Determination of Drug-to-Antibody Ratio for Antibody-Drug Conjugates Purified from Serum

Using Automated Affinity Purification, LC/MS analysis, and Novel DAR Calculation Software

Application Note

Authors

Jing Chen, Michael Bovee, and Steve Murphy

Agilent Technologies, Inc.

Introduction

Antibody-drug conjugates (ADCs) are an emerging class of biotherapeutics designed to provide targeted drug delivery by linking drugs to monoclonal antibodies. Unlike small molecule drugs, ADCs are not single molecular entities, but are instead a heterogeneous population of antibodies that vary by the number of drugs on each antibody and by variability in post-translational modifications. The drug-to-antibody ratio (DAR) is the average number of drugs coupled to an ADC. The DAR is a critical quality attribute of an ADC that is optimized and closely monitored during ADC development, as it can affect efficacy and toxicity[1,2].

The DAR for an ADC can change over time in circulation due to drug release[1,3]. Therefore, it is critical to have a robust solution for ADC DAR determination of samples from pharmacokinetic (PK) studies. To determine ADC DAR, first the ADC must be purified from the serum, then analyzed by LC/MS. The sample preparation and data analysis in this workflow are typically labor intensive and, therefore, susceptible to variability and human error.

This Application Note presents a solution for determining the DAR of ADCs in serum samples that uses the Agilent AssayMAP Bravo Platform to automate ADC affinity purification, an Agilent 1290 Infinity UHPLC coupled to an Agilent 6550 Q-TOF mass spectrometer to acquire accurate intact protein mass data, and Agilent MassHunter BioConfirm and DAR Calculator software to determine the ADC mass and DAR. This workflow decreases the labor, variability, and the probability of human error associated with ADC DAR determination, while simultaneously allowing the number of samples to be scaled with minimal additional effort.

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Experimental

Materials

Recombinant human HER2 extracellular domain (ECD) was purchased from ACRO Biosystems (Newark, DE). EZ-Link Sulfo-NHS-LC-Biotin and Pierce Biotin Quantitation Kit were purchased from Thermo Fisher Scientific (Grand Island, NY). Rat serum was purchased from BioreclamationIVT (Hicksville, NY). AssayMAP Streptavidin cartridges (SA-W) were from Agilent Technologies, Inc. (Santa Clara, CA). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Biotinylation and immobilization of ADC antigen

HER2 ECD was biotinylated using the EZ-Link Sulfo-NHS-LC biotin kit per manufacturer's instructions. The molar ratio of biotin to HER2 ECD was determined to be 9.0 by the Pierce Biotin Quantitation Kit per manufacturer's instructions. Two micrograms of biotinylated HER2 ECD were immobilized on each AssayMAP SA-W cartridge using the Agilent AssayMAP Bravo controlled by the Immobilization application. Briefly, SA-W cartridges (microchromatography cartridges packed with approximately 5 µL of packed resin to which streptavidin was covalently coupled) were primed and equilibrated with 1 % formic acid, then washed with HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) using the Immobilization application default settings for Prime, Equilibrate, and Internal Cartridge Wash 1. All other steps in the Immobilization application were turned off. Priming and equilibrating with 1 % formic acid purges the entrained air from the cartridges, and acts as a stringent wash to remove streptavidin monomers that dissociate from the solid support in the low pH condition experienced in the final elution. The wash step prepares the cartridges to bind the biotinylated antigen. Next, each SA-W cartridge was loaded with 2 µg of biotinylated antigen in a 100 µL HEPES buffer at a flow rate of 5 µL/min followed by one wash with HEPES buffer using the AssayMAP Bravo controlled by the Immobilization application. In this case, the default settings for the Sample Load and Internal Cartridge Wash 1 were used, and all other steps of the application were turned off. Following this procedure, 2 µg of biotinylated ADC antigen were coupled to each SA-W cartridge thereby generating ADC affinity cartridges.

ADC affinity purification

Commercially obtained lyophilized ADC was reconstituted in deionized (DI) water to 1 mg/mL, aliquoted, and stored at –80 °C until used. ADC was spiked into rat serum and serially diluted to prepare ADC concentrations of 20, 10, 5, 2.5, 1.25, and 0.625 µg/mL. Rat serum without ADC spiked was also included as a control. These samples were further diluted 1:1 with HEPES buffer immediately before loading the samples on ADC affinity cartridges. Affinity purification was performed using the AssayMAP Bravo controlled by the Affinity Purification application. Briefly, 100 µL of the diluted rat serum containing ADC was loaded onto each ADC affinity cartridge (n = 4 for each ADC concentration) at a flow rate of 3 µL/min, followed by four 50 µL washes (HEPES buffer, 1 M NaCl in HEPES buffer, 0.003 % formic acid, and water) at a flow rate of 10 µL/min. Finally, the purified ADC was eluted with 15 µL of 1 % formic acid per cartridge into an existing volume of 15 µL 0.5 % ammonium hydroxide to neutralize the purified ADC.

LC/MS analysis

LC/MS analyses were conducted on an Agilent iFunnel Accurate Mass 6550 Q-TOF (Santa Clara, CA) equipped with a Dual Agilent Jet Stream ESI source coupled with an Agilent 1290 Infinity UHPLC system (Santa Clara, CA). Table 1 and Table 2 list the LC/MS parameters used. Three microliters ($1/10^{th}$ of the total sample volume) of the ADC purified from all the serum samples were injected for MS only analysis; additionally, 10 µL ($1/3^{rd}$ of the total sample volume) of the ADC purified from serum samples containing ADC concentrations of 2.5, 1.25, and 0.625 µg/mL were injected for MS only analysis. One hundred nanograms of commercially obtained ADC were injected (n = 4) as a control.

Data analysis

Raw data files were analyzed by Agilent MassHunter BioConfirm software. Spectra were extracted and averaged between 2.2 and 3.2 minutes, and deconvoluted. The DAR of ADC and the percentage of ADC with each drug load were calculated using the Agilent MassHunter DAR Calculator and the deconvoluted spectra. Table 3 shows the deconvolution parameters.

Table 1. Liquid chromatography parameters.

Parameter	Agilent 1290 Infinity UHPLC System	
Column	Agilent PLRP-S 1000Å 8 µm 150 × 2.1 mm (PL1912-3802)	
Sample thermostat	5 °C	
Mobile phase A	0.1 % Formic acid in water	
Mobile phase B	0.1 % Formic acid in acetonitrile	
Gradient (segmented)	**Time (min)**	**%B**
	0–0.5	25
	0.5–1.5	25–35.5
	1.5–3.5	35.5
	3.5–5.0	35.5–50
	5.0–5.5	50–25
	5.5–7.5	25
Stop time	7.5 minutes	
Column temperature	80 °C	
Flow rate	0.4 mL/min	

Table 2. Mass spectrometer parameters.

Parameter	Agilent 6550 Q-TOF Mass Spectrometer
Ion mode	Positive ion mode
Source	Agilent Dual Jet Stream
Drying gas temperature	225 °C
Drying gas flow	14 L/min
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
Nebulizer	40 psi
Capillary voltage	4,500 V
Nozzle	1,500 V
Fragmentor voltage	250 V
Oct RF Vpp	750 V
Acquisition parameters MS mode	High (10,000 *m/z*) mass range, Extended mass range (2 GHz), MS only mode, Mass range 1,000–4,500 *m/z*.

Table 3. MassHunter BioConfirm parameters.

Parameter	Agilent MassHunter BioConfirm Deconvolute (MS): Protein
Deconvolution algorithm	Maximum entropy
Deconvolution settings	Mass range: 140–160 KDa Mass step: 1.0 Da
Use limited *m/z* range	2,000–4,500 *m/z*
Baseline	Subtract baseline Baseline factor 3.50
Adduct	Proton
Isotope width	Automatic

Results and Discussion

A solution for determining the DAR of ADCs from serum samples was developed. This solution includes automated affinity purification of ADCs from serum using ADC affinity cartridges on the AssayMAP Bravo, acquisition of MS spectra of intact ADCs using a 1290 Infinity UHPLC and a 6550 Q-TOF mass spectrometer, deconvolution of MS spectra using Agilent MassHunter BioConfirm, and calculation of DAR using Agilent MassHunter DAR Calculator (Figure 1).

The first step of this workflow is the automated purification of ADC from serum. To accomplish this step, custom AssayMAP affinity cartridges were generated by immobilizing biotinylated ADC antigen on AssayMAP SA-W cartridges using the AssayMAP Bravo controlled by the Immobilization application, which is specifically designed to generate custom affinity cartridges. Two micrograms of antigen were immobilized per cartridge, which represents approximately a 4-fold molar excess of antigen compared to the maximum amount of antibody to be purified in this Application Note. Optimization studies demonstrated that a 4-fold molar excess was sufficient for efficient capture of the target ADC (data not shown), at the flow rate used in this Application Note. The flow rate and level of molar excess are related, and both can be altered depending on the experimental design. It should be noted that the amount of antigen used was not limited by the capacity of the SA-W, which is approximately 75 µg for this particular antigen, but was instead chosen to minimize the amount of antigen consumed by these experiments.

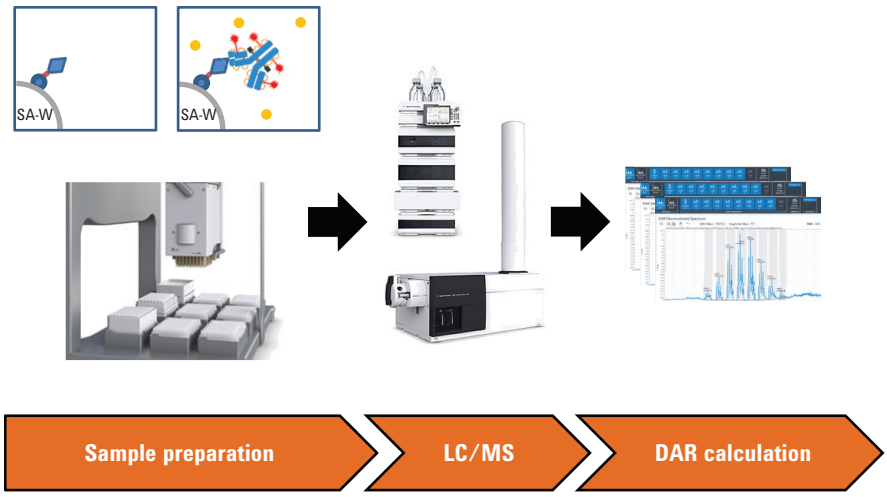

Figure 1. ADC DAR determination workflow.

Commercially obtained ADC was spiked into rat serum at a concentration of 20 µg/mL, then affinity purified using the ADC affinity cartridges and the AssayMAP Bravo controlled by the Affinity Purification application. One hundred nanograms of the commercially obtained ADC (Figure 2A) and 100 ng of ADC affinity purified from the serum samples (Figure 2B), assuming 100% recovery, were analyzed by LC/MS. The major peak, observed between 2.2 and 3.2 minutes, represents the elution of the ADC. Minor additional peaks were observed in the affinity purified ADC between 1 and 1.5 minutes, representing residual salts and between 4.7 and 5.5 minutes, representing copurified proteins from rat serum with the mass envelope centered around 1,500 *m/z*. These residual copurified proteins do not interfere with the ionization of the ADC as they are completely separated by the fine-tuned LC gradient. Figures 2C and 2D show that the extracted spectra over the ADC elution window are nearly identical. After deconvolution, seven peak groups representing DAR values of 0–7 were observed in the deconvoluted mass spectra of both the commercially obtained ADC (Figure 2E) and the affinity purified ADC (Figure 2F), with four major peaks in each peak group, representing the ADC with various glycoforms (that is, G0F/G0F, G0F/G1F, G1F/G1F or G0F/G2F, and G1F/G2F). MassHunter DAR Calculator was used to calculate DAR using areas under the seven peak groups. Similar DARs were calculated (DAR = 3.5) for both conditions. These results demonstrate the high recovery and high purity that can be achieved with automated ADC purification on the AssayMAP Bravo Platform.

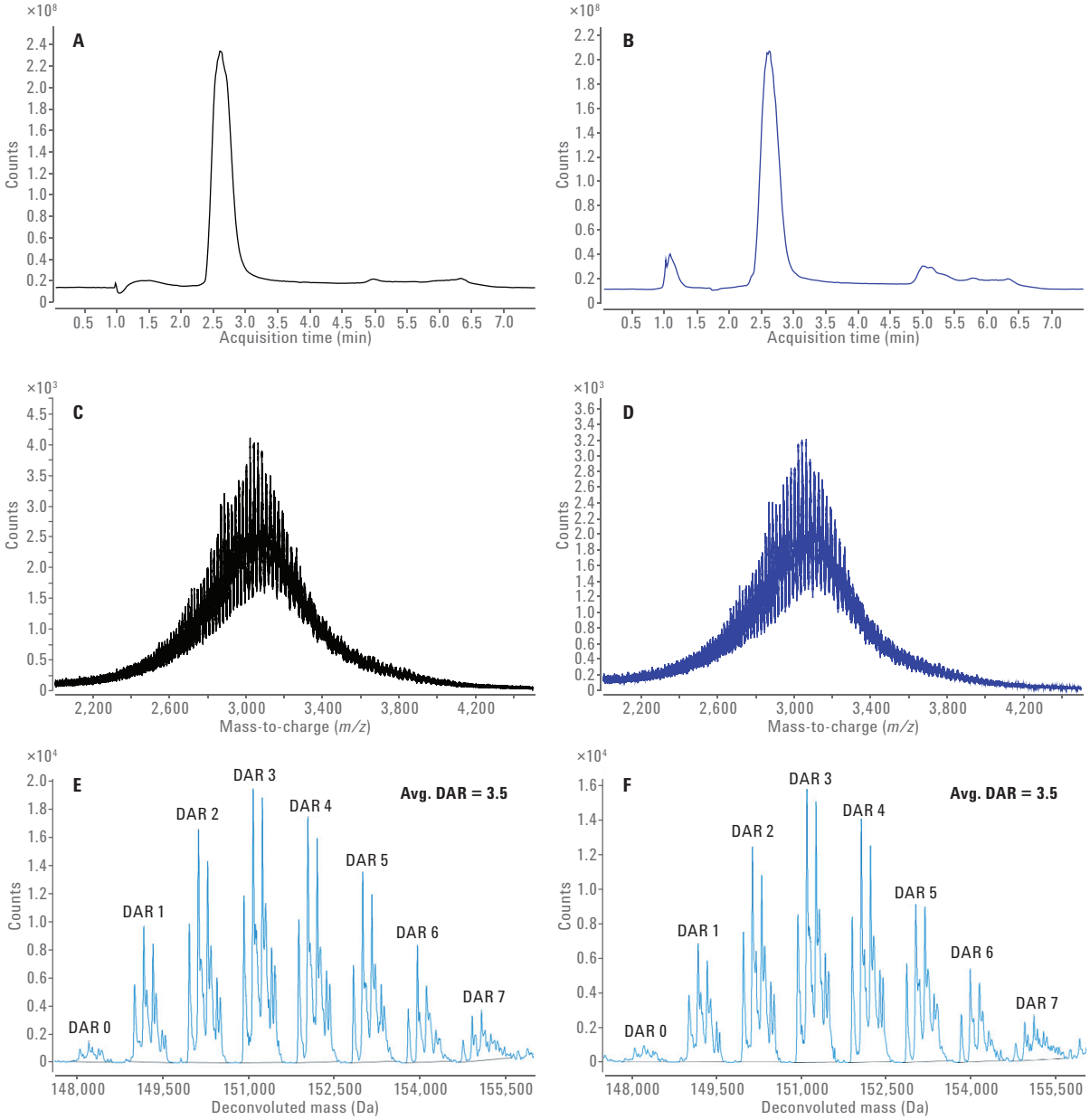

Figure 2. Representative total ion chromatograms (TICs), extracted spectra, and deconvoluted spectra from commercially obtained ADC and the affinity purified ADC. A) TIC of commercially obtained ADC. B) TIC of affinity purified ADC. C) Extracted spectra of commercially obtained ADC. D) Extracted spectra of affinity purified ADC. E) Deconvoluted spectra with DAR calculation of the commercially obtained ADC. F) Deconvoluted spectra with DAR calculation of affinity purified ADC.

To assess the suitability of the workflow for PK studies, the commercially obtained ADC was spiked into and serially diluted in rat serum to 20, 10, 5, 2.5, 1.25, and 0.625 µg/mL and purified as described above. Rat serum samples containing no ADC were also purified as a control. The amount of ADC in the serum sample loaded onto each cartridge was 1,000, 500, 250, 125, 62.5, 31.25, and 0 ng, respectively. Ten percent of the eluate was injected for mass spectrometry analysis for each affinity purified sample, corresponding to 100, 50, 25, 12.5, 6.25, 3.125, and 0 ng ADC on-column assuming 100 % recovery. Figure 3 shows the extracted ion chromatograph (EIC) over the mass envelope of the ADC (2,000–4,500 *m/z*). Peaks between 2.2 and 3.2 minutes were integrated and the coefficients of variation (CVs) were calculated for each ADC concentration. Excellent reproducibility was achieved with CVs less than 10 % for purified ADCs from serum samples with concentrations higher than 1.25 µg/mL. Note that the EIC of affinity purified serum control (no ADC spiked) are essentially flat, demonstrating the absence of copurified proteins in the ADC elution window. This specificity was achieved with the low nonspecific binding of the SA-W cartridge, the high specificity of the ADC for the antigen, and the fine-tuned wash conditions. No detergent was needed for removal of copurified proteins.

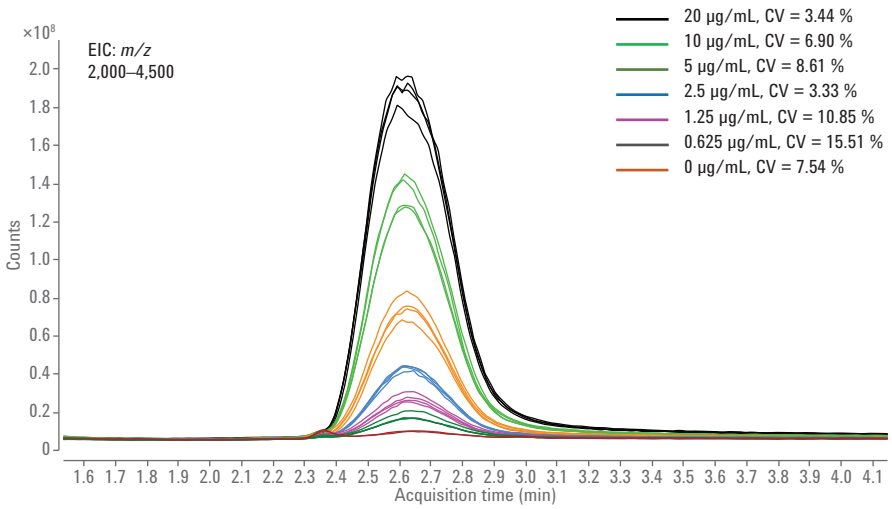

Figure 3. Extracted ion chromatographs (EIC) of affinity purified ADC at various serum concentrations. Fifty microliters of rat serum containing 20, 10, 5, 2.5, 1.25, 0.625 and 0 µg/mL ADC (n = 4 for each concentration) was affinity purified with ADC affinity cartridges using Agilent AssayMAP Bravo. Ten percent of the purified ADC from serum samples was injected for mass spectrometry analysis for each affinity purified sample. EICs (2,000–4,500 *m/z*) were extracted. Peaks between 2.2 and 3.2 minutes were integrated and coefficients of variation (CV) were calculated for the samples at each ADC concentration.

Figure 4 shows the deconvoluted spectra and DAR of affinity purified ADC from rat serum samples with 20, 10, 5, 2.5, 1.25, and 0.625 µg/mL ADC. The signal-to-noise ratio (S/N) decreases as the amount of ADC analyzed decreases so 33 % of the purified ADC from serum samples with ADC concentrations of 2.5, 1.25, and 0.625 µg/mL were analyzed compared to 10 % of the purified ADC from serum samples with ADC concentrations of 20, 10, and 5 µg/mL. This allowed higher quality deconvoluted spectra to be obtained from the samples with lower ADC concentrations. Comparable deconvoluted spectra and DARs were obtained for all ADC concentrations tested (Figure 4, Table 4). The percentages of ADC with various drug loads (D0-D7) were comparable for ADCs purified from serum samples with ADC concentrations of 20, 10, 5 and 2.5 µg/mL, and closely resemble those of the commercially obtained ADC directly injected for MS analysis. The percentages of the various drug loads were less well aligned to the commercially obtained ADC for ADCs purified from serum samples with ADC concentrations of 1.25 and 0.625 µg/mL (Table 4, Figure 5). To improve spectra quality and decrease detection/quantitation limit of the workflow, a higher percentage of the eluate may be injected for LC/MS analysis. Additionally, deglycosylation may be performed before elution of the ADC to simplify spectra, and to increase the signal. These results demonstrate that the presented workflow can reproducibly determine DAR and the percentages of ADC with different drug loads at low serum ADC concentrations.

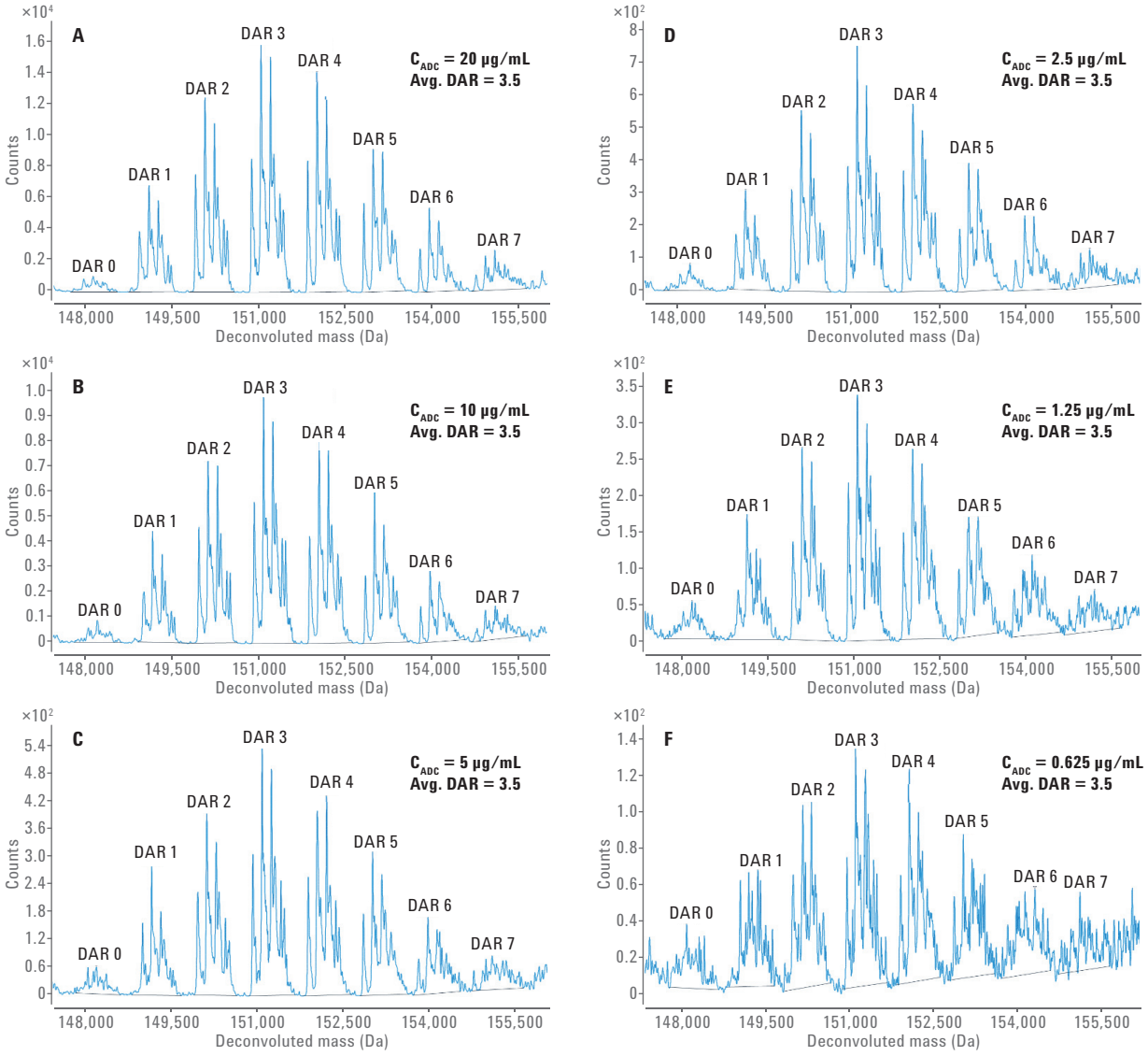

Figure 4. Representative deconvoluted spectra and DAR of affinity purified ADC from rat serum. Fifty microliters of rat serum containing 20, 10, 5, 2.5, 1.25, 0.625, and 0 µg/mL ADC (n = 4 for each concentration) was affinity purified with ADC affinity cartridges using Agilent AssayMAP Bravo. Ten percent of the purified ADC from serum samples containing ADC at 20 µg/mL (A), 10 µg/mL (B), and 5 µg/mL (C) were injected for MS analysis; 33 % of the purified ADC from serum samples containing ADC at 2.5 µg/mL (D), 1.25 µg/mL (E), and 0.625 µg/mL (F) were injected for MS analysis. C_{ADC}: Concentration of ADC in rat serum.

Table 4. The DAR and the percentage of ADC for each drug load.

Sample	DAR	Percentage of ADC for each drug load (%)							
		DAR 0	DAR 1	DAR 2	DAR 3	DAR 4	DAR 5	DAR 6	DAR 7
Commercially obtained (n = 4)	3.50±0.02	2.11±0.75	9.9±1.08	17.35±0.33	22.82±1.69	19.88±1.81	15.35±0.47	7.97±0.93	4.65±0.77
Affinity purified from 20 µg/mL serum samples (n = 4)	3.49±0.02	2.4±0.68	9.45±1.05	17.32±0.25	23.32±1.45	20.17±1.63	14.97±0.59	7.58±0.69	4.8±0.88
Affinity purified from 10 µg/mL serum samples (n = 4)	3.46±0.02	2.54±0.19	9.72±0.24	17.38±0.18	23.87±0.47	19.73±0.13	14.36±0.13	7.47±0.24	4.95±0.38
Affinity purified from 5 µg/mL serum samples (n = 4)	3.49±0.03	3.47±0.21	9.67±0.74	16.6±0.16	22.3±0.1	19.83±1.08	14.41±0.21	8.49±0.26	5.24±0.74
Affinity purified 2.5 µg/mL serum samples (n = 4)	3.43±0.06	3.08±0.26	9.94±0.22	17.83±0.53	23.21±0.59	19.55±0.18	13.64±0.23	8.01±0.65	4.73±0.54
Affinity purified 1.25 µg/mL serum samples (n = 4)	3.4±0.1	4.43±0.22	11.31±1.68	17.96±0.89	20.73±0.65	18.17±0.94	12.55±1.17	9.08±0.5	5.78±0.66
Affinity purified 0.625 µg/mL serum samples (n = 4)	3.48±0.08	6.49±0.66	10.95±1.45	16.43±0.8	17.9±0.98	16.61±0.49	12.67±1.29	11.91±0.71	7.05±0.43

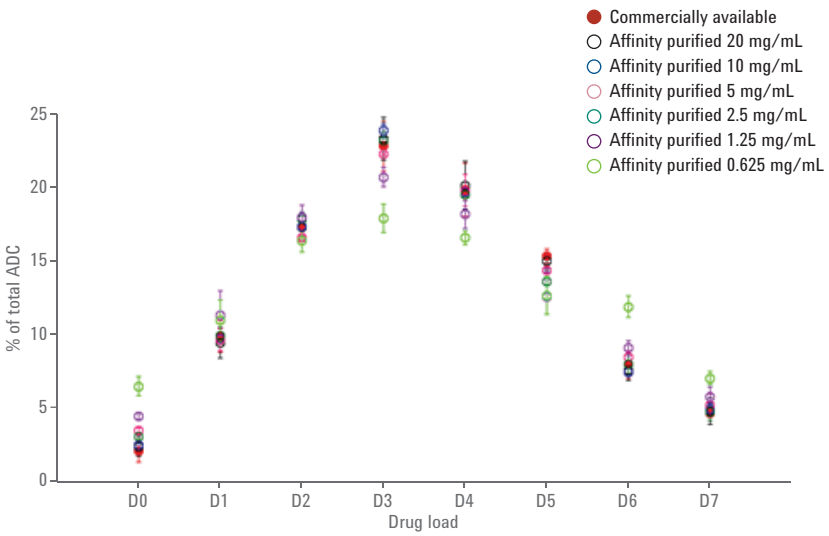

Figure 5. Percentage of ADC for each drug load.

Conclusions

Agilent provides a complete ADC DAR determination solution that includes automated affinity purification with the Agilent AssayMAP Bravo, LC/MS acquisition with the Agilent 1290 Infinity UHPLC and an Agilent 6550 Q-TOF, deconvolution with Agilent MassHunter BioConfirm software, and DAR determination with Agilent MassHunter DAR Calculator. This ADC DAR determination solution:

- Reduces manual labor, variability, and the probability of human error
- Purifies ADCs from serum with high yield and purity
- Generates high resolution spectra
- Provides easy DAR calculation

References

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Published in the USA, March 30, 2016
5991-6621EN

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