Meeting the Challenges of Antibody Drug Conjugate Characterization by LC-MS/(MS)

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ABSTRACT

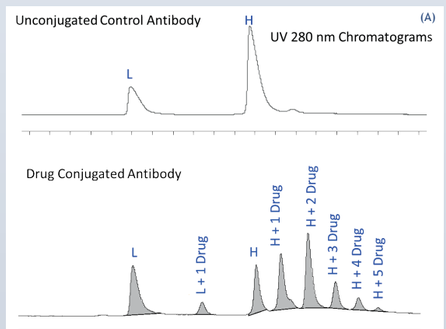
Antibody-drug conjugates (ADCs) are potent biopharmaceuticals comprised of a cytotoxic agent attached to an antibody or antibody fragment via a chemical linker. As complex three part (bio)molecules, ADCs require analytical considerations intersecting the sometimes disparate modes of small and large molecule investigations, and mass spectrometry plays a key role in their characterization. We describe LC-MS/(MS) approaches addressing various ADC attributes, including drug-antibody ratio (DAR) determination, mapping of drug conjugation sites, and accounting for various drug/linker chemistries including associated impurities. Selected MS-based methodologies are designed based on the class of ADC molecule, the conjugation chemistry, and any requirements for fine-scale structural understanding.

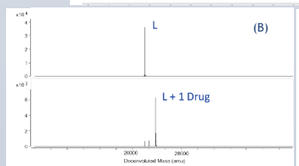
Analysis of ADCs with attachments through cysteine, lysine, or other sites may require significantly different strategies. The application of native vs. denaturing LC-MS, including instrument optimization in response to various linker chemistries, is discussed. For characterization of site-to-site drug conjugation, sequence-dependent selection of appropriate endopeptidases and application of appropriate MS/MS fragmentation approaches is considered. Impurity characterization is carried out by accurate mass and MS/MS analysis to a significant degree, and the limitations of MS-based methodologies are kept in mind with regard to the known challenges of small molecule structural characterization.

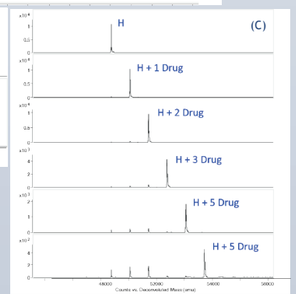
Results and Discussion

Characterization of Reduced Antibody Drug Conjugates with LC-MS

Figure 1. (A) UV 280 chromatograms of reduced unconjugated antibody and drug conjugated antibody separated on a hydrophobic interaction column (HIC) using an MS-friendly mobile phase. Deconvoluted mass spectra of (B) light and (C) heavy chains obtained with a high resolution mass QTOF spectrometer.







ADCs were deglycosylated, reduced, and the light and heavy chains, with varied degrees of lysine-conjugation, were well separated on a HIC column with the eluate analyzed by QTOF MS.

Drug-Antibody Ratio Determination via Intact LC-MS

Figure 2. DAR Analysis at the intact mass level under denaturing conditions: Acetonitrile /Water with TFA.

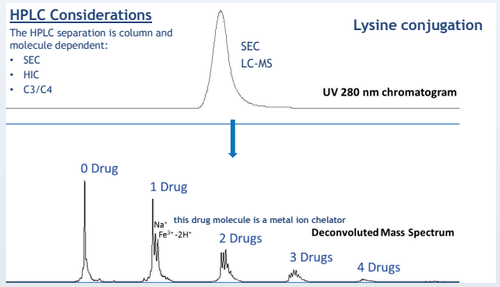
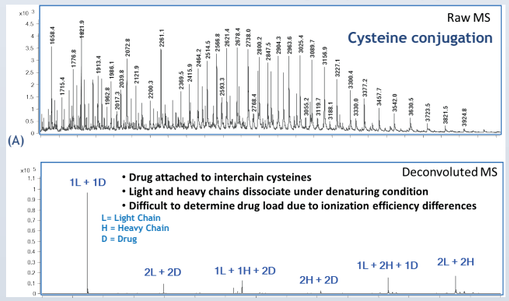
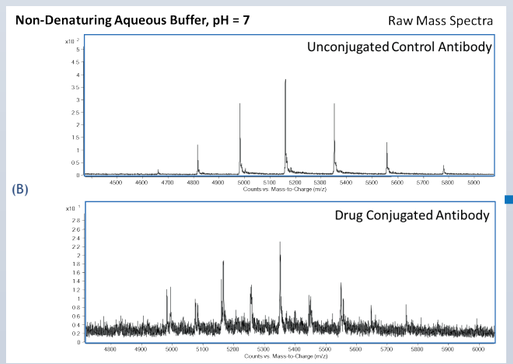


Figure 3. Analysis of Intact ADCs under denaturing vs. non-denaturing LC-MS conditions: (A) denaturing, (B) non-denaturing for cysteine conjugations.



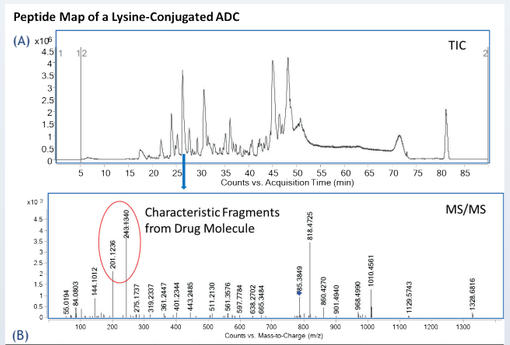


CONCLUSIONS

Mass Spectrometry is a key tool for ADC characterization, including DAR determination, drug linked site mapping and investigations of drug-linker chemistry including related impurities.

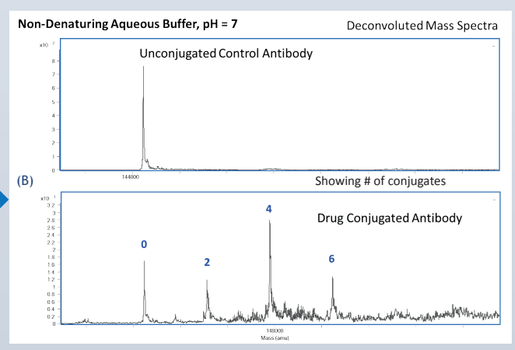
DAR and Conjugation Site Analysis via LC-MS Peptide Mapping

Figure 4. Endopeptidase digestion followed by QTOF LC-MS/MS.
(A) Identification of drug-linked peptides, (B) DAR tabulation.



(B)

Seq Location	Sequence	Theor. Mass (Da)	Obs. Mass (Da)	Delta ppm	RT (min)	Abundance	Observed Drug-Conjugations	Conjugated/ Unconjugated Ratio
1-27	XXXXXXXXXXXKKXX XXXXKXXXKXXXX	xxxx.yyyy	xxxx.yzzz	0.27	26.2	40911846	None	0.08
1-27	XXXXXXXXXXXKKXX XXXXKXXXKXXXX	…	…	-0.29	34.7	853617	1*Drug-conjugation	
1-27	XXXXXXXXXXXKKXX XXXXKXXXKXXXX			0.15	35.3	579500		
1-27	XXXXXXXXXXXKKXX XXXXKXXXKXXXX			0.28	36.0	1009113		
1-27	XXXXXXXXXXXKKXX XXXXKXXXKXXXX			-0.03	37.0	912150		
80-97	YKYYYYKYYYYYYY YY			0.52	27.6	7817319	None	0.18
80-97	YKYYYYKYYYYYYY YY			0.01	39.3	1409249	1*Drug-conjugation	
131-145	ZZZKZZZZZZZZZ			0.35	25.1	7653427	None	0.13
131-145	ZZZKZZZZZZZZZ			0.10	38.3	996768	1*Drug-conjugation	



ACKNOWLEDGMENTS

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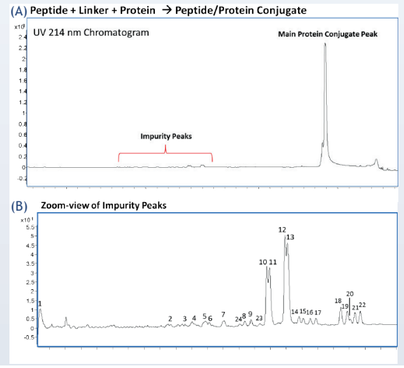
[1]: Currently at Alnylam Pharmaceuticals Inc.

Impurity Characterization

Figure 5. Characterization of impurities in a peptide/protein conjugate.
(A) Full-view C4 LC-MS chromatogram, (B) Zoom-in of impurities,
(C) Identification by accurate mass, (D) by MS/MS, and (E) isotope patterns.

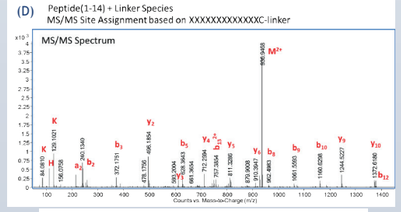


(C) **Peak Identification**

Species	Theoretical Monoisotopic Mass (Da)	Observed Monoisotopic Mass (Da)	Delta ppm	Retention Time (min)	Peak #	Peak Area	Peak Area (%)*
Peptide(1-4)	xxxx.xxxx	xxxx.yyyy	0.58	2.1	1	131	0.32
Peptide(5-14) + Linker	…	…	1.24	16.8 & 17.0	10 & 11	609	1.51
Peptide(5-14) + Linker			-1.17	15.1	24	29	0.07
Peptide(1-14) + Linker			1.02	17.9 & 18.1	12 & 13	890	2.21
Peptide(1-14) + Linker			0.21	16.4	23	10	0.02
Peptide(5-14) + Linker - H_2O			0.89	13.1 & 13.4	6	30	0.07
Peptide(5-14) + Linker - H_2O			0.07	15.3 & 15.8	8 & 9	97	0.24
Peptide(5-14) + Linker - H_2O			0.52	21.5 & 21.9	18 & 19	177	0.44
Peptide(1-14) + Linker - H_2O			0.70	18.8 & 19.1	14 & 15	88	0.22
Peptide(1-14) + Linker - H_2O			-1.57	19.5 & 19.9	16 & 17	98	0.24
Peptide(1-14) + Linker - H_2O			-0.65	22.4 & 22.7	21 & 22	178	0.44
Peptide(5-14) + Linker + Sugar - H_2O			1.30	12.9	5	65	0.16
Peptide(1-14) + Linker + Sugar - H_2O			0.59	14.1	7	55	0.14
Peptide(5-14) + Linker + H_2O			-1.01	10.7	2	11	0.03
Peptide(5-14) + Linker + H_2O			0.65	11.6	3	10	0.02
Peptide(1-14) + Linker + H_2O			0.95	12.2	4	20	0.05
Peptide(1-14) + Linker + H_2O			0.53	13.1	6	30	0.07
Peptide(1-14) + Linker + Peptide(1-4) - H_2O			-0.89	22.1	20	30	0.07

* % relative to the main protein peak.

(D) Peptide(1-14) + Linker Species
MS/MS Site Assignment based on XXXXXXXXXXXXC-linker



(E) Molecular formula confirmation by isotope pattern matching