Orthogonal techniques for LCMS characterization of a lysine-conjugated ADC

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SCAN FOR POSTER

Introduction

Lysine-based conjugation is a widely used non-specific conjugation strategy for the development of Antibody Drug Conjugates (ADCs). Conjugation at lysine residues results in highly heterogeneous products that pose challenges to current characterization methods. These challenges include:

Accurate drug to antibody ratio (DAR) calculation.

Site specific localization.

Quantification of site occupancy.

The last two are quality attributes which often need to be determined bybottom-up MS approaches and typically use lysine specific proteases (trypsin or LysC). As lysine conjugation results in variable modification, the use of such proteases results in complex mixtures of peptides which can be challenging when quantifying site occupancy.

Here we present an analytical strategy utilizing the arginine specific protease RgpB (GingisREX®, Genovis AB).

Intact reconstruction from peptide mapping data was used to determine the level of correlation between the orthogonal techniques of intact mass and peptide mapping.

Methods

DAR determination at the intact level

Trastuzumab emtansine was deglycosylated in an automated fashion using PNGaseF immobilized on magnetic beads for 30 min at 37°C in PBS. The resulting samples were analyzed at the intact level by reversed-phase LC-MS on a Waters™ BioAccord™ system equipped with a Waters™ BioResolve™ RP mAb column (2.1 x 50 mm).

Trypsin digestion of ADC samples

Samples were reduced and denatured in 5 M Gnd-HCl, 3 mM DTT for 30 min at RT, followed by alkylation with 7 mM IAA for 20 min. IAA was quenched by addition of 14 mM DTT and the solution was diluted to 0.6 M Gnd-HCl with Rapizyme Trypsin digestion buffer (Waters). RapiZyme Trypsin (Waters) was added at a ratio of 1:5 followed by digestion for 2 h at 37°C. The reaction was stopped by addition of 0.1% acetic acid. The resulting peptides were separated on a Waters™ ACQUITY Premier CSH C18 column (2.1 x 150 mm) and analyzed by MS/MS on a Bruker Impact II QTOF mass spectrometer.

Arg-specific digestion of ADC samples using RgpB

Samples were reduced and denatured in 7.9 M urea, 5 mM DTT for 30 min at 30°C, followed by alkylation with 10 mM IAA for 30 min. IAA was quenched by addition of 11 mM DTT and the concentration was adjusted to 1 mg/ml antibody and 6 M urea. RgpB was added at a ratio of 1:50 followed by digestion for 4 h at 30°C. The reaction was stopped by addition of 1% formic acid. The resulting peptides were separated on a Waters™ BioResolve™ RP mAb column (2.1 x 150 mm) and analyzed by MS/MS on a Bruker Impact II QTOF mass spectrometer.

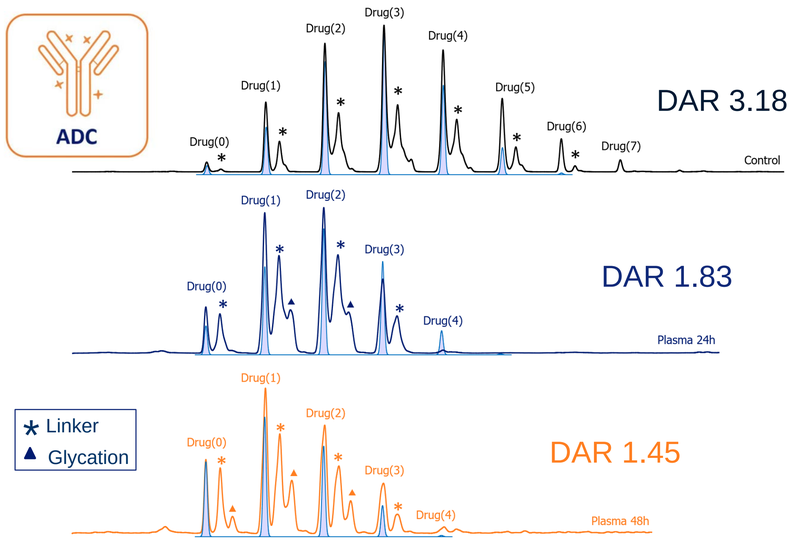
In vitro plasma stability assay

Trastuzumab emtansine was incubated for 24-48 hours at 37°C in bovine plasma at an ADC concentration of 0.2 mg/ml. The ADC was isolated by affinity capture and prepared for MS analysis according to the protocols above.

Data Processing

Raw data files from intact and peptide analyses were processed in Protein Metrics Byos® (vendor neutral software), using the ADC and PTM workflows respectively.

Intact Analysis and DAR Calculation



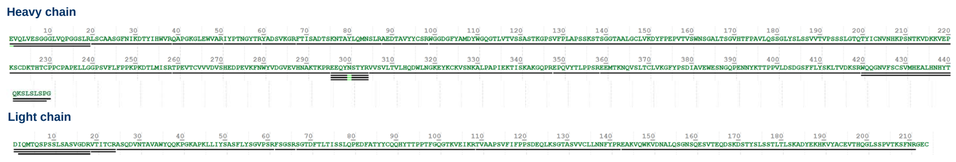
Deglycosylated trastuzumab emtansine raw data files were processed using the ADC workflow in Byos. This workflow was used to perform intact deconvolution and mass matching. DAR calculations were automatically generated in the report.

A decrease in DAR was observed following incubation in plasma.

Shaded blue areas show reconstruction of the intact data from peptide mapping data.

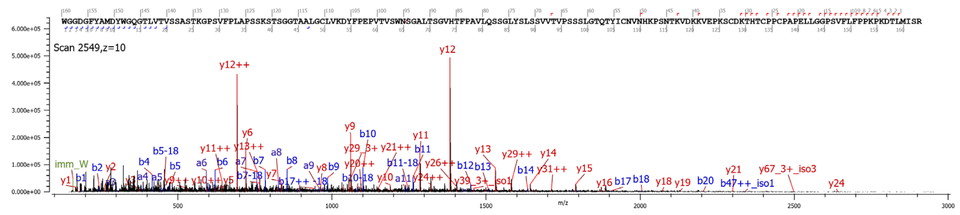
Cleavage specificity

In order to assess the specificity of RgpB as well as the ability to accurately detect and identify the digestion products, a control digest was performed using unconjugated trastuzumab.

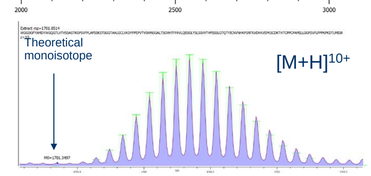


Full coverage was observed from arginine terminating peptides only and no cleavage at lysine was observed, showing high specificity.

The PTM workflow in Byos was used to identify peptides. This workflow utilizes the Byonic™ search engine to identify peptide based on MS2 spectra. All arginine terminating peptides were confidently identified based on MS2 fragments.

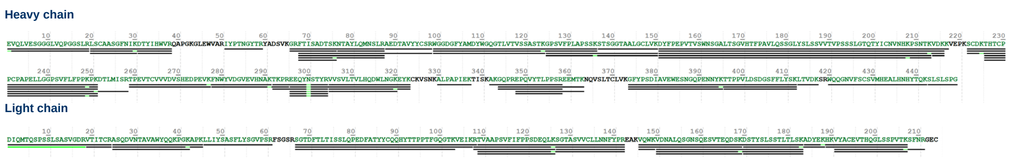


The largest peptide was identified based on the annotated MS2 spectra shown above. This identification was further validated by the MS1 isotope plot. Green bars show the theoretical (averagine based) isotope distribution.



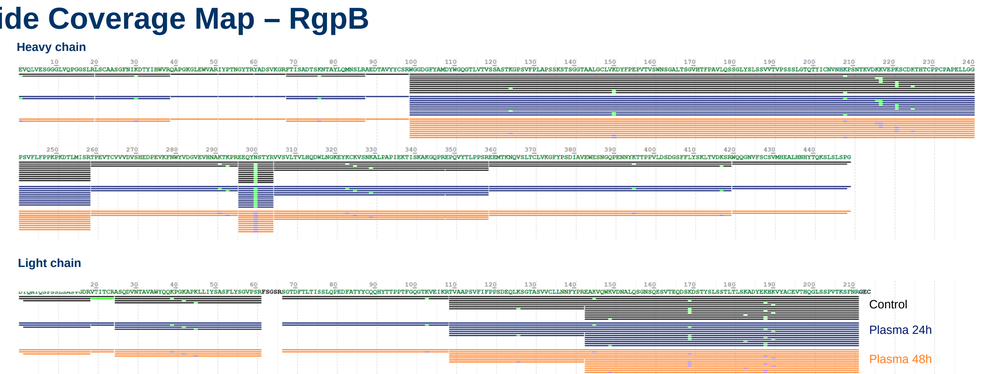
Comparison of tryptic and RgpB digestion

Peptide Coverage Map – Tryptic digest



Tryptic digestion resulted in incomplete coverage of lysine containing peptides. Furthermore, since conjugation prevents cleavage at lysine, this creates an issue for traditional peptide quantification methods, as the corresponding unconjugated or differentially conjugated peptides will not be present.

Peptide Coverage Map – RgpB



The RgpB digest yielded complete coverage of lysine containing peptides over all three samples. Moreover, the digestion profile was simplified, as different peptidoforms are present consisting of unconjugated, conjugated with one drug (at multiple sites) and conjugated with two drug molecules.

Comparison of relative peptide conjugation levels

The table shows a comparison of normalised XIC areas of conjugated (not site specific) and unconjugated peptides, between control and incubated plasma samples. For reporting purposes percentages of unconjugated peptides are hidden.

			MS Alias name ←	Control (%)	Plasma 24h Plasma D1 (%)	Plasma 48h Plasma D2 (%)
Sequence (unformatted)	{Missed Cleavage?} ↑	Mod. Names				
ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR	No	Drug/956.3100		5.07	2.37	1.32
EAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR	No	Drug/956.3100		48.1	26.8	14.9
		Drug/956.3100; Drug/956.3100		4.46	1.56	0.456
EEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR	No	Drug/956.3100		24	16.2	9.31
FTISADTSKNTAYLQMNSLR	No	Drug/956.3100		1.86	0.963	0.542
LSCAASGFNIKDTYIHWVR	No	Drug/956.3100		4.6	2.44	1.15
SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR	No	Drug/956.3100		3.15	1.78	1.02
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR	No	Drug/956.3100		4.3	5.05	3.46
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	No	Drug/956.3100		3.91	2.38	1.12
TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNR	Yes	Drug/956.3100		49.7	35.5	20.5
VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSR	Yes	Drug/956.3100		9.69	6.11	3.17
WGGDGFYAMDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR	No	Drug/956.3100		34.9	27.4	15.5
		Drug/956.3100; Drug/956.3100		1.85	1.33	0.892

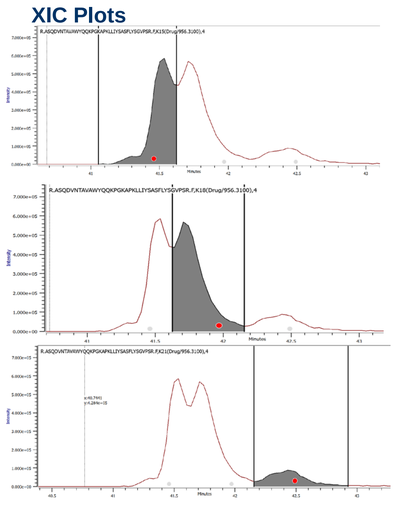
This quantitative information was used in the intact ADC workflow to perform a reconstruction of the intact data. In this process, conjugations at the amino acid level are convolved against each other to reconstruct a theoretical Intact mass spectrum. Comparing the theoretically reconstructed Intact mass spectra against the experimental spectra provides a way to compare peptide mapping data against Intact mass spectrometric analysis. The differences between the two spectra can then be used to compare how well each method correlates with the other.

Site occupancy ratio quantification

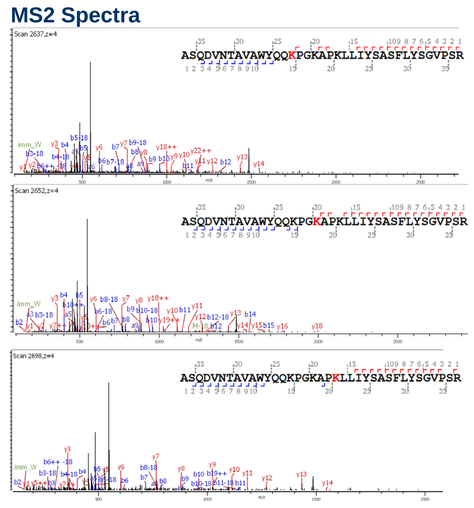
Peptide **R3** on the light chain of the antibody:

Row#	MS Alias name	Sequence	Score	Mod. Summary	Delta Mod. Score
1	Control; Plasma 24h; Plasma 48h	R.ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR.F	1389.20		206.9 - 1389.2
1.1	Control	R.ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR.F	1207.96		206.9 - 1208.0
1.2	Plasma 24h	R.ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR.F	1389.20		333.7 - 1389.2
1.3	Plasma 48h	R.ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR.F	1324.50		325.1 - 1324.5
2	Control; Plasma 24h; Plasma 48h	R.ASQDVNTAVAWYQQKPGKApKLLIYSASFLYSGVPSR.F	560.00	K21(Drug/956.3100)	1.9 - 49.1
2.1	Control	R.ASQDVNTAVAWYQQKPGKApKLLIYSASFLYSGVPSR.F	560.00	K21(Drug/956.3100)	32.8
2.2	Plasma 24h	R.ASQDVNTAVAWYQQKPGKApKLLIYSASFLYSGVPSR.F	491.10	K21(Drug/956.3100)	49.1
2.3	Plasma 48h	R.ASQDVNTAVAWYQQKPGKApKLLIYSASFLYSGVPSR.F	362.54	K21(Drug/956.3100)	1.9
3	Control; Plasma 24h; Plasma 48h	R.ASQDVNTAVAWYQQKPGiAPKLLIYSASFLYSGVPSR.F	656.68	K18(Drug/956.3100)	1.2 - 26.2
3.1	Control	R.ASQDVNTAVAWYQQKPGiAPKLLIYSASFLYSGVPSR.F	639.65	K18(Drug/956.3100)	1.2
3.2	Plasma 24h	R.ASQDVNTAVAWYQQKPGiAPKLLIYSASFLYSGVPSR.F	656.68	K18(Drug/956.3100)	26.2
3.3	Plasma 48h	R.ASQDVNTAVAWYQQKPGiAPKLLIYSASFLYSGVPSR.F		K18(Drug/956.3100)	
4	Control; Plasma 24h; Plasma 48h	R.ASQDVNTAVAWYQQiPGKAPKLLIYSASFLYSGVPSR.F	717.05	K15(Drug/956.3100)	3.9 - 50.4
4.1	Control	R.ASQDVNTAVAWYQQiPGKAPKLLIYSASFLYSGVPSR.F	690.72	K15(Drug/956.3100)	3.9 - 25.2
4.2	Plasma 24h	R.ASQDVNTAVAWYQQiPGKAPKLLIYSASFLYSGVPSR.F	717.05	K15(Drug/956.3100)	37.0 - 37.0
4.3	Plasma 48h	R.ASQDVNTAVAWYQQiPGKAPKLLIYSASFLYSGVPSR.F	674.29	K15(Drug/956.3100)	50.4 - 50.4

XIC Plots



MS2 Spectra



Some peptides contain multiple lysines (i.e., potential conjugation sites), however in several peptides, Byonic allowed accurate determination of the conjugation sites. In this example, three peptidoforms were confidently identified and accurately quantified.

The Delta Mod. Score confirms the localization of the modification on the determined site. The score is calculated by the presence of MS2 fragment ions between two possible sites. The table shows the relative amounts of each peptidoform based on normalized XIC area.

			MS Alias name ←	Control (%)	Plasma 24h Plasma D1 (%)	Plasma 48h Plasma D2 (%)
Sequence (unformatted)	Mod. Names	Var. Pos. Peptide ↑				
ASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSR	Drug/956.3100	15		2.4	1.16	0.599
		18		2.17	0.932	0.554
		21		0.499	0.279	0.164
			Level 1	**5.07**	**2.37**	**1.32**
			Totals			

Conclusions

The work here demonstrates two orthogonal techniques for the quantification of lysine conjugation on trastuzumab emtansine.

Digestion of the ADC using the arginine specific protease RgpB resulted in a simpler digestion profile that facilitated clearer determination of peptide quantification and, in some cases, site occupancy ratio determination.

One challenge in analytical chemistry is the need to compare orthogonal methods in a systematic fashion. Intact reconstruction visually confirms that the quantitative data obtained from peptide mapping correlates to the intact analysis.

Conflict of interest statement

Some of the authors are employees and/or shareholders of Genovis which has commercialized the enzyme described here.