Application of a MS in QC method for characterization and attribute monitoring in Antibody-Drug Conjugates

Michael A. Blank, Stephane Houel, Aaron O. Bailey, and Jonathan Josephs; Thermo Fisher Scientific, San Jose, CA, 95134

ABSTRACT

Determination of the lysine drug occupancy is a critical part of process evaluation and lot release for antibody drug conjugates. Using the multi-attribute method as a high confidence lot release strategy the relative abundances and amount of conjugation at each lysine site was performed in a routine and Part 11 compliant fashion for an ADC using a Trypsin and GluC digest. Data were acquired at ultra-high resolution and with the further ability to add additional sites to be monitored as needed without reacquisition. To increase confidence, a sophisticated scoring metric was applied to the processing method. Mass accuracies of less than 10 ppm without external calibration or dopants combined with isotopic correlation evaluations made for confident assignments of peptide identify. Finally, the use of high resolution (120k @ 200m/s) did not compromise cycle time, with more than 20 points across each peak

INTRODUCTION

The multi-attribute method is a novel lot release strategy that leverages another dimension of separation, namely high-resolution accurate mass spectrometry, to resolve and directly monitor critical quality attributes of biologic drug products. In simultaneously replacing numerous conventional lot release and characterization protocols (e.g. CEX, HILIC, etc. with UV spectroscopy) this GMP-compliant method provides a broad and highly specific knowledge of a biologic drug product such as a monoclonal antibody and can even be extended to highly modified macromolecule products such as lysine linked antibody drug conjugates (ADC).

MATERIALS AND METHODS

A lysine linked ADC was prepared both by a tryptic SMART digest following the manufacturers recommendations or according to a previously published optimized digestion protocol. Briefly, the sample was diluted to 1 mg/mL using 7M Guanidine HCl and 100 mM Tris then reduced and alkylated using 500 mM DTT and 500 mM IAA respectively. The alkylation was quenched and the sample was buffer exchanged into 50 mM Tris using a size exclusion spin column. This was followed by digestion for >4 hours at 37 C with 1:10 GluC.

Peptides were separated and eluted using a Thermo Scientific™ Vanquish™ Horizon uHPLC system and a Thermo Scientific™ Accucore™ 1.5 um particle 2.1 x 150 mm column before being electrospray ionized and analyzed by a Thermo Scientific™ Q Exactive™ HF or Q Exactive™ Plus all mass spectrometer under the control of the Thermo Scientific™ Chromeleon™ 7.2 data system. Approximately 3 ug was loaded on column. A linear gradient from 10% to 35% B over 70 minutes was employed, where mobile phase A comprised 0.1% formic acid in water and mobile phase B comprised 0.1% formic acid in acetonitrile. A second gradient from 10% to 45% over 8 minutes was performed to blank the column between injections.

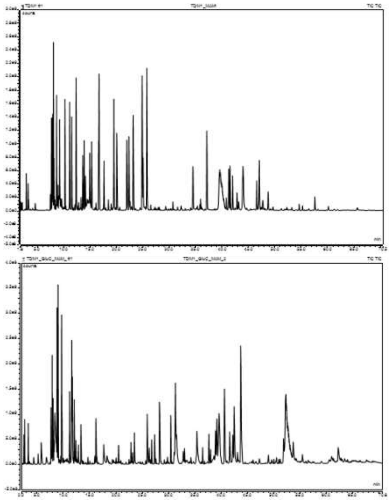
Two Chromeleon instrument methods were developed. The first used a Top 5 ddMS2 acquisition scheme with resolutions of 120k and 30k and respectively for MS1 and MS2 scans. The settings for the Q Exactive Plus were similar using 140k and 17.5k resolution. This was employed to build a peptide map and determine critical quality attributes. The data were processed using Thermo Scientific™ BioPharma Finder™ 2.0 software . These results were exported directly to Chromeleon and a second MS1-only method was used to monitor and quantify key peptide sequences of import (e.g. those containing conjugated lysine residues). An automated processing, scoring, and reporting method was configured to enable confident routine analysis and the detection of relative abundance of lysine conjugation with the drug under the two digestion conditions.

RESULTS

Following quantitative analysis of the trypsin digest it became apparent that some regions of the primary sequence would be inaccessible as the peptides would be far too short (e.g. three amino acid residues) to confidently assign and use for comparison against the modified sequence. Additionally, similar to the challenges inherent to the relative quantitation of lysine glycation, the primary sequence of the peptide modified by the linker, drug compound, or both would differ from the unmodified sequence due the presence of a missed cleavage at the conjugated lysine residue. While not strictly necessary as part of a lot release strategy as relative abundances would be measured in exactly the same way between lots, a GluC digest was performed to provide orthogonal peptide coverage and assess the effect of differing ionization efficiencies by quantifying the conjugated peptide versus the same unmodified sequence (Figure 1).

The use of GluC generally resulted in much larger peptides given the placement of glutamic and aspartic acid residues, but the use of the 140,000 resolution setting proved sufficient resolving power for on-the-fly determination of charge state and base line separation of isotopes meaning even very large peptides could be assigned. As a result, 100% sequence coverage on both the heavy chain and light chain of the NISTmAb was obtained allowing for the detection and quantification of a set of lysine residues complimentary to the results of the tryptic digest. Indeed many peptides inaccessible to quantitation by trypsin digest were able to be quantified only in the GluC digest and vise versa.

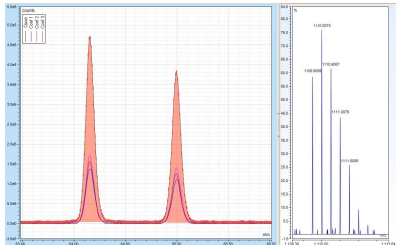
Figure 1: Trypic and GluC Digest Total Ion Chromatograms



A clear disparity in the Total Ion Chromatogram (TIC) is observable between the tryptic digest (top) and the GluC digest (bottom). Given the placement of glutamic and aspartic acid residues in the sequence some very large peptides can be observed to elute late in the GluC MS chromatogram. Total intensities were similar at 3.0e9 and 3.5e9 respectively. Drug conjugated peptides can be seen to elute as a second cluster of peaks late in the run.

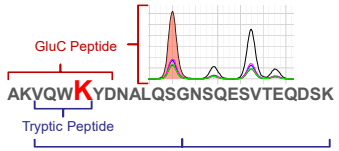
One major advantage to using the GluC digest was that the same peptide sequence was conserved between the modified and unmodified peptide, simplifying data processing and analysis as well as limiting the effect of differences in ionization efficiency or varied charge states. Drug conjugated peptides typically displayed a doublet peak as well as a chlorine containing isotopic envelope for both digests (Figure 2). In some cases, the presence of two lysine residues on the same GluC peptide both of which were drug conjugated resulted in a "doublet of doublets" as expected. One such example can be seen in Figure 3.

Figure 2. Extracted Ion Chromatogram and +2 charge state isotopic envelope for the GluC peptide NNYKTTPPVLD with drug payload



A characteristic double peak is seen for the drug conjugated peptide., Even on the Q Exactive Plus at the 140,000 resolution setting, an excess of 25 data points (red ticks) were acquired across each ≈15 second peak. The presence of a chlorine atom is evident from the isotopic profile (note the high number of isotopes for +2). The use of high resolution enabled clear distinction between the isotopes and interfering ions.

Figure 3. Digestion scheme and relative abundance for the overlapped peptides containing a modified LC lysine-148 from both a tryptic and GluC digest



GluC

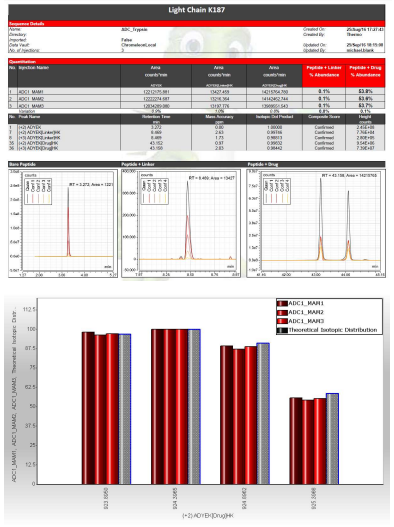
Peak Name	Ret.Time	Peak Area			
	min	counts*min			
First Injection	ADC1_Gluc ADC1_GI	ADC1_Gluc ADC1_GluC_MAM_2	ADC1_GluC_MAM_3	ADC1_GluC_MAM_4	
MS Quantitation Peak	MS Quantit	MS Quant	MS Quantit	MS Quantitation Peak	MS Quantitation Peak	MS Quantitation Peak
AKVQWKVD	8.786	8.839	8.863	1299446802.934	1280725552.731	59102163.555
AKVQWK[Drug]VD	56.202	56.254	56.299	1074236.575	1022164.343	442051.726
AKVQWK[Drug]VD_2	56.893	56.944	56.987	775596.578	733392.969	313714.656
				1317944.55.1	1296281192	59859119.37
		Drug		1.4%	1.4%	1.3%

Trypsin

Peak Name	Ret.Time	Peak Area			
	min	counts*min			
First Injection	ADC1_MA	ADC1_MA	ADC1_MA	ADC1_MAM1	ADC1_MAM2	ADC1_MAM3
MS Quantitation Peak	MS Quantit	MS Quantit	MS Quantit	MS Quantitation Peak	MS Quantitation Peak	MS Quantitation Peak
VQWK	8.691	8.686	8.893	5309326.644	5948539.730	4958069.131
VQWKVDNALQSGNSQESVTEQDSK	16.704	16.726	16.763	43120.214	47864.782	35519.444
AQWK[Drug]VDNALQSGNSQESVTEQDSK	56.868	56.891	56.936	73616.677	71263.095	62981.684
				5423257.535	5164147.607	5056441.059
		Drug		1.4%	1.4%	1.2%

In this case, the quantitation of relative abundance of drug payload at LC lysine-148 (light chain) using both trypsin and GluC were highly similar, although complicated by a complex doublet pattern for the GluC digest or varying charge states for the trypsin digest.

Figure 4. Customized Chromeleon quantitation report for light chain lysine-187 by proxy of the ADYEK and ADYEKHK peptides



This customized report template shows the occupancy of both the drug and dead linker as percent relative abundance compared to the total intensity of all forms of this peptide. Interpretation of the results is further complicated by the fact that for this peptide, significant missed cleavage abundance only results from the presence of either the linker or the drug, necessitating the comparison between the tryptic peptide and a missed cleavage peptide. The use of isotopic correlation was beneficial to the scoring algorithm given the unique isotopic envelope of the chlorine containing drug.

Very low statistical variance was observed between technical replicates for both the GluC and tryptic digests indicating a high degree of systematic reproducibility in the measurement and LCMS system. Confidence in the determined site occupancies was further bolstered by good mass accuracies of less than 10 ppm and the correlation between the observed and theoretical isotopic ratios, which are especially diagnostic in the case of this ADC whose drug payload contains a chlorine atom. A composite scoring model was made to take both of these and numerous other factors into account. Customized extraction windows enabled the both peaks of the characteristic doublet to be automatically integrated.

In most cases the abundance of the unmodified missed cleavage peptide could be ignored as the area was several orders of magnitude below the specific cleavage peptide and equal to or less than the amount of peptide with linker-only. For some peptides almost no dead linker was observed, while for others an unconjugated and linker only variant were detected but a drug conjugated form of the peptide was not (data not shown). By far the majority of drug conjugated sites quantified were unique to a particular digest, further indicating the importance of using two enzymes.

Table 1. Relative abundance values for linker and drug payloads at several lysine residues

Residue	Linker	Drug	Stand. Dev.
HC K291	0.02%	0.5%	0.05%
HC K320	0.02%	0.1%	0.00%
HC K337	ND	0.4%	0.05%
HC K395	0.01%	1.2%	0.20%
HC K64	0.10%	4.3%	0.00%
LC K148	ND	1.4%	0.06%
LC K187	0.10%	53.6%	0.10%
LC K206	ND	17.6%	0.30%

CONCLUSIONS

Overall, the use of two digestion methods allowed quantitation of potential conjugation sites inaccessible to a single method and even showed good agreement for at least one overlapped site.

While there can be some argument about comparing peptides of different sequence and charge state, particularly with the tryptic digest, nevertheless this is still an appropriate approach as the basic tenet of the multi attribute method is comparison to a reference standard-so long as the quantitation is performed in the same way.

The ability to score based on isotopic dot product was tremendously advantageous here given the unique elemental composition of the drug.

High resolution was also of great value given the complex nature of the mixture. Despite the use of the high resolution setting, even on the slower of the two instruments, more than 25 data points across the peak were collected contributing in no small part to sensitive detection of low abundant species and the high reproducibility between measurements.

Finally, a tremendous variability in drug conjugation was seen between lysine residues. Some were almost completely bare while others showed orders of magnitude greater abundance. One must be wary comparing peptides to peptides, but *prima facie*, the light chain lysine residues were among the most conjugated.

REFERENCES

1. R. Rogers, et al. *MAbs* 2015, 7, 881-890

TRADEMARKS/LICENSING