Analysis of Antibody-Drug Conjugates Using Size Exclusion Chromatography and Mass Spectrometry

Application Note

Biologics and Biosimilars

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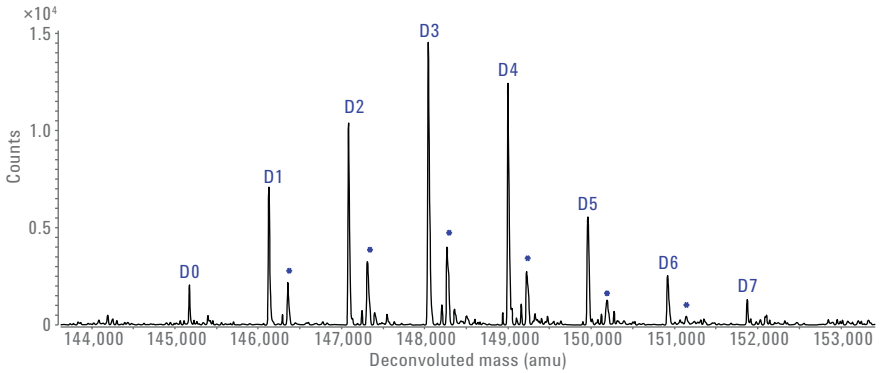
Abstract

Recombinant monoclonal antibodies (mAbs) have emerged as important therapeutic agents for the treatment of different diseases. This technology has been extended by the development of antibody drug conjugates (ADCs). ADCs are mAbs to which cytotoxic payloads are covalently attached, enabling the delivery of potent drugs to a specific target. This Application Note describes the analysis of an mAb (trastuzumab) and an ADC (trastuzumab emtansine) by size exclusion chromatography with online mass spectrometry (SEC-MS) using the Agilent 1290 Infinity II LC system with an Agilent 6530 Accurate Mass Q-TOF LC/MS system. We demonstrate that SEC is a straightforward way to introduce mAbs and ADCs into the LC/MS system, requiring little method optimization. From the MS data collected for the ADC, it was possible to calculate a drug-to-antibody ratio (DAR) of 3:2.



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Introduction

Antibody drug conjugates (ADCs) are monoclonal antibodies (mAbs) conjugated to cytotoxic drug molecules using various available linker chemistries. The combination of highly potent drugs with antibodies targeted to specific antigens is expected to enhance therapeutic action by the sensitive discrimination between healthy and diseased tissue. The advantage of ADCs as therapeutic agents is the increased control over the pharmacokinetics of the drug[1]. Delivery to the target diseased tissue is improved while the exposure of healthy tissues to toxic drugs is minimized, leading to selective elimination of tumor cells expressing the target antigen.

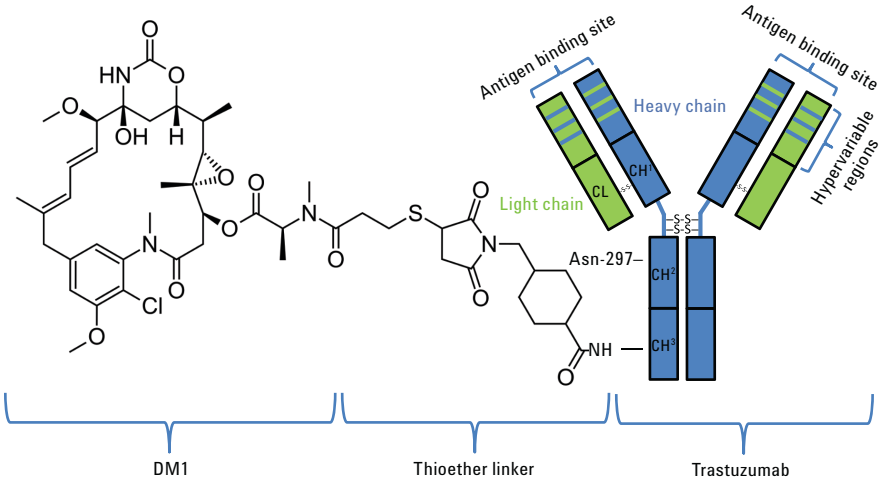

Figure 1. Structure of trastuzumab emtansine.

Figure 1 shows the structure of the ADC trastuzumab emtansine (T-DM1, Kadcyla). It consists of three components:

- mAb (trastuzumab)
- A bifunctional linker ([N-maleimidomethyl]cyclohexane-1-carboxylate, MCC)
- Small molecule drug (DM1, derivative of maytansine)

Trastuzumab emtansine is typically administered for the treatment of human epidermal growth factor receptor 2 (HER2) positive breast cancer. The mAb part of trastuzumab emtansine binds selectively to HER2-positive cells, leading to internalization of the ADC-antigen complex into the cell. Inside the cell, the complex is degraded, releasing the cytotoxic drug, which destroys the cancer cell[2]. Emtansine is the combination of the cytotoxic maytansinoid DM1 (which inhibits tubulin polymerization to induce cell-cycle arrest and cell death) and a bifunctional MCC linker. MCC is chemically linked on one side to the antibody through lysine residues, and on the other side bound to DM1 by nonreducible thioether linkages[3]. The resulting ADC is typically heterogeneous regarding both the distribution and the loading of the cytotoxic drug species on the antibody. Therefore, physicochemical characterization of ADCs is more complex compared to the analysis of the corresponding mAb[4].

Two types of ADCs are currently available on the market – lysine linked ADCs (such as T-DM1), and cysteine linked ADCs[5]. Some cysteine conjugates can be analyzed using hydrophobic interaction chromatography (HIC) to determine the drug-to-antibody ratio (DAR). The analysis of lysine conjugates, however, is not feasible using HIC. Due to the higher heterogeneity, UV/VIS spectroscopy and MS are generally the methods of choice to determine the DAR. In contrast to cysteine conjugates, lysine conjugated ADCs do not dissociate under acidic or high organic conditions (such as those commonly used in MS) due to their intact interchain disulfides. The analysis of ADCs using reversed phase (RP) liquid chromatography (LC) coupled to MS detection can be problematic due to irreversible binding of the proteins to the stationary phase, especially for large proteins[4,6]. Elevated column temperatures (60 to 80 °C) and propanol-containing buffers can reduce these effects. However, these conditions might lead to problems regarding temperature-sensitive proteins. SEC is usually carried out at room temperature or slightly above without the need for high temperatures.

This Application Note describes the SEC-MS analysis of trastuzumab emtansine as well as trastuzumab using an Agilent 1290 Infinity II LC coupled to quadrupole time-of-flight mass spectrometry (Q-TOF-MS).

Experimental

Instrumentation

The SEC-MS experiments were carried out on an Agilent 1290 Infinity II LC with online Q-TOF detection comprising the following modules:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B)
- Agilent 6530 Accurate Mass Q-TOF LC/MS system

The offline SEC experiments were carried out on an Agilent 1260 Bio-Inert Infinity Quaternary LC comprising following modules:

- Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A)
- Agilent 1260 Infinity High-Performance Bio-Inert Autosampler (G5667A)
- Agilent 1290 Infinity Thermostat (G1330B) for sample cooling
- Agilent 1290 Infinity Column Compartment (G1316C) with bio-inert solvent heat exchanger
- Agilent 1260 Infinity Diode Array Detector VL (G1315D) with a 10 mm bio-inert standard flow cell

Column

Agilent Bio SEC-3, 300 Å,
7.8 × 300 mm, 3 µm (p/n 5190-2511)

Software

- Agilent MassHunter Workstation Software with Bioconfirm Software, Version B.07.01
- Agilent DAR Calculator Version B.01.00 - Build 1.15.2.0

Sample

Trastuzumab emtansine (T-DM1, Kadcyla) and trastuzumab (Herceptin)

Chemicals

All solvents used were LC grade. LC/MS grade acetonitrile and formic acid (FA) were purchased from Sigma-Aldrich, St. Louis, Missouri, U.S. N-glycanase and MS friendly deglycosylation (Tris) Buffer was purchased from Prozyme, Hayward, CA, USA). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample preparation

Both samples (mAb and ADC) were deglycosylated by adding 25 mU of N-glycanase to 100 µL of 1 mg/mL protein solution (in Tris) and incubated overnight at 37 °C.

Column preparation

Agilent Bio SEC-3 columns are shipped in 0.1 M sodium phosphate buffer, pH 7.0. Sodium phosphate buffer is not compatible with MS analysis, as it is a nonvolatile salt. The column was extensively flushed with mobile phase for approximately 48 hours before LC/MS analysis.

Table 1. Chromatographic and MS conditions.

Parameter	Value
Mobile phase	49 % Water, 50 % ACN, 1 % FA
Flow rate	0.8 mL/min
Stop time	17 minutes
Needle wash mode	Standard Wash
Injection volume	25 µL
Column temperature	30 °C
MS detection	
Acquisition mode	MS1
Minimum range	100 *m/z*
Maximum range	7,000 *m/z*
Ion polarity	Positive
Source parameters	
Gas temperature	300 °C
Gas flow	13 L/min
Nebulizer	45 psig
Sheath gas temperature	400
Sheath gas flow	12
Scan source parameters	
Vcap	5,000 V
Nozzle voltage	2,000 V
Fragmentor	170 V
Skimmer 1	65 V
Octopole RF peak	750

Results and Discussion

Typically, SEC of mAbs is carried out in a physiological buffer such as phosphate buffered saline (PBS) with a pH of approximately 7.4. The buffer maintains the native structure of the protein, and reduces undesired binding of the proteins to the column. However, PBS is incompatible with MS analysis. Instead, for the SEC-MS analysis, a PBS-free system (Agilent 1290 Infinity II Binary LC) was used together with a solvent mix of 50 % ACN, 49 % water, and 1 % FA. Figures 2A and 2B show a comparison of a typical PBS run on the 1260 Infinity Bio-Inert Quaternary LC (2A) together with the TIC signal of a LC/MS run with MS-friendly buffer (2B). The mAb peak using PBS buffer is sharp and intense using DAD detection, but would not be visible in the MS. Using an MS-friendly buffer, the MS signal-to-noise ratio (S/N) is excellent, with only minor loss in regards to the peak shape.

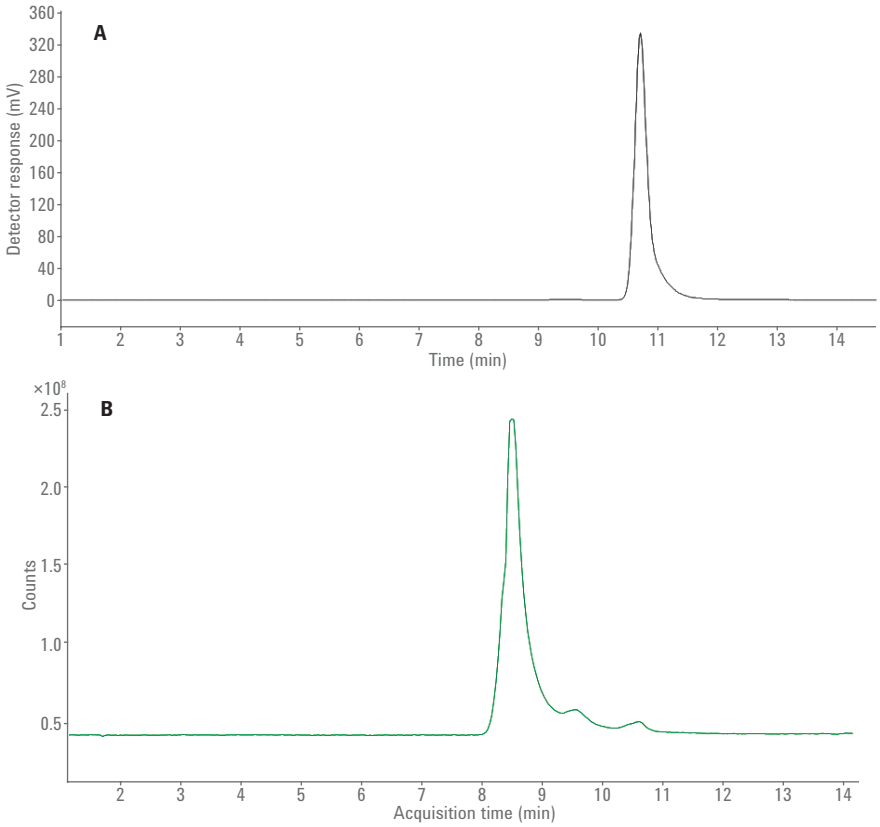

Figure 2. Comparison of SEC analysis of trastuzumab A) with PBS and Agilent 1260 Infinty Bio-Inert Quaternary LC and DAD detection, and B) with ACN/water/FA (50/49/1) and the Agilent 1290 Infinity II LC and MS detection (TIC signal).

Figure 3 displays the SEC-MS analyses of intact and deglycosylated trastuzumab as deconvoluted spectra. The insets show the Gaussian distribution of the charge state envelope for the mAbs. The spectra were deconvoluted using the maximum entropy deconvolution algorithm in Agilent MassHunter BioConfirm Software. The deconvoluted spectrum of the intact trastuzumab (Figure 3A) shows multiple mass peaks corresponding to different glycoforms of the mAb. As a comparison, Figure 3B shows the deconvoluted spectrum of the deglycosylated trastuzumab, observed as a single main peak with a mass of 145,173 Da. Using size exclusion chromatography before MS analysis, adduct peaks (which would arise from buffer salts when performing static infusion experiments) are minimized[6].

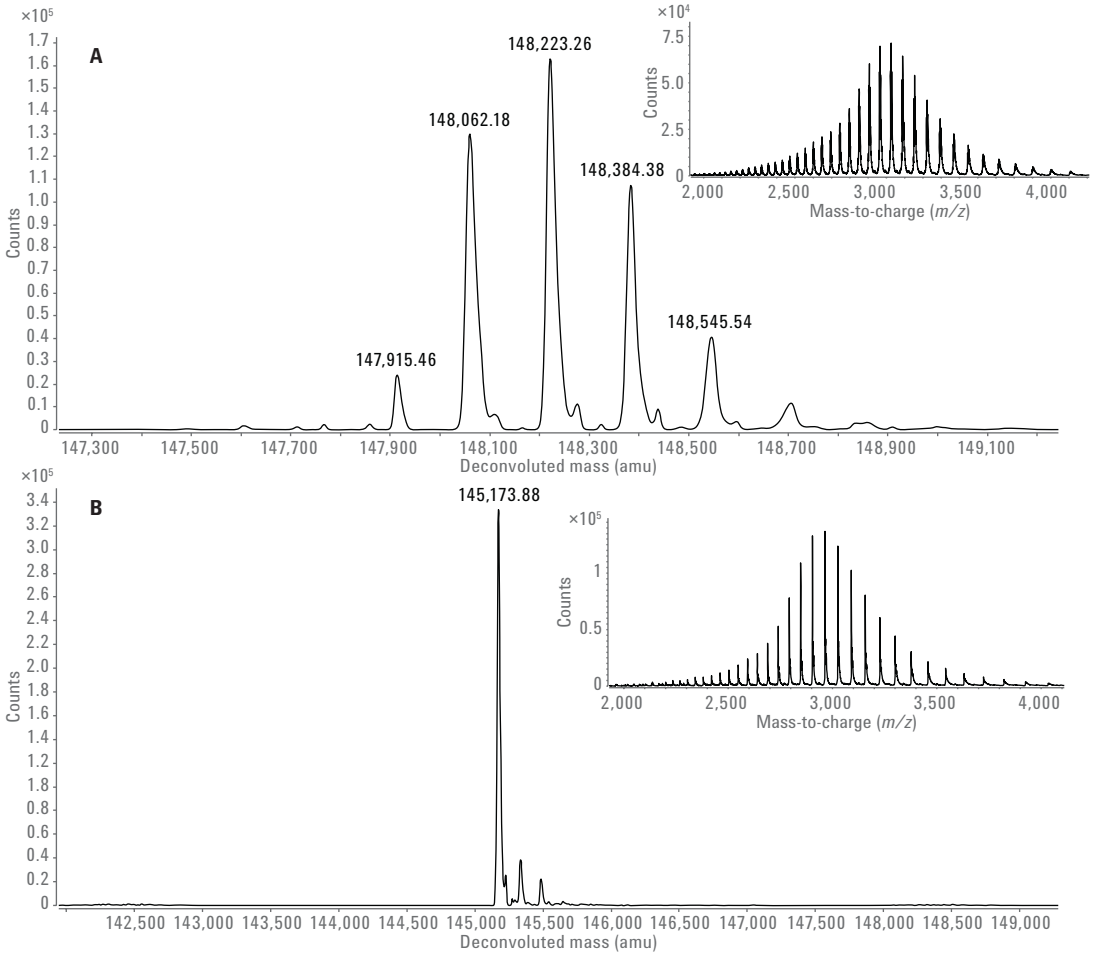

Figure 3. A) Deconvoluted spectrum of intact trastuzumab. B) Deconvoluted spectrum of deglycosylated trastuzumab. The inset shows the raw mass spectra of the analyte.

Mass spectra of ADCs are even more complex due to the combination of both glycosylation and drug loading heterogeneities, which poses a challenge for interpretation, see Figure 4. Therefore, the ADC was deglycosylated prior to further SEC-MS analysis to reduce spectrum complexity.

The SEC-MS analysis of the deglycosylated T-DM1 is shown in Figure 5A (TIC signal), 5B (raw mass spectrum), and 5C (deconvoluted spectrum). The spectrum was also deconvoluted using the maximum entropy algorithm in Agilent MassHunter BioConfirm Software. The spectrum of the deglycosylated ADC is much easier to interpret, and the resulting deconvolution (Figure 5C) is considerably simpler. The ADC spectrum is comprised of eight equally spaced groups of peaks, where the masses of the most intense peaks are separated by 959.7 Da. This mass corresponds to the mass of one DM1 drug (738.3 Da) and one MCC linker (221.4 Da), representing the drug load species[7]. The first peak in the deconvoluted ADC spectrum matches the mass of the deglycosylated trastuzamab in Figure 3B (145,173 Da). Hence, D0 (trastuzumab with zero drug load) can be assigned to the first peak of the spectrum. The following peaks represent species with DARs ranging from 1 to 7. A second minor series of peaks, marked with an asterisk, is separated from the main peaks with a mass of about 221.7 Da higher (Figure 5C). These peaks can be assigned to species with an attached linker, but without conjugated DM1, as shown by Marcoux *et al.*[5]. The average DAR was calculated using the Agilent DAR Calculator as approximately 3.2, matching previously determined values for this molecule[7].

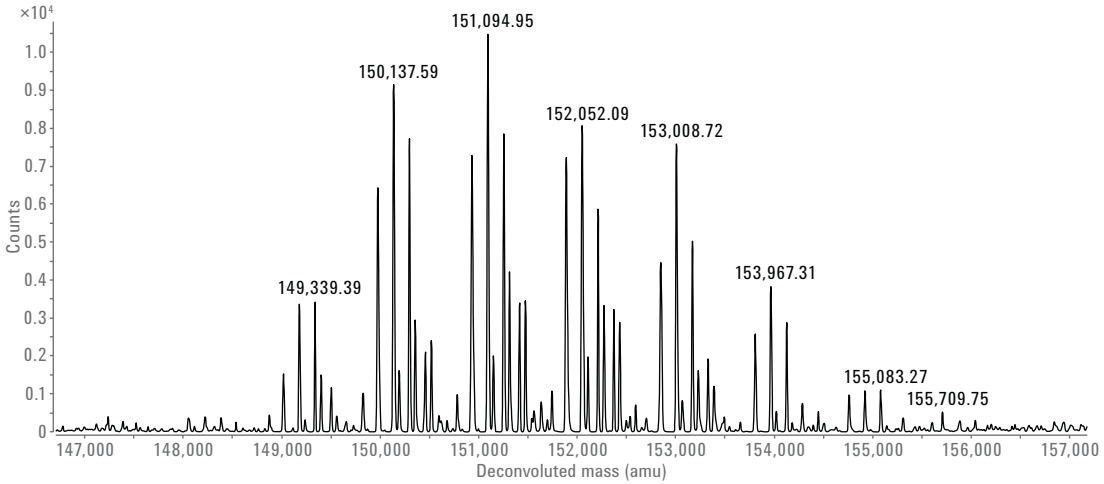

Figure 4. Deconvoluted spectrum of trastuzumab emtansine (T-DM1).

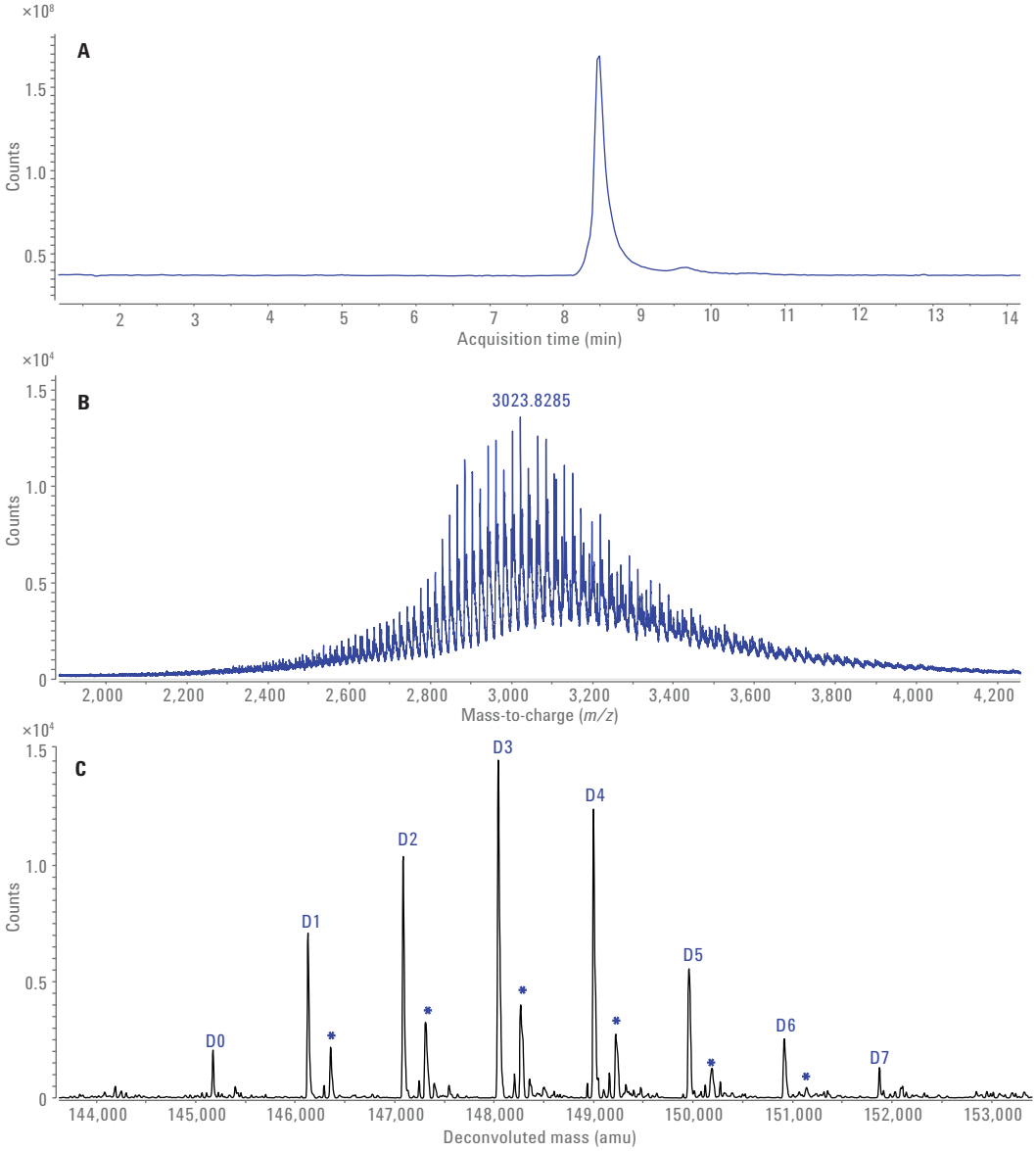

Figure 5. SEC-MS analysis of the deglycosylated T-DM1: A) TIC signal, B) raw mass spectrum, and C) deconvoluted spectrum representing DAR species D0 to D7 plus satellite peaks (marked with an asterisk) representing trastuzumab + linker signals.

Conclusion

SEC-MS using MS compatible buffer (solvent mix of 50 % ACN, 49 % water, and 1 % FA) was performed for the analysis of trastuzumab and trastuzumab emtansine on the Agilent 1290 Infinity II LC system with Q-TOF detection. SEC prior to MS was useful because mAbs and ADCs can easily be desalted before introduction into the MS ion source. The spectrum of trastuzumab revealed multiple mass peaks corresponding to different glycoforms of the mAb. The spectrum of trastuzumab emtansine revealed a complex peak pattern due to both glycosylation and drug loading heterogeneities. Deglycosylation of trastuzumab and trastuzumab emtansine permitted a more facile interpretation of the data. The deconvolution of the deglycosylated T-DM1 revealed eight equally spaced peaks, which were assigned to eight versions of trastuzumab with zero to seven molecules of DM1 attached. The average DAR was 3.2, consistent with values described in the literature.

References

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