Measuring Drug-to-Antibody Ratio (DAR) for Antibody-Drug Conjugates (ADCs) with UHPLC/Q-TOF

Application Note

Biopharma

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Abstract

In this paper, we are investigating an antibody drug conjugate (ADC) with a cysteine linker. The Agilent 1290 Infinity II liquid chromatography system connected to a PLRP-S reversed phase liquid chromatography column was used to separate restored light and heavy chains and light and heavy chains of the corresponding drug linkers. Each chromatographic peak was verified through the Agilent 6530 High-resolution quadrupole time of flight (Q-TOF) LC-MS system. The peak area percentage of each light and heavy chain in the UV absorption spectrum was integrated and combined with the number of drug conjugates corresponding to each peak, and the weighted average drug to antibody ratio (DAR) was calculated for ADC. The Agilent DAR Calculator was used to calculate the DAR values for the mass spectral deconvolution data at mean time, and the results were the same as the UV absorption spectrum calculations.

Introduction

Antibody-Drug Conjugates (ADC) are a type of new anticancer drug that links target antibodies with strongly cytotoxic drugs via a specific chemical coupling[1]. ADC combine the specific targeting capability of antibodies with the high cytotoxicity of small-molecule drugs. They can deliver cytotoxic drugs directly to lesion tissue, while limiting toxicity in non-target tissues. They are less toxic than small-molecule drugs and have a longer half-life than small-molecule drugs. For this reason ADC are considered promising as a potential cancer treatment[2].

Adcetris is a type of cysteine linked ADC in which the disulfide bond in the antibody undergoes partial reduction to become a free cysteine residue. This subsequently links with small molecule drugs to form a mixture composed of 0-8 antibody-drug complexes as shown in Figure 1.

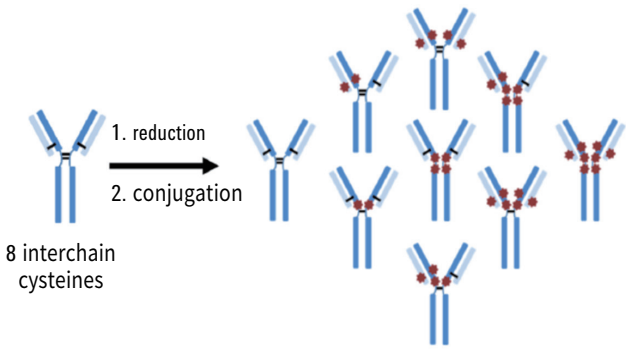


Figure 1. The schematic of cysteine linked ADC.

Drug to Antibody Ratio (DAR) is the average number of drugs linked to each antibody. DAR is a key property used to measures the quality of ADC because it can significantly affect ADC efficacy. It is essential to measure DAR because low DAR value may indicate decreased efficacy, while a relatively high DAR value may negatively impact safety.

In this paper, we investigated Adcetris using reversed phase liquid chromatography and a quadrupole time of flight LC-MS system. Each component in the reduced ADC was separated chromatographically and verified with mass spec and a diode array detector. In addition, the DAR values of the various ADC were calculated using UV absorption spectrum results and mass spectral deconvolution data.

Experimental

Material

Adcetris is an ADC with a cysteine linker; dithiothreitol was purchased from Fluka; formic acid was purchased from Dikma; trifluoroacetic acid was purchased from Dikma; acetonitrile was purchased from Merck Millipore.

Instrumentation

Experiments were conducted using the Agilent UHPLC/Q-TOF system, composed of the Agilent 1290 Infinity II Liquid Chromatography system equipped with a PLRP-S reversed-phase column and diode array detector (DAD) and the Agilent 6530 High-resolution quadrupole time of flight (Q-TOF) LC-MS system equipped with dual Agilent jet stream electrospray ionization (ESI+).

Sample preparation

50 µg of ADC sample was diluted to a concentration of 1 mg/mL, and freshly prepared dithiothreitol (DTT) stock solution was added to make a final DTT concentration of 50 mmol/L. The DTT solution was incubated at 37°C to produce the reduced ADC samples.

Reverse phase LC conditions

Column:	Agilent PLRP-S 1000Å, 5um, 2.1 × 50 mm (PN: PL 1912-1502)	
Mobile phase:	A: 100% water, with 0.1% formic acid and 0.025% trifluoroacetic acid	
	B: 100% acetonitrile, with 0.1% formic acid and 0.025% trifluoroacetic acid	
Flow rate:	0.25 mL/min	
Gradient:	Time (min)	%B
	0	27
	3	27
	25	49
	26	95
	31	95
	31.5	27
	45	27
Temperature:	70 °C	
UV Detection:	280 nm	

MS conditions

Gas temperature:	325 °C
Gas flow:	7 L/min
Nebulizer pressure:	35 psi
Sheath gas temperature:	200 °C
Sheath gas flow:	4.5 L/min
Capillary voltage:	3,500 V
Nozzle Voltage:	500 V
Fragmentor voltage:	175 V
MS scan rate	2 spec/s
MS mass range:	500 m/z-5,000 m/z

Data analysis

Agilent MassHunter Bioconfirm (version: B.07.00 Service pack 1) was used to deconvlute the original mass spectrometry results. Deconvolution results were imported into the Agilent DAR Calculator (Agilent DAR Calculator 1.0), and first entered/selected the mass numbers for D0 and the drug/linker, the DAR calculator then automatically selected, labeled, and integrated the mass spectral peak groups for ADCs based on various drug loading amounts, and subsequently calculated the average DAR and generated the peak list.

Results and Discussion

In this article, DTT was used to reduce Adcetris and dissociate it into light chains with 0 to 1 drugs and heavy chains with 0 to 3 drugs. In addition, a reverse phase chromatography system based on variations in hydrophobicity was used to efficiently separate the reduced light and heavy chains and the light and heavy chains of the linked drug. Each peak of the reduction ADC was identified by mass spectrometry using the reverse phase chromatography - mass spectrometry technique. The original results obtained were then deconvoluted using Agilent MassHunter Bioconfirm and imported into the Agilent DAR calculator to get the weighted average for the DAR of the ADC. Figure 2 shows the total ion chromatogram for the reduced ADC separated via reversed-phase chromatography, where the mass chromatogram for each peak was extracted and deconvoluted. Figure 3 shows the mass spectrometry deconvolution results for peaks a and b. Peak a correspond to the light chain of ADC (LC). In the deconvolution results for peak b, the peak with a mass number of 225041.4 Da is the light chain linked with one small molecule drug (LC-1d), and the peak with a mass number of 24279.29 Da is the light chain linked with the chemical unit, i.e., the linker (LC-linker). This may be due to the fact that during the ionization process, some small molecule drugs were partially detached from the sample. For this reason peak b corresponds to the light chain linked with one small molecule drug (LC-1d). Deconvolution of c, d, e, and f peaks in the mass chromatogram (Figure 4) shows that they correspond respectively to the ADC heavy chain (HC), heavy chain with one small molecule drug (HC-1d), heavy chain with two small molecule drugs (HC-2d), and heavy chain with three small molecule drugs (HC-3d). Deconvolution results for the small peaks d', e' and f' on the mass chromatogram have a few more Daltons than the results for the primary peak. This is due to the fact that a small part of the in-chain disulfide bond underwent reduction.

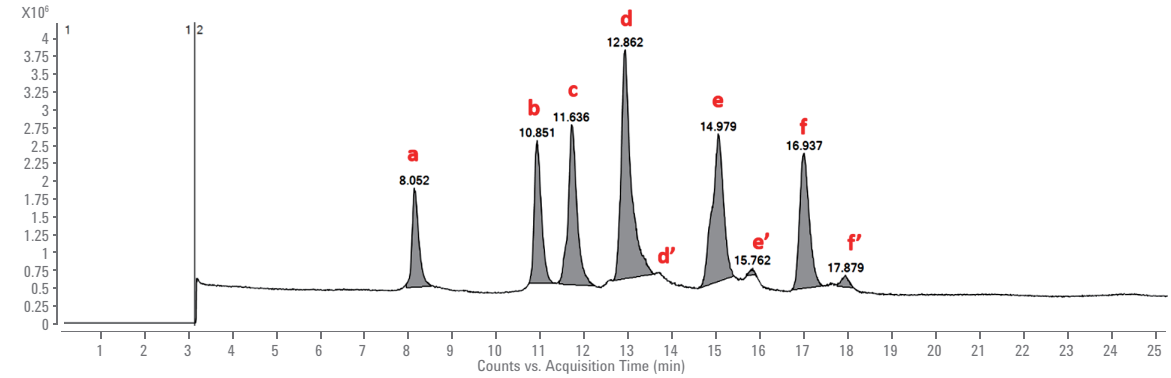


Figure 2. Total ion chromatogram for the mass chromatogram of reduced ADC separated with reversed phase chromatography.

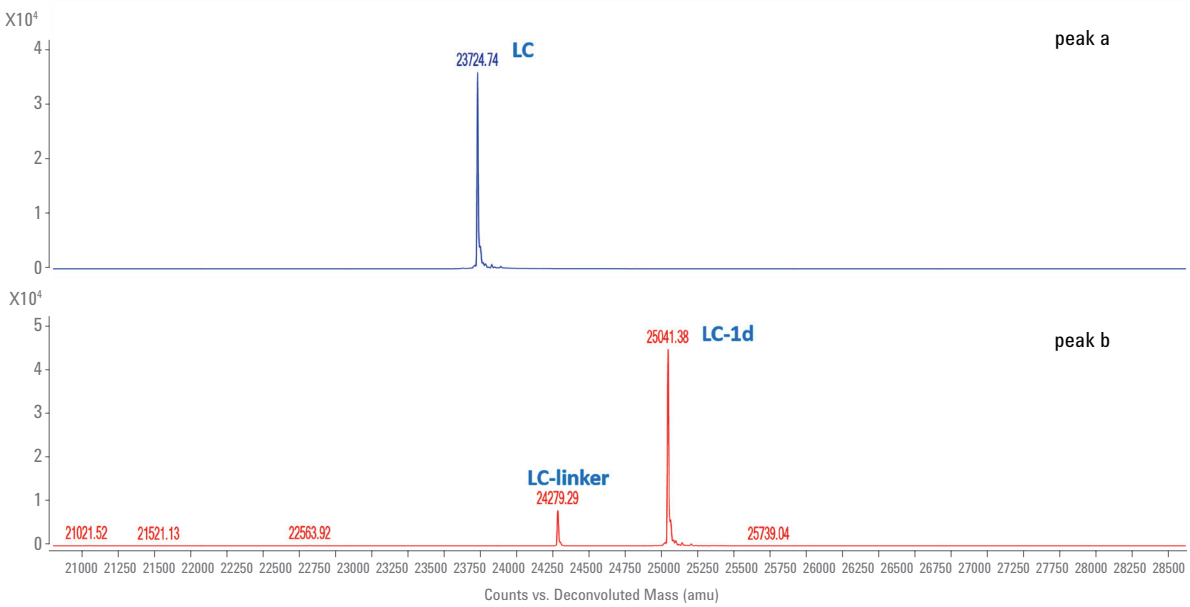


Figure 3. Deconvolution results for peaks a and b in the mass chromatogram.

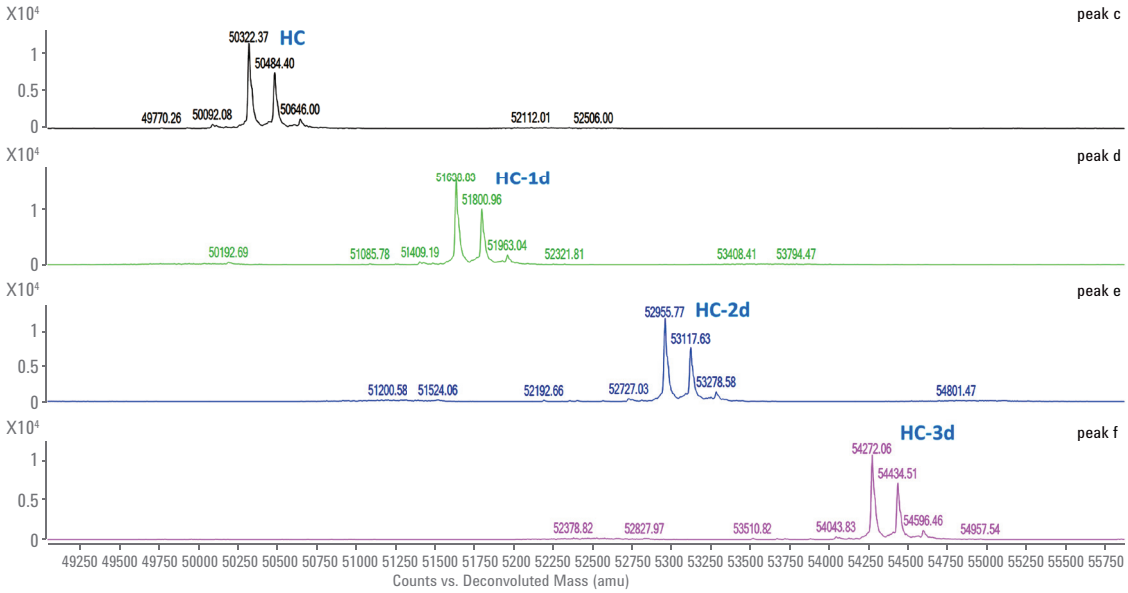
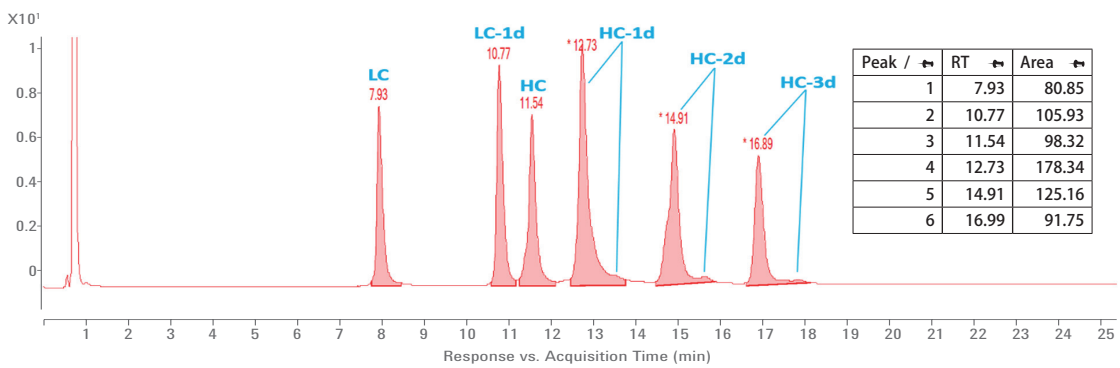


Figure 4. Deconvolution results for peaks c, d, e and f.

The identity of each peak in the UV absorption spectrograph for reduced ADC separated by reverse phase chromatography can be determined through mass spectrometry (Figure 5). The proportion of light chain peak area was calculated and the sum of the peak area proportions totaled 100%. The proportion of the peak area for the heavy chain peak was calculated and the sum of the peak area proportions totaled 100%. According to the formula DAR = 2 X (Σ weighted peak area of heavy chain + Σ weighted peak area of light chain) / 100[3], the weighted average of DAR for ADC-Adcetris was calculated to be 4.0 (as shown in Table 1).



Peak	RT	Area
1	7.93	80.85
2	10.77	105.93
3	11.54	98.32
4	12.73	178.34
5	14.91	125.16
6	16.99	91.75

Figure 5. UV absorption spectrum at 280 nm wavelength for reduced ADC separated by reversed phase chromatography and integrated areas for each peak (Top right).

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

Peak name	Drug load	Peak area proportion (%)	Weighted peak area (drug load X peak area)/%
LC	0	43.3	0
LC-1d	1	56.7	56.7
HC	0	19.9	0
HC-1d	1	36.1	36.1
HC-2d	2	25.4	50.8
HC-3d	3	18.6	55.8
Weighted average DAR	4.0		

* Calculated based on the peak area of the chromatogram

The chromatograms of light and heavy chains from mass spectrometry were deconvoluted and the results were imported into the Agilent DAR Calculator to automatically calculate the DAR of ADC. As shown in Figure 6, the peaks of DAR 0 and DAR 1 for light chains and DAR 0–DAR 3 for heavy chains were selected manually, and the DRA of light chains and heavy chains were calculated automatically with software to yield the final weighted average of DAR for ADC. The weighted average of DAR for ADC was to be 4.0 using the Agilent DAR Calculator. This was consistent with the liquid chromatography results.

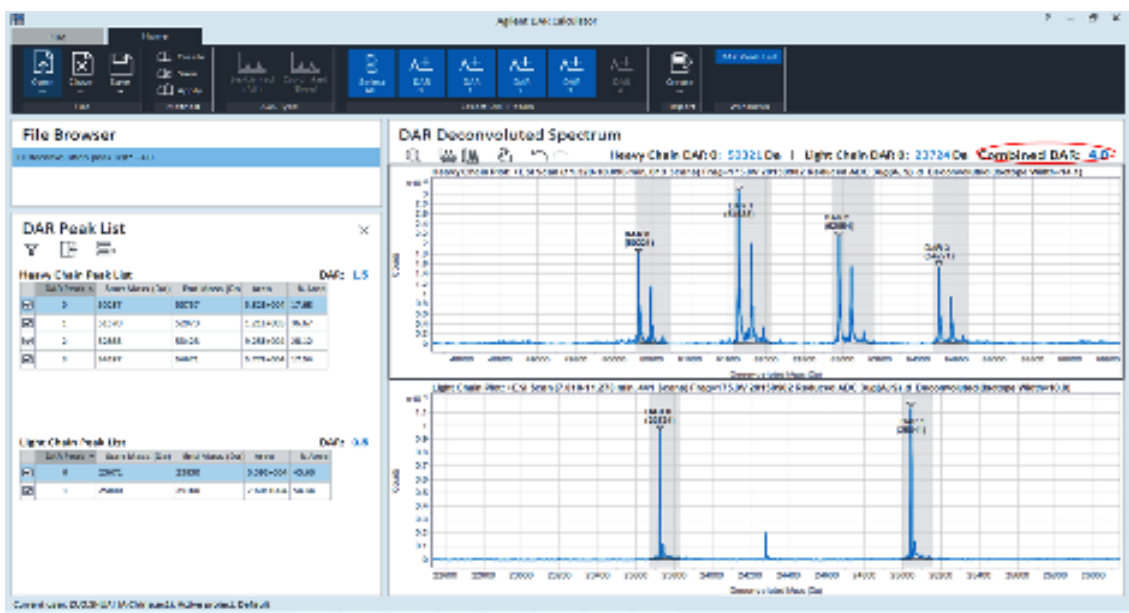


Figure 6. The DAR of ADC calculated using the Agilent DAR Calculator.

Conclusion

In this article, a reverse phase LC-MS system was used to separate and measure each fragment of reduced ADC in order to calculate the weighted average of DAR for ADC. PLRP-S columns can easily separate each fragment of reduced ADC and The LC-MS technique can be used to identify each peak. This is accomplished by calculating the peak area proportion for each peak in the UV absorption spectrum results for ADC. The weighted average of DAR for ADC can then be obtained based on mass spectrometry results and the weighted average of DAR for ADC can be calculated using the Agilent DAR Calculator. Unlike with traditional HIC method, the RP-HPLC method does not require the use of high salt concentrations that tend to contaminate conventional LC systems. Because this method uses a mobile phase that is also compatible with mass spectrometry, it meets both the qualitative demands of MS and the quantitative requirements of LC. This method is considered a complement to HIC, and the two methods can be used in conjunction to reconfirm results. The Agilent DAR Calculator can be used to automatically calculate the DAR of ADC. The results are consistent with the results from the liquid phase method, and the calculation process is much simpler.

References

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