LC/MS HILIC Analysis for Glycosylation and Deamidation of Peptides and mAbs

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Outline

- N-linked Glycopeptides are better resolved using HILIC compared to reversed phase HPLC
- Analysis of synthetic peptides and protein digests with trypsin fragments is readily accomplished using Hydrophilic Interaction Liquid Chromatography (HILIC), exhibiting resolution and efficiencies that are improved over reversed phase HPLC.
- A mixture of synthetic peptides representing a site of IgG that is known to be prone to asparagine deamidation, and subsequently to form a mixture of aspartate and iso-aspartate peptides, has been examined by HILIC LC/MS, as have asparagine and aspartate containing proteolytic fragments derived from a humanized monoclonal IgG1 biotherapeutic.
- A variety of deamidation and iso-aspartate sensitive sites are detected in peptide fragments from this IgG, and several show significant changes in relative amounts when the protein is stressed for 7 days by incubation at pH 9, at a temperature of 37°C.

Introduction

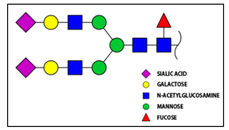
Post-translational modifications (PTMs) include biochemical changes to the protein, such as changes to the amino acid side chain functional groups, the N-termini, and peptide hydrolysis. Many PTMs are polar by nature and lend themselves to improved analysis via hydrophilic interaction liquid chromatography (HILIC) because these modified peptide and protein forms are often better retained and resolved compared to reversed-phase (RP). PTMs must be characterized and controlled for effective and safe biotherapeutic development, such as monoclonal antibody treatments. As additional mAb biotherapeutics and biosimilars enter development, the focus on PTM characterization is expanding and can lead to better understanding their impact on biotherapeutics. Here we specifically investigate glycosylation and deamidation modifications.

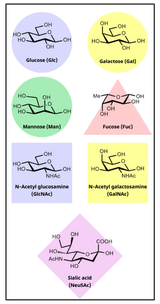
Materials and Methods

mAb IgGs and standard proteins were commercially obtained and dissolved at 1-2 mg/mL in either 0.1% formic acid (FA) or trifluoroacetic acid (TFA). Columns of HALO 160 Å ES-C18, HALO 90 Å Penta-HILIC, and HALO 90 Å Glycan all 2.7 µm diameter silica particles (Advanced Materials Technology, Inc., Wilmington, DE) were compared for MS spectral quality, column retention, peak widths and tailing factors. 2.1 mm or 0.2 mm ID columns were eluted using increasing acetonitrile or increasing water, modified with ammonium formate/formic acid. ESI/MS detection used the Thermo Velos Pro LTQ Orbitrap hybrid instrument, with XCalibur data capture, and Chromeleon (XIC) and PD4.0 (deconvolution) for analysis.

Importance of Glycosylation

- Impacts the safety/immunogenicity
- Influences the efficacy and clearance
- Impacts the stability and solubility
- As a critical quality attribute, must be characterized

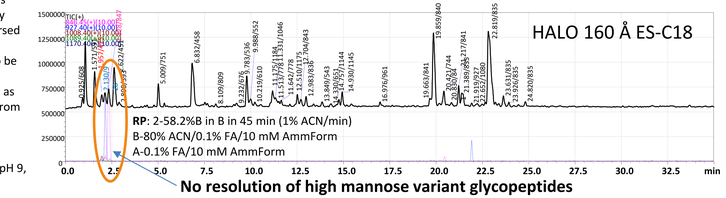


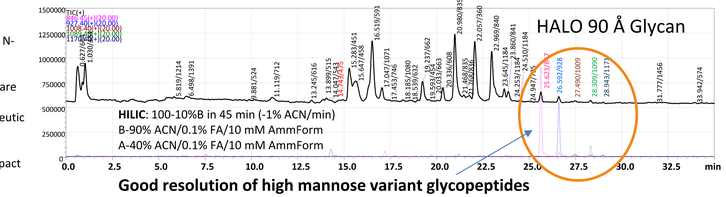


Source: https://www.glytech-inc.com/glycan/what-are-glycans/

HILIC Strongly Retains and Resolves N-linked Glycopeptides

2.1 mm ID x 100 mm, 0.35 mL/min, 40 °C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s
20 µg Bovine Ribonuclease B tryptic digest (CAM)

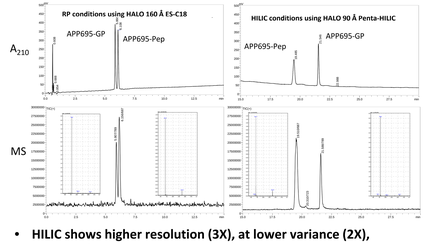




RP and HILIC of Peptide/Glycopeptide Pairs

Table 1. Paired comparison of RP and HILIC for Glycopeptide/Peptide LC/MS. *The 26 peptides shown were analyzed by LC/MS using 2.1 x 100 mm columns, with a flow rate of 0.4 mL/min at 60°C. Gradient conditions: A – 0.1% formic acid/10 mM ammonium formate; B – 90% Acetonitrile in A. 500 pmol was injected, with detection at 210 nm, MS operated +4.5 kV, with 0.45 s scan from 500 to 2000 m/z. RP Gradient - 4% to 34% AcN/30 min (1%/min); HILIC Gradient - 90% to 60% AcN/30 min (-1%/min). The sequences were selected based on known O-HexNAc modified sites in a variety of proteins. Peak widths; means were 0.092 min. for RP and 0.090 min. for HILIC. Average and variance in Rt for the modes were similar.*

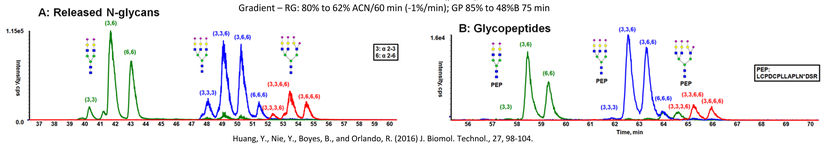
Peptide Description	Sequence	Mass (neutral)	Rt RP (min)	Δ Rt RP (GP-P)	Rs RP	Rt HILIC (min)	Δ Rt HILIC (GP-P)	Rs HILIC
APP695-14Glyp	VPTTQGGLNALSAASTPDAVDK	1574.8	5.87			21.55		
APP695-14Pep	VPTTAASTPDAVDK	1371.7	6.11	-0.24	1.90	19.49	2.07	9.41
MUC5AC	GTTSPSPVPTTSTTSAP	1501.6	9.28			16.41		
MUC5AC-3	GTTJOGaNAcJPSPVPTTSTTSAP	1704.6	8.45	-0.83	6.88	18.68	2.27	13.40
MUC5AC-13	GTTPSPVPTTSTTJOGaNAcJSAP	1704.6	8.53	-0.75	5.82	18.51	2.10	10.72
MUC5AC3/13	GTTJOGaNAcJPSPVPTTSTTJOGaNAcJSAP	1905.7	7.76	-1.52/2	11.84	20.48	4.07/2	23.35
GP-41	Ac-CSTFRPRTJOGlcNAcJSSNAST	1758.8	7.09			18.59		
P-42	Ac-CSTFRHPTSNAST	1535.7	7.03	0.06	0.44	17.03	1.56	11.58
GP-78	Ac-CQHPPVTJOGlcNAcJNGDTVK	1639.8	6.47			20.32		
P-84	Ac-CQHPPVTNGDTVK	1436.7	6.56	-0.10	0.66	18.72	1.61	11.23
GP-79	Ac-CKIADFGLSDGGLNAcJKVEHQ	1932.0	19.36			19.15		
P-85	Ac-CKIADFGLSKVEHQ	1728.9	20.80	-1.44	8.16	17.21	1.94	14.76
GP-17s	CTLHTKASGMAcLLHQ	1854.9	11.62			17.29		
P-20s	CTLHTKASGMAcLLHQ	1651.8	14.23	-0.61	3.06	15.15	2.14	15.38
GP-15	Ac-CFELLPTJO-GlcNAcJPPLSP	1557.8	25.16			5.64		
P-18	Ac-CFELLPTPPLSP	1354.7	27.16	-2.00	8.88	2.71	2.93	20.11
GP-46	Ac-CRSSHYGSSJOGlcNAcJLPNVNQI	1975.9	12.48			17.32		
P-47	Ac-CRSSHYGSSLPNVNQI	1772.8	12.96	-0.48	3.83	15.43	1.89	13.91
GP-51	Ac-CSALNRTSJOGlcNAcJSDSALHT	1806.8	9.08			17.23		
P-52	Ac-CSALNRTSDSALHT	1603.7	9.55	-0.47	3.85	15.55	1.69	12.42
GP-16	Ac-CKIPGQSJOGlcNAcJTPQTL	1487.7	16.41			13.27		
P-19	Ac-CKIPGVSTPQTL	1284.6	16.98	-0.58	3.74	10.59	2.68	21.63
GP-1-p53	Ac-CQLHVISQGGNAcJTPPPG	1543.7	16.43			12.72		
P-1-p53	Ac-CQLWVDSTPPPG	1340.6	17.66	-1.23	7.23	10.41	2.31	10.28
GP-17r	Ac-CLHTKASQDGlcNAcJGMALL	1488.7	16.21			10.59		
P-20r	Ac-CLHTKASQMALL	1285.6	16.98	-0.77	2.79	7.45	3.14	24.73
Average			13.01	-0.73	4.93	15.29	2.17	15.21
Standard Deviation			5.95	0.54	3.32	4.74	0.47	5.13
% RSD			45.7	74.3	67.3	31.0	21.8	33.7



- **HILIC shows higher resolution (3X), at lower variance (2X), compared to RP**
- **Driven by better separation selectivity of this polar modification**

Comparison of Released N-glycans and Glycopeptides of Fetuin

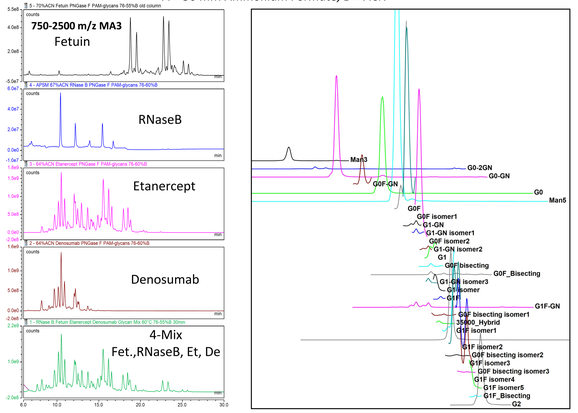
Conditions: Column – 2.1 x 150 mm HALO Penta-HILIC, 0.4 mL/min; 60°C.
Gradient Eluents: A –50 mM ammonium formate (pH 4.4)/5% ACN; B – ACN
Gradient – RG: 80% to 62% ACN/60 min (-1%/min); GP 85% to 48%B 75 min



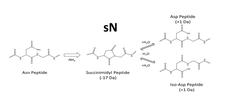
Huang, Y., Nie, Y., Boyes, B., and Orlando, R. (2016) J. Biomol. Technol., 27, 98-104.

Released N-Glycans from mAbs and Other Proteins

HALO 90 Å Glycan, 2.7 µm, 2.1x150mm, 76-55%B in 30 min @ 60°C 0.4mL/min
A = 50 mM Ammonium Formate; B = ACN

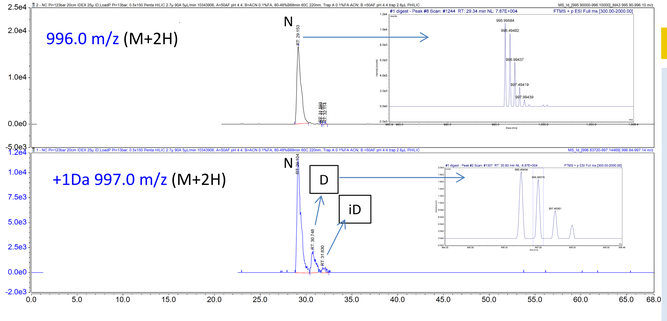


HILIC for Deamidation and Isomerization Detection in Peptides & mAbs



Trastuzumab Light Chain			
25	ASQDVNTAVAWYQQKPGK	42	$N^{30}T$
Trastuzumab Heavy Chain			
76	NTAYLQMNSLR	86	$N^{83}S$
99	WGGDGFYAMDYWGQGTLVTVSSASTK	124	$D^{102}G$
279	FNWYVDGVEVHNAK	292	$D^{284}G$
306	VVSVLTVLHQDWLNGK	321	$N^{319}G$
375	GFYPSDIAVEWESNGQPENNYK	396	$N^{388}G$--$NN^{393}N^{394}Y$
421	WQQGNVFSCSVMHEALHNHYTQK	443	$N^{425}V$--$N^{438}H$

ASQDVNTAVAWYQQKPGK

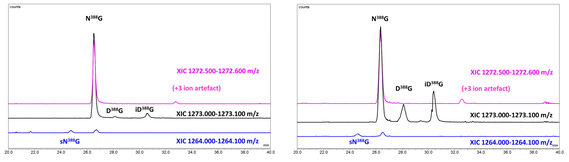


Deamidation and Isomerization in IgG Tryptic Digests

Trypsin digestion of the mAb IgG for native protein (as obtained for pharmaceutical purposes), and on samples that were "stressed" by incubation in Tris-HCl pH 9.0 for 7 days at 4 mg/mL. Reduced and alkylated proteins were digested at 1:30 protein to enzyme for 4hrs in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by formic acid acidification and direct injection on to capillary LC/MS using the Orbitrap/IT. Extracted ions at the monoisotopic masses of the target sequences were integrated; the reported sequences were confirmed in CID MS/MS fragmentation, with the exception of sN sequences of low abundance ($sN^{388}G$ confirmed, but not sN sequence eluting at 26.7 min.). Not all deamidation products are determinate as iD or D residues, due to the similarity of CID fragmentation. ETD studies are ongoing. Relative values of modifications compared to native total Asn or Asp sequences reproducibly obtained, and retention positions for all confidently identified modifications are noted, with comparison to retention position predicted by the current model.

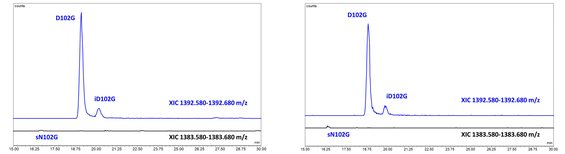
GFYPSDIAVEWESNGQPENNYK in Trastuzumab Digest

Native ***Stressed***



WGGDGFYAMDYWGQGTLVTVSSASTK in Trastuzumab Digest

Native ***Stressed***



- 9 sites of potential modification were analyzed
  - 3 Asn sites with significant deamidation, and subsequent isomerization.
  - 2 sites showed presence of D/iD pairs, neither were strongly affected by "stress"
- All susceptible sites exhibited the cyclic intermediate sN, whether formed from D or N in the native sequence.

Conclusions and Future Directions

- HILIC separations show increased resolution of polar PTMs of mAbs compared to reversed-phase separations
- HALO 90 Å Glycan is well suited for separations of glycovariants and analysis of site occupancy
- Glycosylation analysis using HALO 90 Å Glycan results in separation of isomeric glycans
- A high efficiency and reliable HILIC separation approach was applied to resolution of modified Asn and Asp peptides and tryptic fragments of intact proteins. Resolution of polar modifications was excellent.
- The combination of high resolution separation with high resolution MS permits effective analysis of deamination and isomerization products, with resolution of Asn, Asp, isoAsp and succinimidyl intermediates.