High Efficiency LC/MS Analysis of O-HexNAc Modified Peptides

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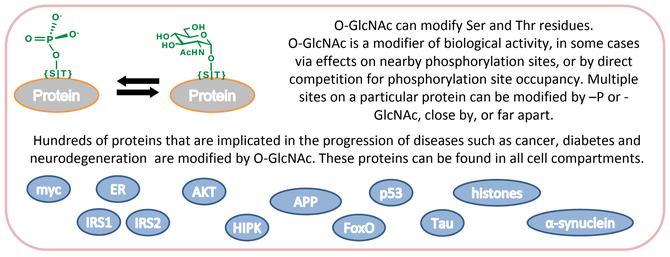
Objectives

- Examine conditions for performing high resolution LC/MS separations of hexosamine modified peptides, particularly the effects of separation mode (RPC and HILIC)
- Evaluate the utility of LC with online MS for identification and purity analysis of hexosamine modified peptides.
- Define the retention and selectivity differences between RPC and HILIC mode of separating hexosamine modified and unmodified peptides
- Explore the possibility of predicting glycopeptide/peptide relative retention, towards directed analysis of novel glycosylation sites

Introduction

Modification of serine or threonine residues of proteins by β-D-N-acetylglucosamine (GlcNAc) has emerged as a significant biological signaling mechanism. O-GlcNAcylation of relevant sites can involve meaningful cross-talk with phosphorylation targets, both nearby and at distant sequences. Similarly modification of polypeptides by α-GalNAc (mucin antigen Tn) is of similar interest. For a variety of purposes, we have prepared 8-30 residue synthetic peptides with and without O-GlcNAc modifications at serine and threonine residues. LC/MS methods to qualify the purity and identities of such peptides using both Hydrophilic-interaction chromatography (HILIC) and reversed-phase chromatography (RPC) are compared. With many peptide/glycopeptide pairs we observe much improved separations and LC/MS features using HILIC, compared to RPC. LC/MS using high performance HILIC permits rapid and sensitive analysis of O-GlcNAcylated peptides. A collection of sequence-matched glycopeptide/peptide pairs are compared for various separation features in RP and HILIC mode, as well as for detection by absorbance at low wavelength (210 nm) and on-line electrospray ionization mass spectrometry (ESI-MS) using the single quadrupole MS analyzer.



Materials and Methods

Columns of HALO Peptide ES-C18 and Halo Penta HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). Both materials employ Fused-Core® silica particles of 2.7 µm diameter, a solid core of 1.7 µm diameter, and a shell thickness of 0.5 µm. The Halo Peptide shell has 160 Å pores, and the Penta HILIC column 90 Å pores. Synthetic peptides were synthesized at the CCRC (Prof. Geert-Jan Boons), or obtained from AnaSpec (Freemont, CA), or from Colin Mant and Prof. R.S. Hodges (U. Colorado, Aurora, CO). The Retention Standard Mix uses the S1-S5 sequences:

S1 RGAGGLGLGK-Am
S2 Ac-RGGGGLGLGK-Am
S3 Ac-RGAGGLGLGK-Am
S4 Ac-RGVGGLGLGK-Am
S5 Ac-RGVVGLGLGK-Am

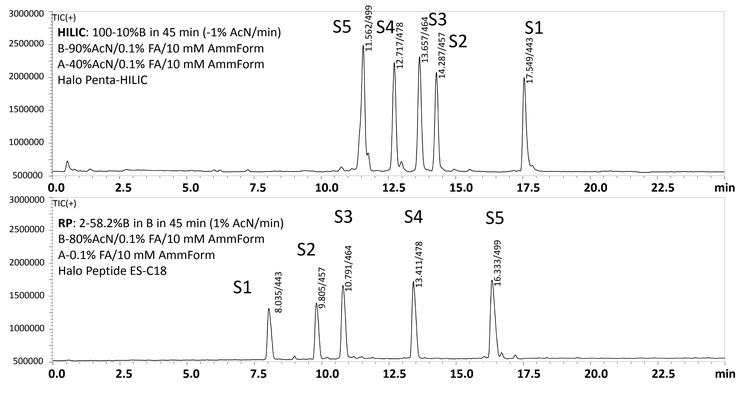
The instrument was the Shimadzu Nexera LC-30 (40 µL mixer), with the SPD20 Absorbance detector, fitted with a high sensitivity semi-micro flow cell inline with the MS-2020 single quadrupole MS (+4.5 kV capillary potential, 300-2000 m/z at 0.3 or 0.45 sec/scan). Chromatographic parameter measures used the absorbance data at 210 nm, generated with LabSolutions v.5.54 software.

LC/MS Conditions for RP and HILIC of Peptides/Glycopeptides

- A mix of Ammonium Formate/Formic Acid is an attractive mobile phase, compared to Formic Acid alone, showing narrow and symmetrical peaks, improved load tolerance (linear isotherm), and good compatibility with online ESI-MS detection (McCalley, J. Chromatogr. A, 1038 (2004), p. 77; Schuster, Boyes, Wagner, Kirkland, J. Chromatogr. A, 1228 (2012), p. 232; Johnson, Boyes, Orlando, J. Biomol. Techn. (JBT), 24 (2013), p. 187.
- RP and HILIC conditions can employ the same mobile phases, with reversal of the gradient elution composition: RP *increases* acetonitrile, HILIC *decreases* acetonitrile (water is the strong solvent).
- Figure 1 presents the TIC chromatograms for a peptide standard separated in RP using the Halo Peptide ES-C18 column and in HILIC mode using the Halo Penta HILIC column. Column efficiencies are comparable, with resolution readily achieved by either mode of separation.
- Retention (Rt), peak widths ($W_{1/2}$), resolution (Rs) and signal intensities in UV absorbance and in ESI-MS total ion currents (TIC) were determined for the 26 peptides shown in Table 1, using conditions similar to those shown in Figure 1. Known mass of glycopeptides and peptides were injected to allow assessment of ESI-MS signals, normalized to UV-derived concentration data.

Figure 1. RP and HILIC Peptide Separations

2.1 mm ID x 100 mm, 0.35 mL/min, 40°C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s



RP and HILIC of Peptide/Glycopeptide Pairs

Table 1. Paired comparison of RP and HILIC for Glycopeptide/Peptide LC.

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 range of Rt for the modes were similar. Values of ΔRt refer to the differences in retention time for glycopeptide and peptide. For bis-glycosylated MUC5A sequence, the values of ΔRt were divided by 2 for calculation of averages.

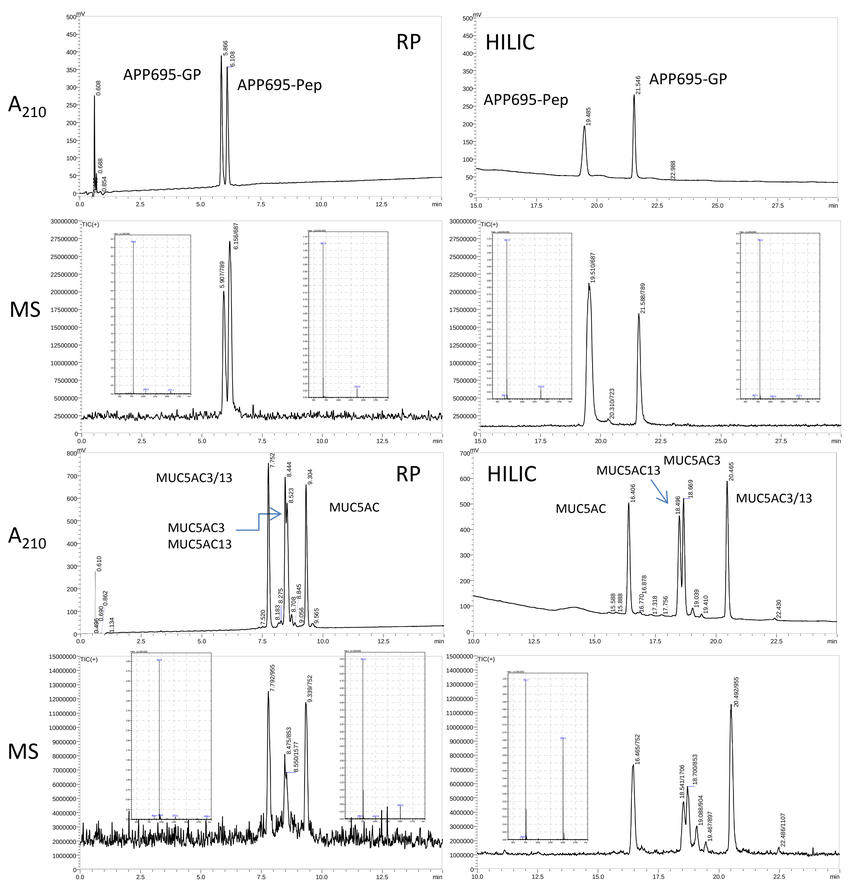
Peptide Description	Sequence	Mass (neutral)	Rt RP (min)	Δ Rt RP (GP-P)	Rs RP	Rt HILIC (min)	Δ Rt HILIC (GP-P)	Rs HILIC
APP695-14GPep	VPTT(OGlcNAc)AASTPDAVDK	1574.8	5.87			21.55		
APP695-14Pep	VPTTAASTPDAVDK	1371.7	6.11	-0.24	1.90	19.49	2.07	9.41
MUC5AC	GTTPSPVPTTSTTSAP	1501.6	9.28			16.41		
MUC5AC-3	GTT(OGalNAc)PSPVPTTSTTSAP	1704.6	8.45	-0.83	6.88	18.68	2.27	13.40
MUC5AC-13	GTTPSPVPTTSTT(OGalNAc)SAP	1704.6	8.53	-0.75	5.82	18.51	2.10	10.72
MUC5AC3/13	GTT(OGalNAc)PSPVPTTSTT(OGalNAc)SAP	1908.1	7.76	-1.52/2	11.84	20.48	4.07/2	23.35
GP-41	Ac-CSTFRPRT(OGlcNAc)SSNAST	1758.8	7.09			18.59		
P-42	Ac-CSTFRPRTSSNAST	1555.7	7.03	0.06	0.44	17.03	1.56	11.58
GP-78	Ac-CQHPPVT(OGlcNAc)NGDTVK	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-CKIADFGLS(OGlcNAc)KIVEHQ	1932.0	19.36			19.15		
P-85	Ac-CKIADFGLSKIVEHQ	1728.9	20.80	-1.44	8.16	17.21	1.94	14.76
GP-17s	CTLHTKAS(OGlcNAc)GMALLHQ	1854.9	13.62			17.29		
P-20s	CTLHTKASGMALLHQ	1651.8	14.23	-0.61	3.06	15.15	2.14	15.38
GP-15	Ac-CFELLPT(O-GlcNAc)PPLSP	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-CRSSHYGGS(OGlcNAc)LPNVNQI	1975.9	12.48			17.32		
P-47	Ac-CRSSHYGGSLPNVNQI	1772.8	12.96	-0.48	3.83	15.43	1.89	13.91
GP-51	Ac-CSALNRTS(OGlcNAc)SDSALHT	1806.8	9.08			17.23		
P-52	Ac-CSALNRTSSDSALHT	1603.7	9.55	-0.47	3.85	15.55	1.69	12.42
GP-16	Ac-CKIPGVS(OGlcNAc)TPQTL	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-2-p53	Ac-CQLWVDS(OGlcNAc)TPPPG	1543.7	16.43			12.72		
P-3-p53	Ac-CQLWVDSTPPPG	1340.6	17.66	-1.23	7.23	10.41	2.31	10.28
GP-17r	Ac-CLHTKAS(OGlcNAc)GMALL	1488.7	16.21			10.59		
P-20r	Ac-CLHTKASGMALL	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

Comparing RP versus HILIC for LC/MS analyses of the collection of paired glycopeptides and peptides (GP and P) reveals:

- As previously observed for unmodified peptides and tryptic digests, RP and HILIC are orthogonal (retention in each mode does not significantly correlate to the other).
- In all cases, glycopeptides were *retained less* in RP, eluting <u>*at or before*</u> the unmodified peptide with the same sequence (ΔRt: -0.7 vs 2.2).
- In HILIC operation, glycopeptides were *retained more*, eluting <u>after</u> the unmodified peptide.
- The difference in retention time (%AcN) between paired GP and P were much larger in HILIC than RP.
- <u>For all pairs of glycopeptides/peptides, resolution is higher in HILIC than RP (Rs: 15 vs. 5).</u>
- TIC signal is slightly lower (-35%) in RP than HILIC, for either GP or P, but variable with sequence. This was the case comparing either all peptides and glycopeptides as groups, or by pair-wise P/GP comparisons. HILIC conditions exhibit decreased MS noise.

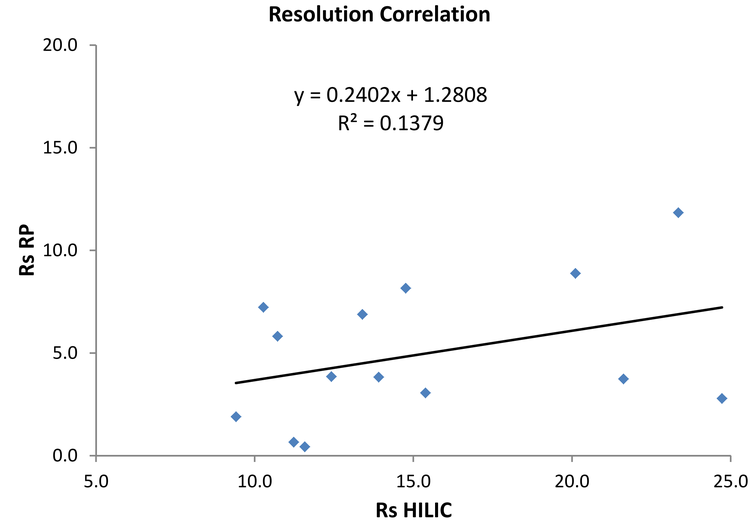
Figure 2. Examples of Paired P/GP Separations by RP and HILIC.

Conditions are described above. For peptide sequences see Table 1. The examples show separations for the human amyloid precursor polypeptide 695 amino acid long variant, with putative glycosylation site, as well as synthetic analogs of the human mucin polypeptide Muc5AC modified by O-GalNAc. Note that the doubly-modified MUC5AC3/13 shows larger effects on retention in both RP and HILIC.



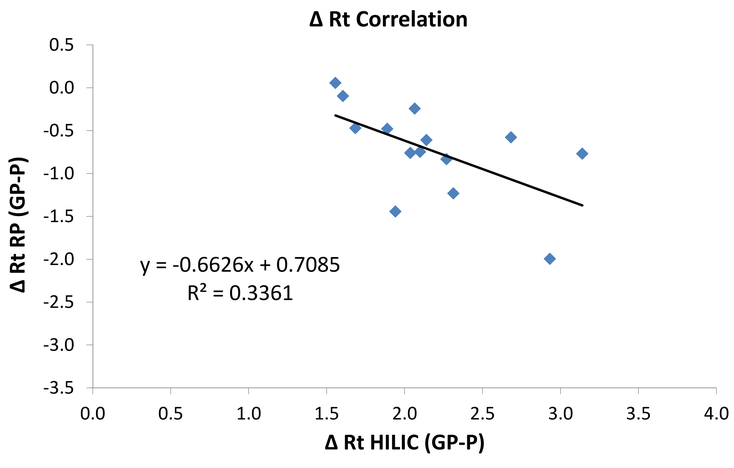
Resolution of Peptide/Glycopeptide Pairs

To uncover new polypeptide glycosylation sites, prediction of RP and HILIC retention effects of protein site occupancy by an O-linked carbohydrate would be useful. As shown below, the correlation of resolution of P/GP pairs using RP and HILIC modes of separation is poor, implying that the mechanisms of separation are very different.



Given that the observed peak widths in these experiments are highly similar for HILIC and RP, improved HILIC resolution of P/GP pairs must be driven by selectivity differences. Comparing the plot of the differences in retention time (ΔRt, representing AcN difference at elution) for each mode, for each peptide pair, also yields a poor correlation.

The poor correlation of ΔRt results, in part, by the greater variability of separation performance in RP mode, and the smaller effect of the HexNAc modification on retention; for RP the mean value of ΔRt (Table 1) is -0.73 min (%AcN, 74% rsd), compared with 2.17 min (%AcN, 22% rsd) for HILIC. Note that the absolute variability is similar for RP and HILIC, but higher HILIC selectivity differences for resolving P/GP pairs leads to less variance in the retention difference resulting from HexNAc addition to a peptide sequence. Prediction of the retention position of the modified peptide, given knowledge of the peptide retention, would thus be more reliable for HILIC than RP.



Conclusions and Future Directions

- High performance HILIC and RP HPLC separations were conducted to define preferred mode of practice to resolve O-GlcNAc and O-GalNAc modified peptides.
- Formic acid/Ammonium formate mixtures with acetonitrile gradient elution were effective for LC/MS analysis of the glycopeptide and peptide mixtures using both RP and HILIC operation.
- HILIC better resolved glycopeptide/peptide mixtures.
- HILIC exhibited potential to resolve O-GalNAc modifications at variant sites (3 and 13) on the MUC5AC peptide sequence.
- Additional glycopeptide/peptide pairs will be examined to improve the retention prediction for O-HexNAc peptide modifications.
- Prediction of retention of the modified sequence could allow verification of novel sites identified by CID/ETD-MS experiments.
- HILIC resolution of "native", O-GlcNAc and phosphorylated sequences could permit analysis of levels of site occupancy, within specific protein targets.

- Supported by NIH NIGMS GM093747.