A standardised LC-MS workflow for rapid DAR determination of thiol-conjugated ADCs across a variety of toxin linkers

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Introduction

Fast, reliable and generic methods are required for drug to antibody ratio (DAR) determination in an ADC development laboratory. In an effort to establish such a method, we have expanded on a previously developed "middle-up" workflow for determination of DAR by liquid chromatography-mass spectrometry (LC-MS). We demonstrate application of the workflow to a range of stochastically conjugated ADCs with DARs from 1 – 8, incorporating auristatin, α-amanitin, duocarmycin, PBD dimer and DX8951 payloads.

Methods

Fc and F(ab')2 subunits of antibody drug conjugates of two IgG_1 antibodies (trastuzumab and MAB013) were generated by digestion and deglycosylation with IdeS (FabRICATOR®, Genovis) and Endo S (Remove-iT, New England Biolabs) for 1 hour at 37°C. The samples were then reduced for 15 min at 37° C to give 2x Fc/2, 2x Fd and 2x Light Chain (LC) subunits. A 2 x 10mm desalting cartridge and 5 min reversed phase gradient served to concentrate, desalt and co-elute ADC subunits into the source of the mass spectrometer. The LC-MS system included an ExionLC™ AD UHPLC system with UV-vis detector, coupled to a SCIEX X500B QTOF mass spectrometer (AB Sciex™ Pte. Ltd.). Instrument control, data acquisition and deconvolution was performed with BioPharmaView 2.0 (AB Sciex™ Pte. Ltd.).

LC-MS "Middle-Up" Workflow

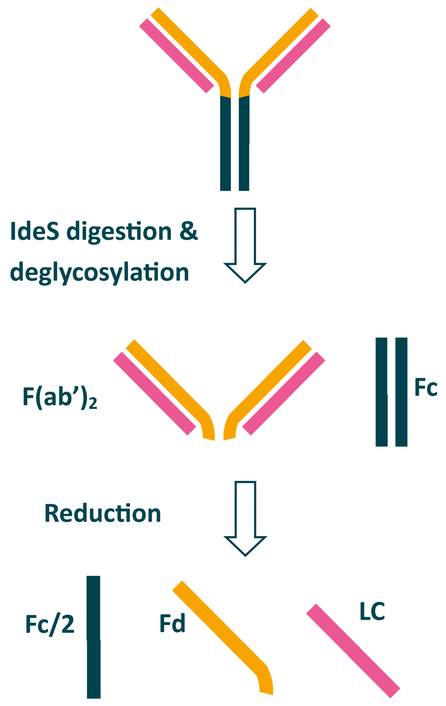


Figure 1: Schematic of the workflow used to generate ADC subunits for LC-MS analysis

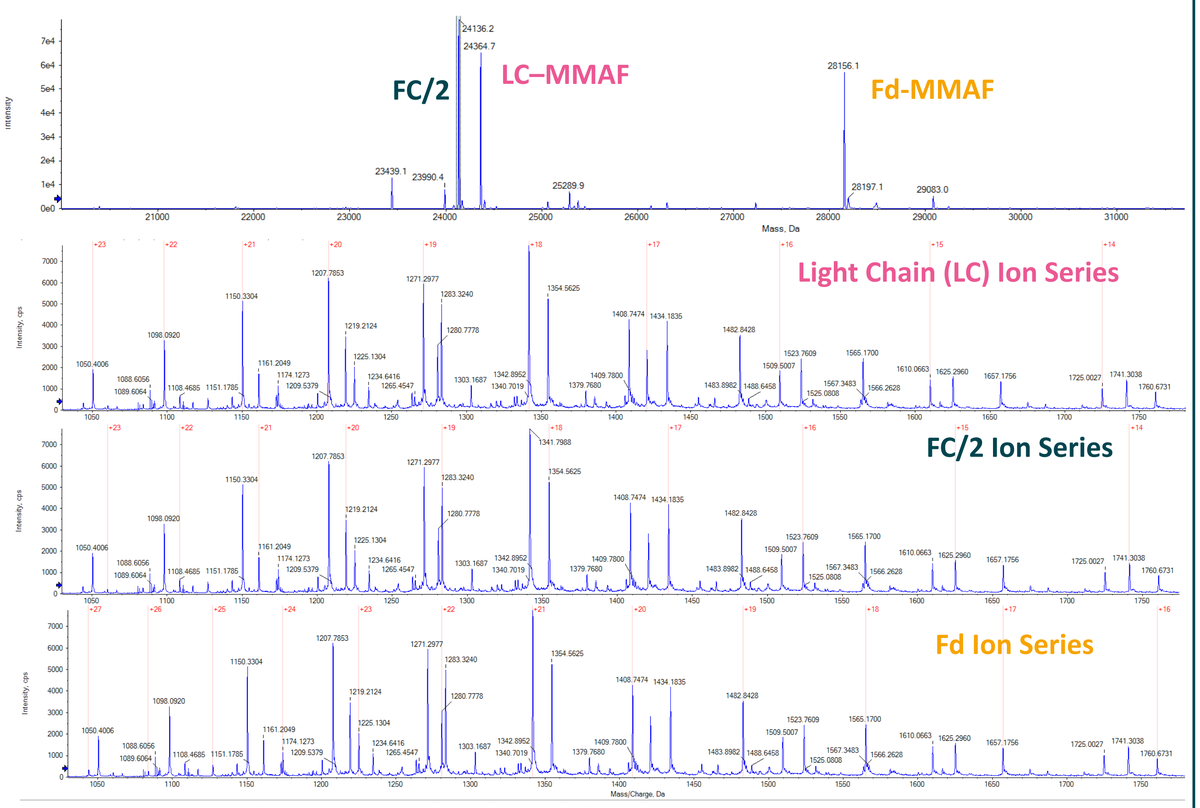


Figure 2: Ion series for the three subunits (LC, FC/2 and Fd) of a trastuzumab-MMAF DAR 7.7 conjugate

Auristatin and α-Amanitin ADCs

Two IgG1 antibodies (trastuzumab and MAB013) were partially reduced with TCEP, using seven different molar equivalents of reductant to antibody. Once fully conjugated, this process would yield a range of conjugates of both antibodies with seven different average drug-to-antibody ratios (DARs), from 1.0 to 8.0. For the conjugation process the reduced antibodies were divided into three aliquots with were conjugated with an excess of either MC-vc-PAB-MMAE, MC-MMAF or MC-vc-PAB-C_6-α-amanitin. DAR calculations were made for the vcMMAE conjugates with a well-developed HIC method. Using the same HIC method for MMAF or amanitin conjugate analysis gave poor resolution. All conjugates were analysed by LC-MS and the DAR calculated, with good correlation between the calculated DAR by HIC and the LC-MS DAR calculation for the ADCs across the DAR range, for each of the three payloads. Ions associated with MMAE and CO_2 loss (-762 Da) from ion-source fragmentation were observed.

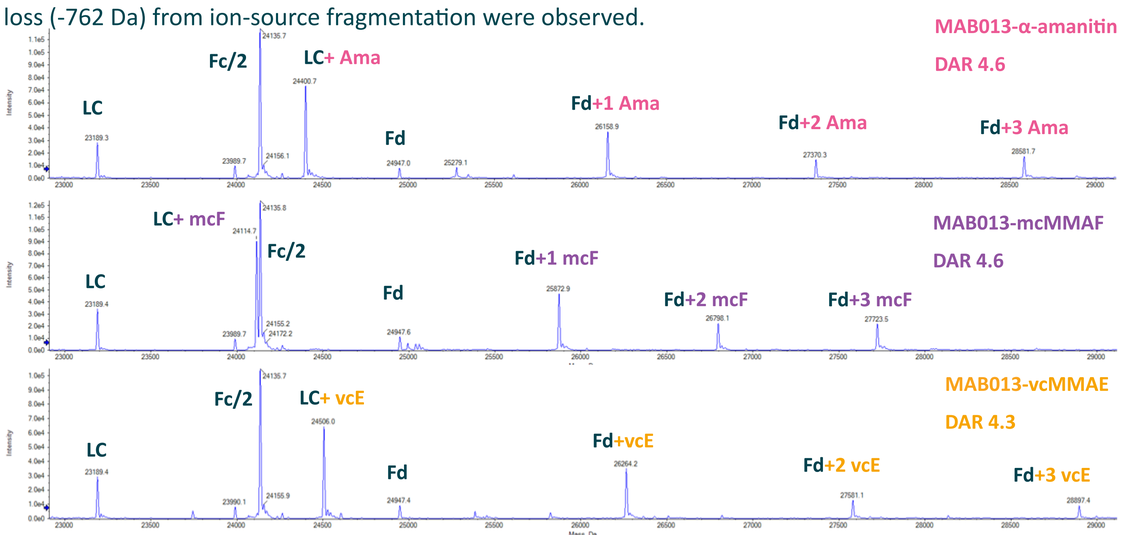
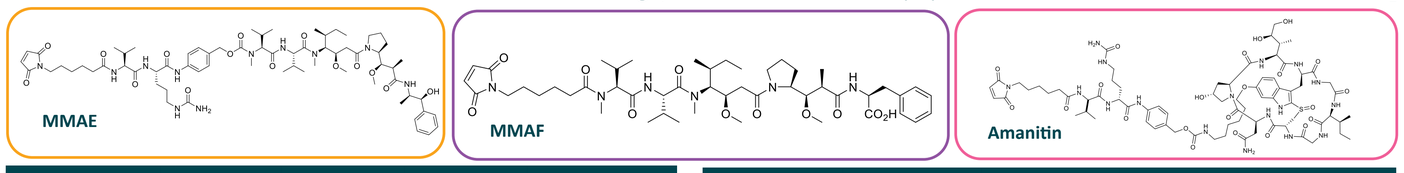


Figure 3: Middle-up deconvoluted MS data for MAB013 conjugates or amanitin, MMAF and MMAE



Antibody	MMAE DAR (HIC)	MMAE DAR (LC-MS)	MMAF DAR (LC-MS)	Amanitin DAR (LC-MS)
Trastuzumab	1.0	1.0	1.0	0.9
	2.0	1.8	2.1	1.8
	2.8	2.7	2.8	2.9
	3.7	3.4	3.9	3.7
	4.5	4.2	4.8	4.6
	6.0	6.0	6.3	6.1
	7.3	7.3	7.1	8.0

Table 1: Summary of DAR calculation results for trastuzumab conjugates of MMAE, MMAF and amanitin by LC-MS

Antibody	MMAE DAR (HIC)	MMAE DAR (LC-MS)	MMAF DAR (LC-MS)	Amanitin DAR (LC-MS)
MAB013	1.0	0.9	1.0	1.0
	2.1	2.0	1.9	2.0
	3.3	3.1	3.3	3.2
	4.4	4.3	4.6	4.6
	4.8	4.5	5.0	4.8
	6.9	6.6	6.9	6.9
	7.8	8.0	7.7	8.0

Table 2: Summary of DAR calculation results for MAB013 conjugates of MMAE, MMAF and amanitin by LC-MS

DX8951 ADCs

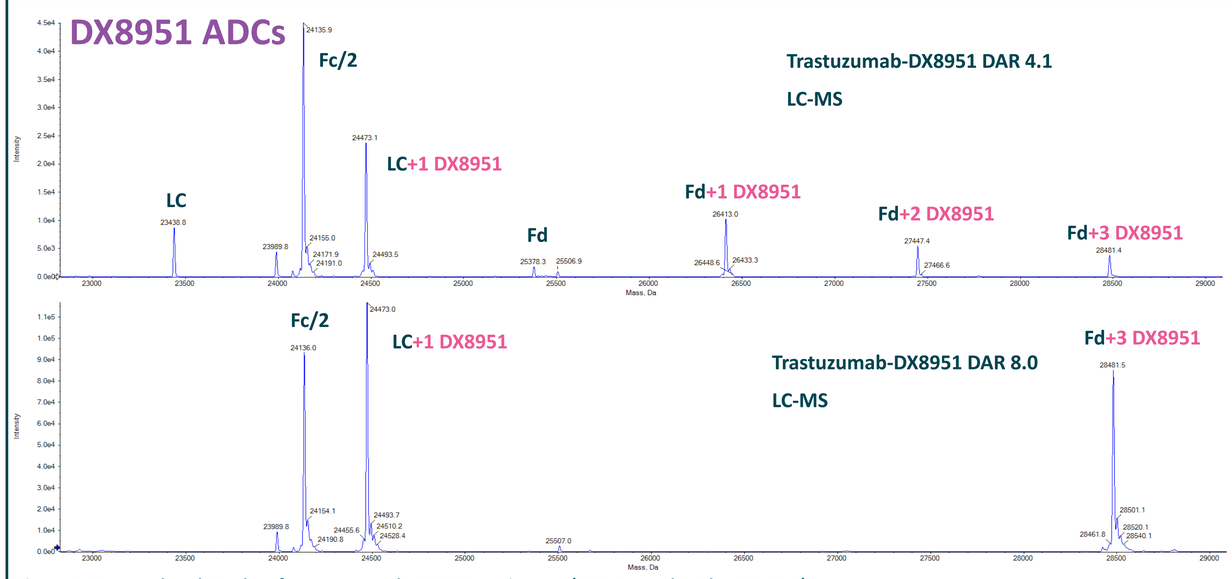


Figure 4: Deconvoluted MS data for trastuzumab-DX8951 conjugates (DAR 4.0 and 7.5 by RP-HPLC)

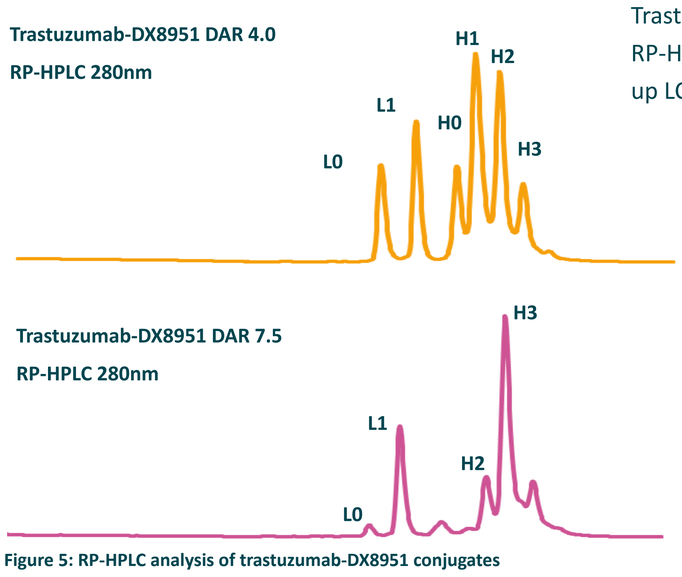
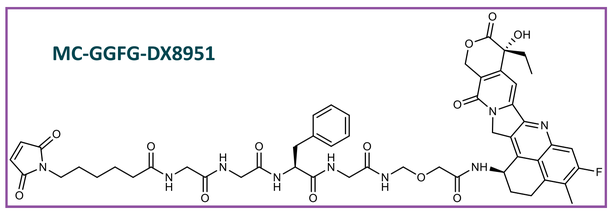


Figure 5: RP-HPLC analysis of trastuzumab-DX8951 conjugates

Trastuzumab conjugates of DX8951 can be well characterised by RP-HPLC and were also found to be compatible with the middle-up LC-MS DAR characterisation method at both DAR 4 and 8.

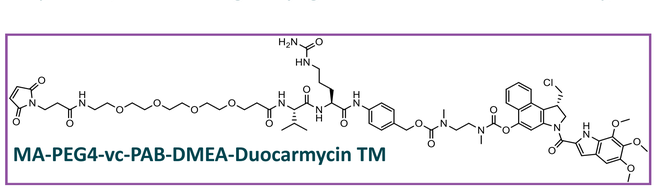


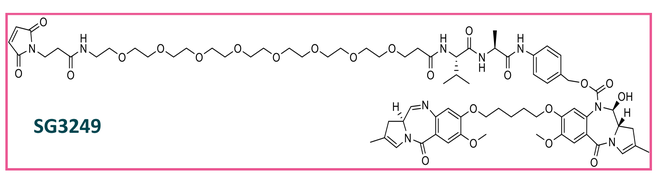
Antibody	Toxin-Linker	DAR (PLRP)	DAR (LC-MS)
Trastuzumab	MC-DX8591	4.0	4.1
		7.5	8.0

Table 3 DAR calculation summary for trastuzumab DX8951 conjugate LC-MS analysis

PBD and Duocarmycin ADCs

The method used for DAR by LC-MS analysis of auristatin, DX8951 and amanitin conjugates was applied to PBD (SG3249) and duocarmycin TM ADC at DAR 2.2—2.6 range. The LC-MS analysis of duocarmycin and PBDs conjugates within this DAR range were found to underestimate the DAR when compared to a RP-HPLC based characterisation. All conjugated Fd subunits for the duocarmycin conjugate were observed, but at lower relative intensities compared to the RP-HPLC analysis. The PBD ADCs gave poor recovery for conjugated Fd ions with >1 PBD per subunit, with no observed signal for Fd + 3 PBD. The poor Fd ion recovery may be part explained by signal dilution as a result of mass adducts; both expected, considering conjugate structure and chemistry, and unexpected as a result of sample preparation artefacts.





Antibody	Toxin-Linker	DAR (PLRP)	DAR (LC-MS)
Trastuzumab	Duocarmycin TM	2.2	2.0
	SG3249	2.3	2.0
	SG3249	2.6	2.1

Table 4: DAR calculation summary for PBD and duocarmycin conjugate LC-MS

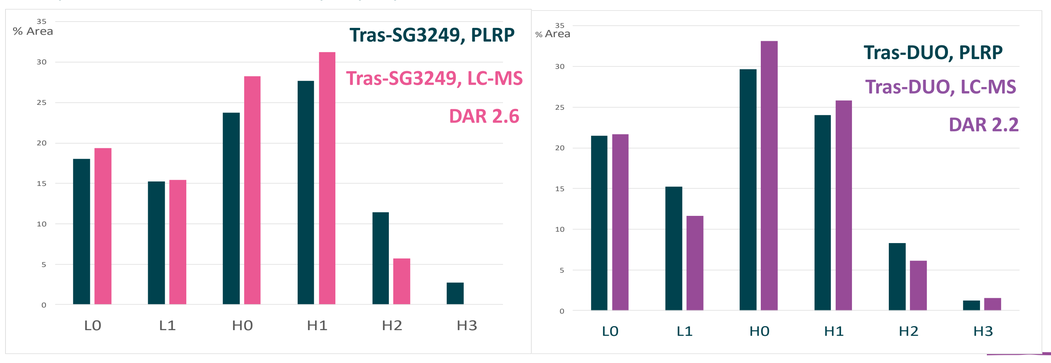


Figure 6: % Area of heavy and light chain species in Tras-SG3249 (left) and Tras-DUO (right) conjugate as analysed by PLRP and LC-MS

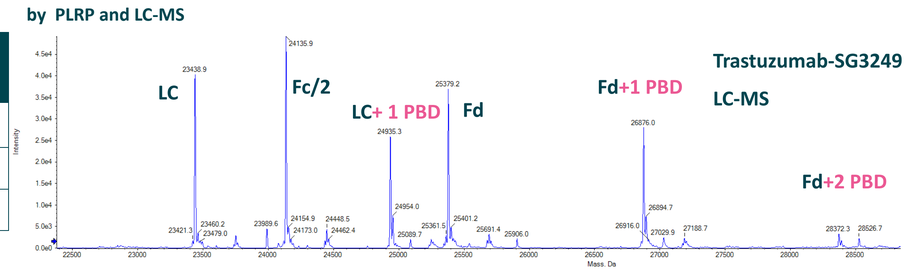
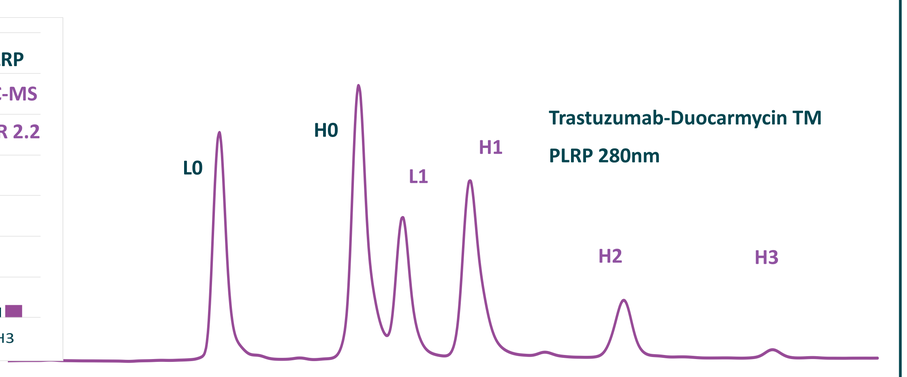


Figure 7: RP-HPLC and deconvoluted subunit MS data of trastuzumab-SG3249, DAR 2.6



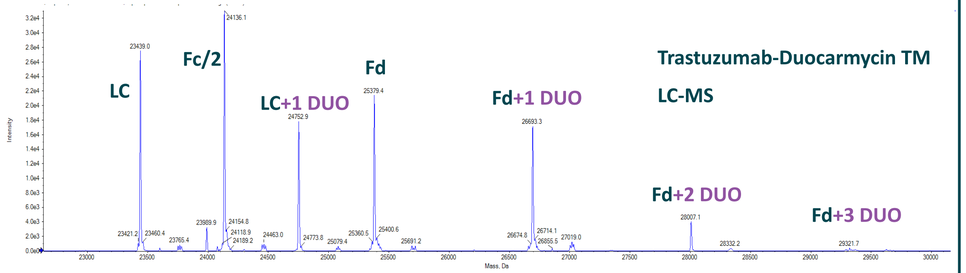


Figure 8: PLRP (above) and deconvoluted subunit MS data of trastuzumab-duocarmycin TM, DAR 2.2

Conclusion

The middle-up approach of LC-MS for DAR characterisation of ADCs has been shown to be applicable for the analysis of IgG_1 antibodies with a range of different payloads, including those hard to characterise by HPLC chromatography (eg. α-amanitin conjugates). The method demonstrated consistency for DAR determination of thiol-conjugated ADCs from DAR 1-8. ADCs with PBD and duocarmycin gave increasing challenges to LC-MS DAR determination. Alternative approaches to sample preparation, including enzymatic digestion strategies and further optimised ionisation strategies, could provide a route to PBD and duocarmycin conjugate analysis.

Acknowledgments: **Sibylle M. Heidelberger, Senior Application Support Specialist, Biologics, AB Sciex™ Ltd.**

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