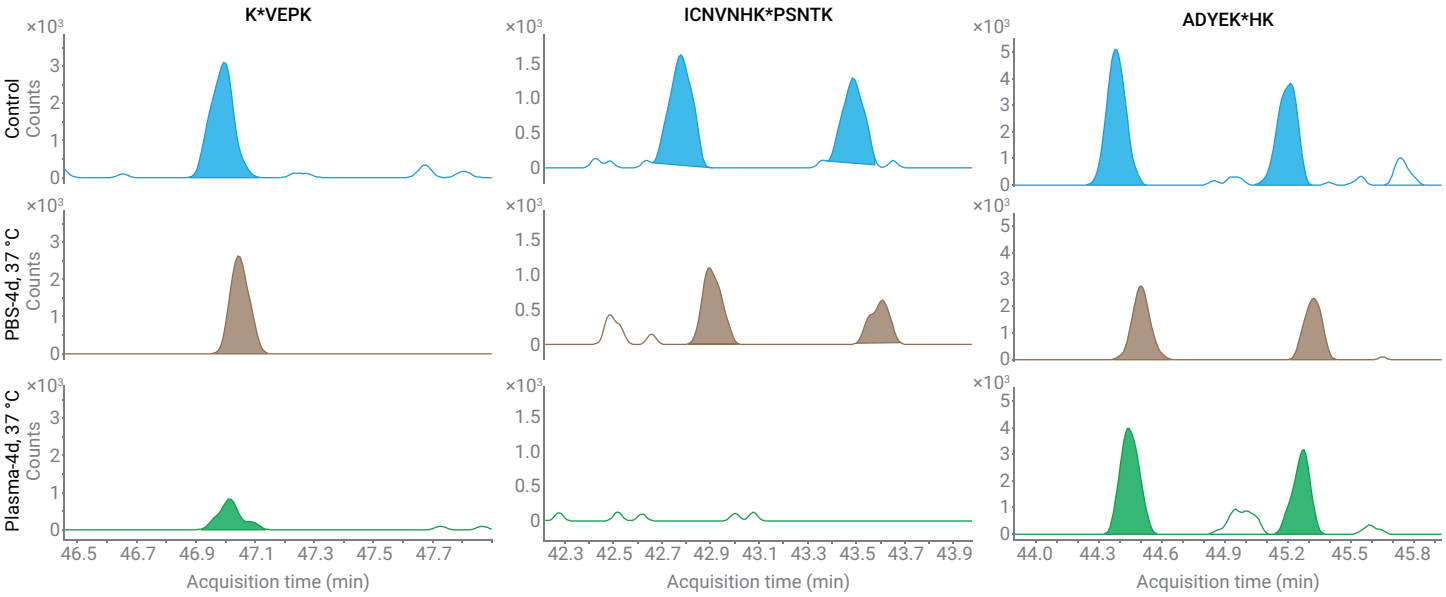

Figure 7. EICs of the exemplary drug conjugated peptides from control (top), PBS-4d (middle), and plasma-4d (bottom) samples. The asterisk marks the conjugation site.

Conclusion

In this application note, we demonstrate the characterization of T-DM1 in vitro transformation at both intact and peptide levels using a ligand binding LC/MS (LB-LC/MS) workflow. This workflow is composed of the Agilent AssayMAP Bravo protein sample prep platform, 1290 Infinity II bio LC, and 6545XT AdvanceBio LC/Q-TOF system.

At the intact level, a series of minor species were well resolved from the major DAR peaks and successfully assigned to various degradation products, particularly in the treated samples. This result highlights the power of the high-resolution 6545XT AdvanceBio LC/Q-TOF in resolving the complex ADC MS spectra. At the peptide level, the overall abundance of conjugated peptides decreased in the treated samples, aligning with the result from the intact level. Peptide-level results also indicate that drug loss at the individual conjugation site may be susceptible to the matrix effect. This phenomenon warrants further investigation.

In summary, this LB-LC/MS workflow is a powerful tool for studying the in vitro transformation of T-DM1. It offers both specificity and sensitivity and holds great promise for future applications in drug discovery and development.

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