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Analysis of Free Drug Content in Antibody-Drug Conjugate Using 2D-LC/Q-TOF/MS

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Introduction

Antibody-drug conjugates (ADCs) are complex therapeutic biomolecules composed of an antibody linked to a potent cytotoxic small molecule drug. The antibody enables targeted delivery of the cancer-killing drug to the tumor site while limiting the toxicity. The drug is conjugated via linker to the specific amino acid sites on the antibody, either cysteine or lysine. Incomplete conjugation results in the presence of unbound drugs within the ADC, potentially leading to heightened toxicity. Therefore, measurement of free drug content is a unique critical quality attribute (CQA) of ADCs.

Reversed-phase (RP) LC can be used for the free drug analysis[1]. However, the samples need to go through protein removal step either by SPE or protein precipitation in order to prevent irreversible binding of protein to stationary phase. These manual and offline procedures are tedious and time-consuming.

In this study, we leveraged on the heart-cutting 2D-LC technology to automate the protein removal procedure. And by coupling the 2D-LC with LC/Q-TOF MS, the free drug analysis in ADC was streamlined and fully automated. The analytical components of the method are shown in Figure 1.

Experimental

Sample preparation

ADC sample was desalted and dissolved in 100 mM ammonium acetate buffer (pH 7.0) at a concentration of 5 mg/mL. DM1(drug) and SMCC-DM1(linker-drug) were separately spiked to the ADC sample at the concentration of 100 µg/mL each[2].

Instrumentation

Key instrument parameters are listed in Table 1 and 2.

Table 1. 2D-LC parameters

Parameters	
First Dimension	
Solvent A	100 mM ammonium acetate
Solvent B	acetonitrile
Gradient	Isocratic 40% B
Flow Rate	0.25 mL/min
UV	252 nm at 20 Hz data rate
Second Dimension	
Solvent A	0.1% formic acid
Solvent B	0.1% formic acid + 95% acetonitrile/H_2O
2D-LC Mode	Heart-cutting
Flow Rate	0.5 mL/min
Sampling Table	9.0 min, Time-based Heart Cut, HiRes 3 x 7.68 s, Multi-Inject Yes
2D Cycle Time	Analysis: 5 min Equilibration: 0.7 min
2D Gradient	38% - 65% B in 5 min
Col. Temp	40 °C

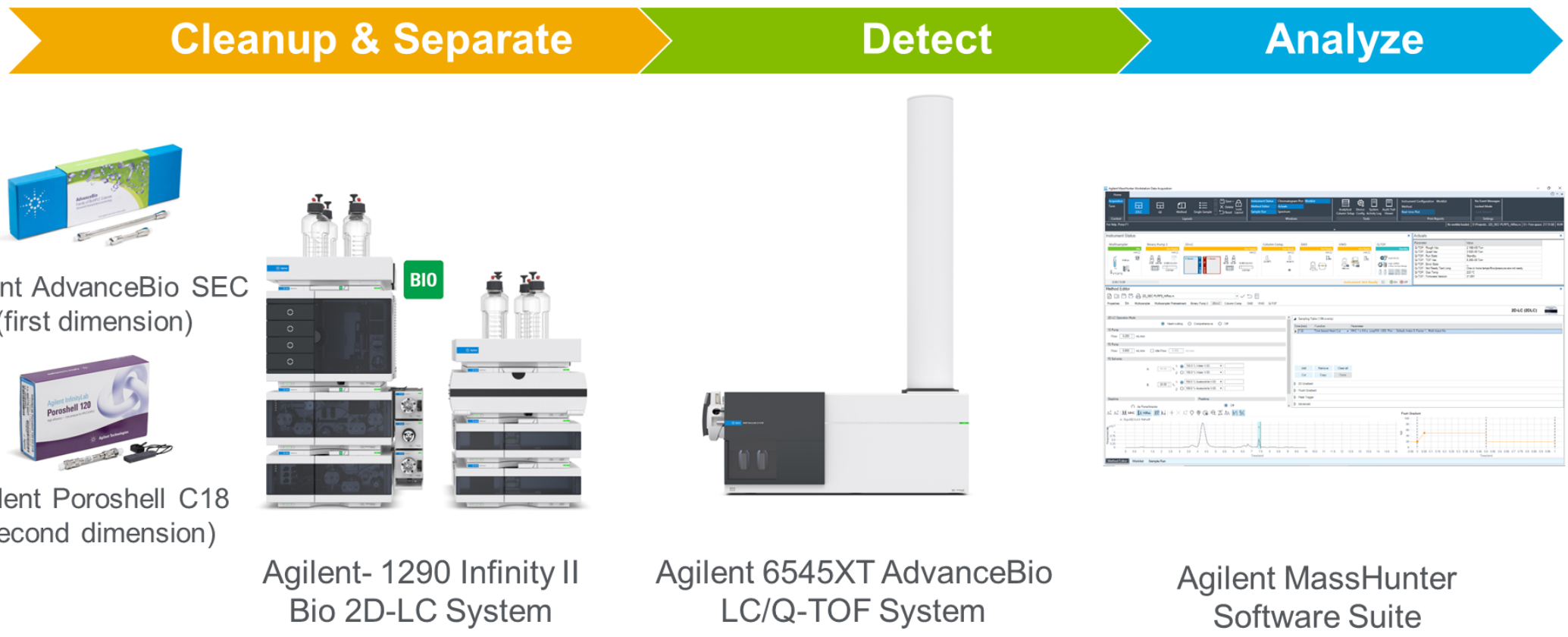

Figure 1. Analytical components of the 2D-LC/Q-TOF analysis of ADC free drug content.

Results and Discussion

First dimension SEC method development

Considering the compatibility with MS detection, ammonium acetate was selected over phosphate buffer as SEC mobile phase in first dimension. Acetonitrile (ACN) was chosen as organic modifier to mitigate the hydrophobic interaction between column stationary phase and the analyte.

The concentration of ACN was scouted from 20% to 40% in 5% increments for the analysis of DM1 standard. Figure 2 showed that 40% acetonitrile rendered best peak shape of DM1. It is safe to use this percentage of organic modifier because it falls within the organic solvent tolerance limit of 50% for the AdvanceBio SEC column.

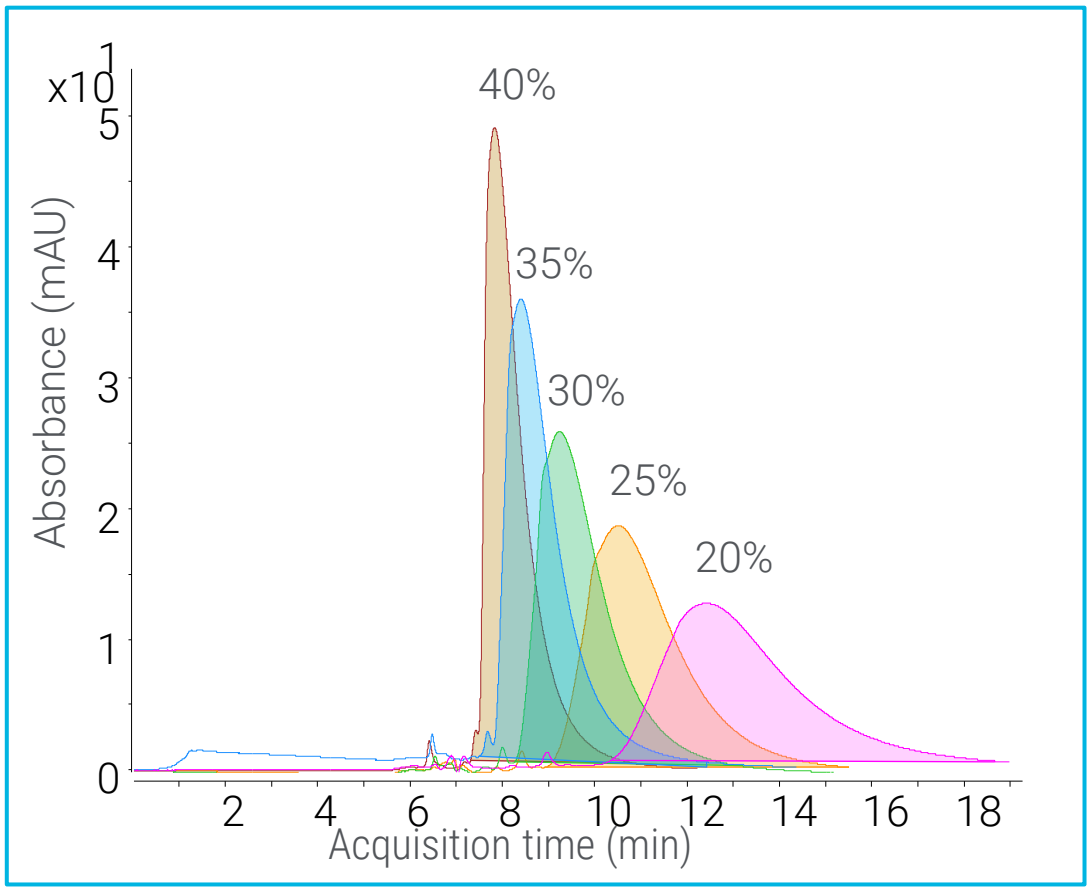

Figure 2. Scouting of ACN concentration in 1D SEC for the DM1 analysis.

Table 2. LC/MS parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Source	Agilent Jet Stream ESI
Polarity	positive
Drying Gas Temp.	300 °C
Drying Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas Temp.	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3500 V
Nozzle Voltage	0 V
Fragmentor	135 V
Mass Range	*m/z* 100 -1700
Acquisition Rate	1 spectra/s

Second dimension sampling

Using the 40% ACN in 100mM ammonium acetate (pH 7.0), the separation between ADC and free drug content was achieved in the spiked sample in the first dimension as illustrated in Figure 3.

However, DM1 and SMCC-DM1 co-eluted as a broad peak from 8 to 10 min in the spiked samples. This is partly because the Mw difference between the two molecules were not significant enough for separation under SEC. Moreover, the spiked DM1 and SMCC-DM1 could have interactions with ADC at the column head during initial stage of separation, which caused the broadening of the peaks.

Three fractions of the broad peak as shaded in Figure 3 were sampled into three loops via multi-inject sampling mode. The three cuts were sequentially transferred to the 2D. The cuts content was subsequently analyzed within one single 2D gradient cycle. The multi-inject mode enables sampling of a broad 1D peak using the standard 40 µL loops without hardware modification. It also reduces the run time by analyzing multiple cuts within one 2D cycle. The total method time was therefore shortened to only 17 min.

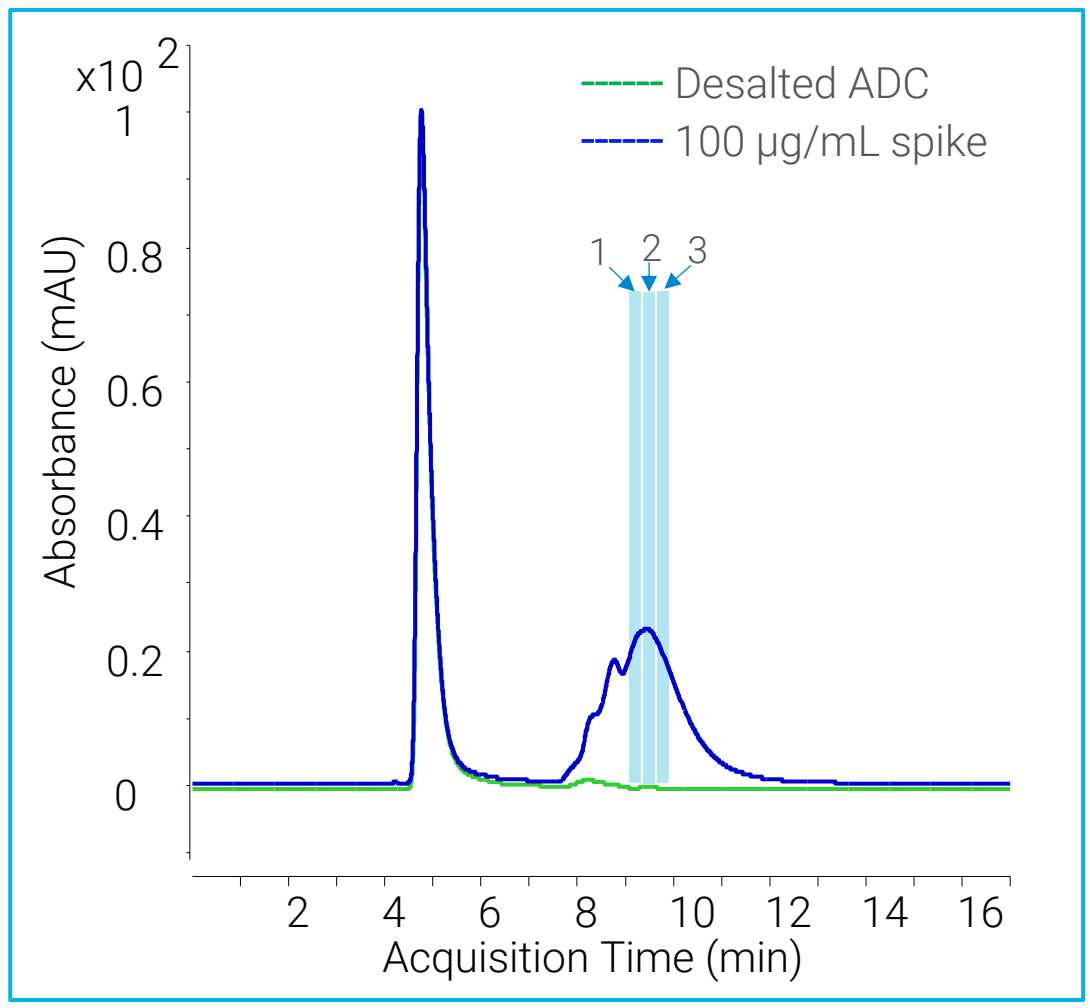

Figure 3. Overlaid UV chromatograms of first dimension SEC separation of desalted ADC (green) and 100 µg/mL spiked desalted ADC (blue). The shaded area represents the three continuous cuts sampled into 2D using multi-inject mode.

Results and Discussion

Second dimension LC/MS analysis of free drug

A total of 96 µL of 1D effluent was transferred onto 2D RP column. Three peaks were detected and separated in both UV and total ion chromatogram (TIC) as shown in Figure 4A.

Peak 1 was confirmed as DM1 based on measured mass of 738.2839 Da. The mass accuracy of it was 2.30 ppm from theoretical mass 738.2822 Da. Sodium and potassium adducts presented high abundance which could be attributed to the use of buffer salt in 1D SEC and subsequently introduced to the 2D during sampling. Neutral loss of water fragment was also detected due to in source fragmentation.

The peak 2 and 2' have identical MS spectra. Their mass of $[M+H]^+$ ion was 1072.3985 Da, which was confirmed as SMCC-DM1. The mass accuracy was 0.18 ppm from theoretical mass of 1072.3987 Da. This doublet peak phenomenon was caused by the presence of a stereocenter in the SMCC-DM1 molecule[3]. The two peaks are diastereomers which have identical mass.

Leveraging on the 2D-LC/Q-TOF technique, the free drug content was separable from ADC in SEC and subsequently the individual components were successfully separated from each other in RP and confirmed by MS.

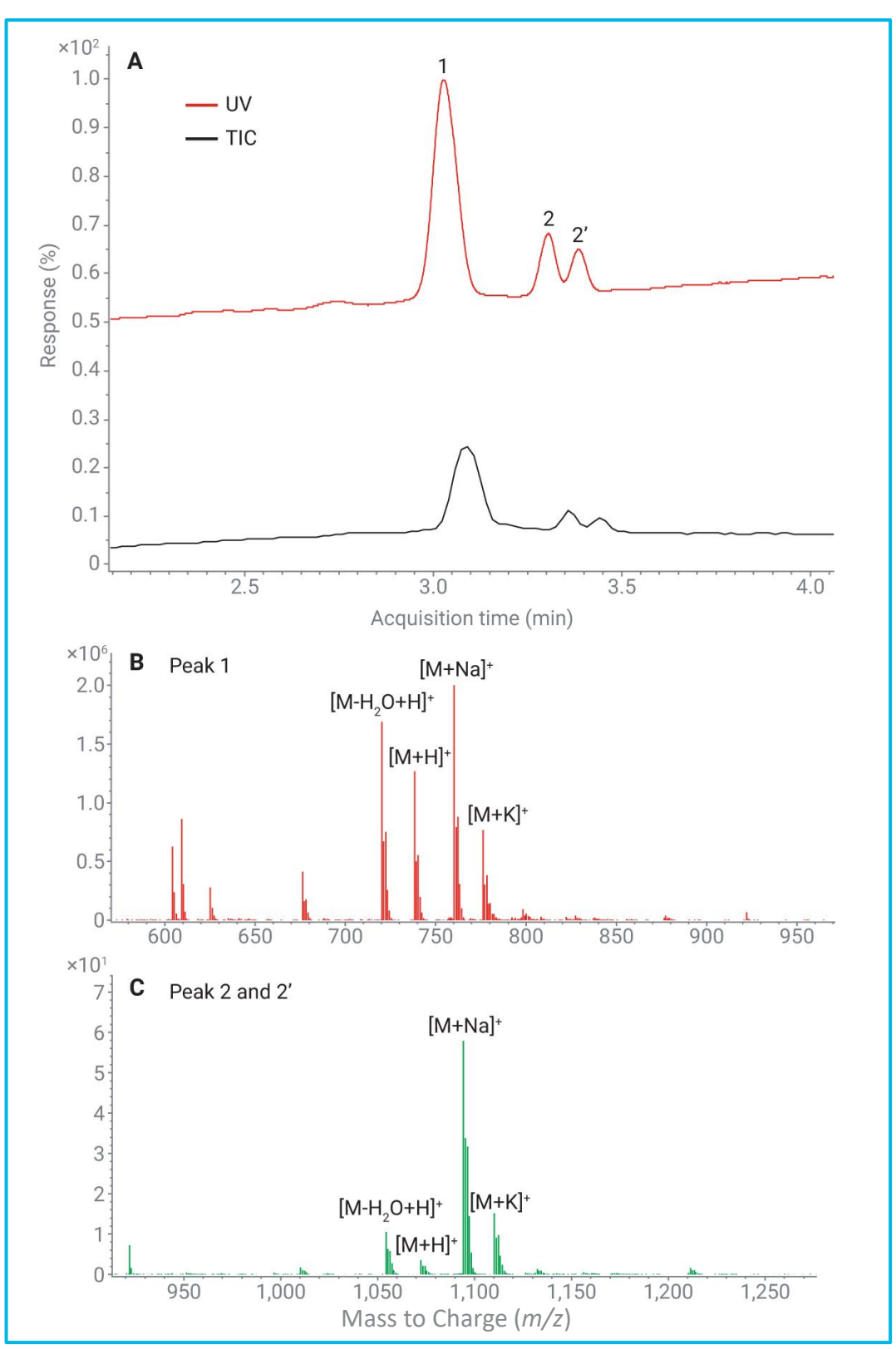

Figure 4. LC/MS analysis of free drug content. (A) UV and TIC (B) Peak 1 MS spectra (C) Peak 2 and 2' MS spectra.

Conclusions

- A streamlined 2D-LC/MS method was developed for the identification of free drug content in ADCs, utilizing the Bio 2D-LC, AdvanceBio SEC column, Poroshell EC-C18 column, and 6545XT AdvanceBio LC/Q-TOF.
- This method effectively integrates protein elimination and free drug identification into a single analysis.
- The automated protein removal process not only saves time but also protects the RP column from deterioration, enhancing the longevity and reliability of the system.
- Additionally, the method achieves superior chromatographic separation between the drug and the linker-drug, ensuring precise and reliable identification through accurate mass detection.
- This comprehensive approach significantly improves the efficiency and accuracy of ADC analysis.

References

1. Li, Y. et.al. Limiting degradation of reactive antibody drug conjugate intermediates in HPLC method development. Journal of Pharmaceutical and Biomedical Analysis 2014, 92, 114–118.

2. Agilent application Note 5994-7182EN for more details

3. Singh R. et.al. A new triglycyl peptide linker for antibody–drug conjugates (ADCs) with improved targeted killing of cancer cells. Molecular Cancer Therapeutics 2016, 15 (6), 1311–1320.

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