Optimizing Chromatography and High Resolution Time-of-Flight Mass Spectrometry for Antibody-Drug Conjugate DAR Characterization

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1. INTRODUCTION

Antibody-drug conjugates (ADCs), aiming to combine the potency of cytotoxic drugs with the high specificity of a monoclonal antibody (mAb), are becoming increasingly important as new targeted therapies in oncology and require designer LC-MS methodologies to characterize them. The ADC product used in this study consists of a cytotoxic agent chemically attached to one of eight cysteine residues involved in inter-chain disulphide bridges. After mild reduction the LC-MS results can reveal heavy and light chain subunits with 1 – 4 payloads, if chromatography optimization efforts lead to fast yet effective separation for accurate drug-antibody-ratio (DAR) calculations.

Subsequently, digestion of the ADC immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS), followed by LC-MS was investigated. IdeS specifically cleaves immunoglobulin G under its hinge region. Under reducing conditions, IdeS digestion of mAbs results in three polypeptide chains of around 25 kDa each; Fd region, LC region, and the Fc/2 region. With minimal sample preparation, and within a single optimized LC-MS analysis, the method can provide efficient LC and MS resolution that potentially results in relevant information on N-glycan profiling, and accurate DAR calculation specifically aimed at payload distribution linked to the Fd region.

2. METHODS Part 1 – LC-MS

ADC product was aliquoted into two preparations; (1) reduced; and (2) IdeS digestion. The reduction was carried out by treating the ADC with 10 mM TCEP for 1 hr at 60C. The ADC was also digested with the IdeS enzyme (Fabricator – Genovis) for 2 hr at 37ºC followed by reduction with 10 mM TCEP for 1 hr at 60ºC

The LC-separation was carried out using a DIONEX Ultimate 3000 RLS-nanoLC system configured with a CTC PAL autosampler operating flow rates at 300 µL/min through an Zorbax Poroshell 300-C8 (1 mm x 75 mm x 300 µm) column heated to 75ºC. The chromatography method is shown as overlays in red on chromatograms in results section for a method of a total of 22 mins. The protein intact mass analysis was carried out use the AB SCIEX TripleTOF® 5600+ system using the optimized parameters listed in Table 1 enabling accurate charge envelope detection of light and heavy chains of the ADC product. Raw data was processed entirely using BioPharmaView™ Software, new from AB SCIEX, providing intact mass deconvolution and values listed for DAR calculations.

Table 1. TripleTOF® System Acquisition Parameters

Parameter Setting	ADC Reduced
Intact Protein Mode (Script Menu)	ON
Curtain Gas	30
Time Bins to Sum	40
Declustering Potential	150
Accumulation Time	2 s per MS scan
Mass Range	600-3200 m/z
CE	15
Source TEMP	300

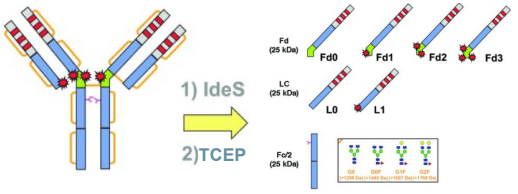
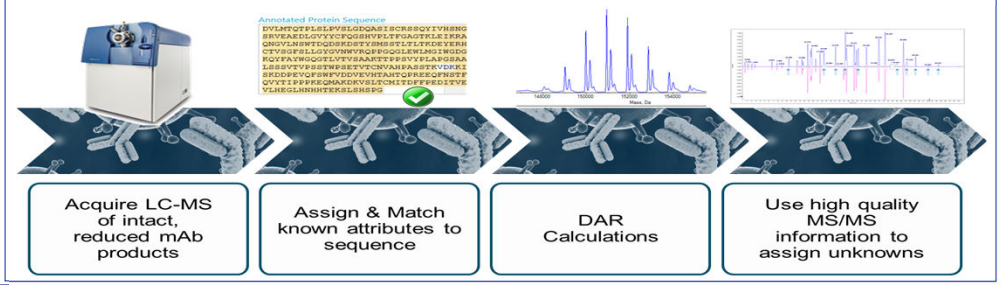
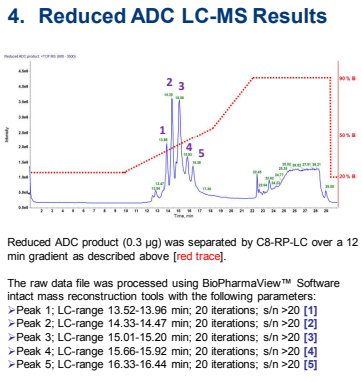


Figure 1. ADC reduction and IdeS digestion technique providing accurate DAR distribution measurements by LC-MS. Picture modified Rousset *et al*, 2013 ; Antibody-drug conjugate model fast characterization by LC-MS following IdeS proteolytic digestion.

3. METHODS Part 2 – BioPharmaView ™ Software Workflow

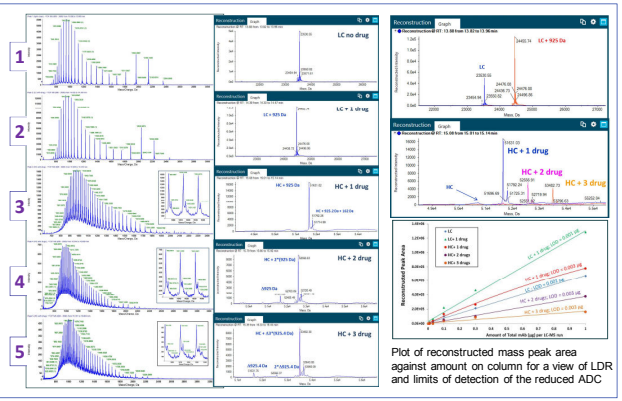


4. Reduced ADC LC-MS Results



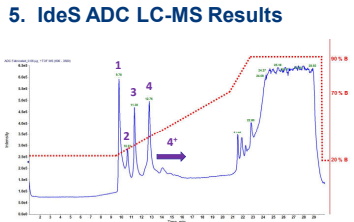
Reduced ADC product (0.3 µg) was separated by C8-RP-LC over a 12 min gradient as described above [red trace].

The raw data file was processed using BioPharmaView™ Software intact mass reconstruction tools with the following parameters:
- Peak 1; LC-range 13.52-13.96 min; 20 iterations; s/n >20 [1]
- Peak 2; LC-range 14.33-14.47 min; 20 iterations; s/n >20 [2]
- Peak 3; LC-range 15.01-15.20 min; 20 iterations; s/n >20 [3]
- Peak 4; LC-range 15.66-15.92 min; 20 iterations; s/n >20 [4]
- Peak 5; LC-range 16.33-16.44 min; 20 iterations; s/n >20 [5]



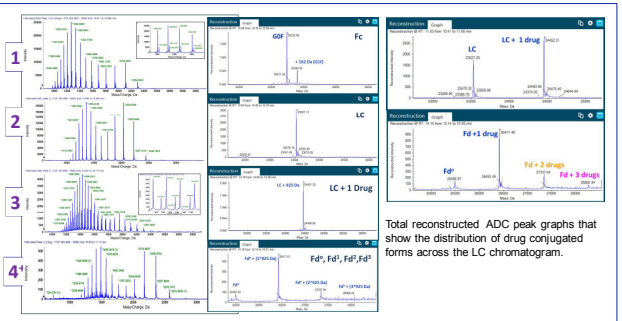
Plot of reconstructed mass peak area against amount on column for a view of LDR and limits of detection of the reduced ADC

5. IdeS ADC LC-MS Results



IdeS TCEP treated with ADC product (0.06 µg) was separated by C8-RP-LC over a 12 min gradient as described above [red trace].

The raw data file was processed using BioPharmaView™ Software intact mass reconstruction tools with the following parameters:
- Peak 1; LC-range 9.65 - 9.79 min; 20 iterations; s/n >20 [1]
- Peak 2; LC-range 10.49-10.70 min; 20 iterations; s/n >20 [2]
- Peak 3; LC-range 14.05-14.30 min; 20 iterations; s/n >20 [3]
- Peak 4; LC-range 14.10-16.21 min; 20 iterations; s/n >20 [4]



Total reconstructed ADC peak graphs that show the distribution of drug conjugated forms across the LC chromatogram.

6. ADC DAR Calculations

Data Analysis Using BioPharmaView™ Software:

1.peak areas of the reconstructed masses for each LC range were exported
2.calculate the percentage peak distribution of light chain so that the total percentage sums to 100
3.calculate the percentage peak distribution of heavy chain so that the total percentage sums to 100
4.calculate weighted peak area of the light and Fd pieces separately, by multiplying the percentage peak area by the drug load
5.calculate the average DAR as shown in the eq[n] in Table 2 &3.

Table 2. DAR calculation from reduced ADC LC-MS analysis after spectra deconvolution

ADC chain/piece	DAR = 2 x (Σ (weighted peak area of light chain) + Σ(weighted peak area of heavy chain))/100		
Reconstructed peak of 0.3 µg total ADC	Drug Load	Percentage Peak Area (%)*	Weighted peak area (drug load x peak area%)
LC	0	34	0
LC + 1 drug	1	66	66
HC	0	< 1	0
HC + 1 drugs	1	60	60
HC + 2 drugs	2	23	46
HC + 3 drugs	3	16	48
$DAR_{average}$ HC			1.53
Average DAR			4.19

*Percentage peak are (%) represents the distribution of drug-loaded LC or HC, for example, LC + 1 drug accounts for 66% of the entire LC forms.

Table 3. DAR calculation from IdeS ADC LC-MS analysis after spectra deconvolution

ADC chain/piece	DAR = 2 x (Σ (weighted peak area of LC) + Σ(weighted peak area of Fd[n]))/100		
Reconstructed peak of 0.06 µg total ADC	Drug Load	Percentage Peak Area (%)*	Weighted peak area (drug load x peak area%)
LC	0	32	0
LC + 1 drug	1	68	68
Fd^0	0	12.6	0
Fd^0 + 1 drugs	1	57.6	57.6
Fd^0 + 2 drugs	2	21.5	43
Fd^0 + 3 drugs	3	8.3	24.9
$DAR_{average}$ Fd			1.44
Average DAR			3.96

*Percentage peak are (%) represents the distribution of drug-loaded LC or HC, for example, LC + 1 drug accounts for 68% of the entire LC forms.

Summary

- The intact mass analysis of a cys-linked ADC as reduce and IdeS digested was successfully carried out, showing a linear dynamic range covering 3.5 orders with detection down to 0.001 µg (low ng levels).
- The drug antibody ratios were calculated to reveal an average DAR of 0.66 to each light chain and 1.5 to each heavy chain resulting in an average DAR of 4.2 for the complete ADC product.