HILIC LC/MS Analytical Approach for Identification of Protein Deamidation and Isomerization Modifications

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Outline

- Resolution of synthetic peptides and protein digests with trypsin fragments is readily accomplished using Hydrophilic Interaction Liquid Chromatography (HILIC), exhibiting resolution and efficiencies similar to reversed phase HPLC.
- Previous results have shown that retention of peptides in HILIC depends primarily on amino acid composition, and that retention can thus be predicted for peptides of known structure (Badgett, et al., *Chromatogr. Today* 8: 39 (2015)). This approach has been recently extended to define the effects of deamidation and oxidation on HILIC separations of peptides (Badgett, et al. *Am. Soc. Mass Spectrom.* 28: 818 (2017)), and the current work.
- 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

Protein therapeutics and protein reagents continue to find expanded use in research and health care. This contributes to a highly active growth in protein analysis by LC and LC/MS. Protein post translational modifications and a variety of chemical modifications of protein amino acyl side chains occur in vivo and in vitro, altering the hydrophobicity of proteins or proteolytic fragments. For example, this could include attachment of a monosaccharide or oligosaccharide, phosphorylation, oxidation of methionine, deamidation and/or formation of aspartyl- or gluatamyl- isomers, amongst others. Although reverse-phase HPLC can resolve many modifications, selectivity can be variable. Hydrophilic interaction liquid chromatography (HILIC) can efficiently resolve peptides and peptides that are modified, with confident modeling of peptide retention effects. We demonstrate the utility of HILIC with online LC/MS analysis of therapeutic mAb IgGs and IgGs in serum to determine the extent of deamidation and isomerization of protein asparagine and aspartate residues.

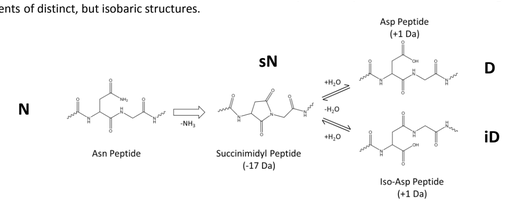
Materials and Methods

Columns of HALO penta-HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 2.7 µm diameter, shell thicknesses of 0.5 µm, and pore sizes of 90 Å. RP separations employed the Halo Peptide C18 column.
Mobile phase modifiers were obtained from Pierce (TFA, FA), Sigma/Millipore (TFA, Formic acid, Ammonium Formate). Acetonitrile was MS grade from JT Baker. Synthetic peptides were from AnaSpec, trypsin from Promega.
Analytical separations used the Shimadzu Nexera LC-30 components (40 µL mixer), with the SPD 20A UV detector and MS-2020 quadrupole MS operated in series at +4.5 kV capillary potential. A special low volume flow cell was obtained from Shimadzu Scientific for this effort, to minimize band dispersion effects. Capillary column separations used the Dionex RSLC 3000 with a trap column, connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface operated at 3.8 kV potential. MS spectra were recorded in the Orbitrap, using 30,000 resolution scans, with CID MS/MS obtained using a Top 4 data dependent regime. MS data were analyzed in Xcalibur Quant Browser (v2.7), or transferred to Chromeleon v. 7.2 for integration. Chromatographic peak widths are reported as half height ($PW_{1/2}$).

Analyses of tryptic fragments employed a 0.5 mm ID x 150mm PentaHILIC capillary column, operated at 12.0 µL/min, and 60°C. A trap column of the same material, of 2.6 µL size (0.5 mm x 12.5 mm) was obtained from Optimize, Inc. Analytical gradient conditions were from 80%B for 4min, then 80-48%B in 64 min. 12 µL/min, with detection at 220nm for absorbance detector.

HILIC for Deamidation and Isomerization Detection in Proteins and Peptides

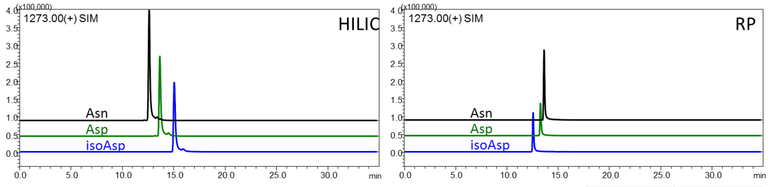
Deamidation of asparagine, as well as glutamine, in both peptides and in intact proteins appears as an increasingly important area of current interest. The basic reactions are shown below, and the rate of deamidation of an asparaginyl-containing sequence is known to depend on many factors, including pH, counter ions present, solution conditions, and both short-range and long-range sequence and conformational effects when the sequences are present in proteins. The initial loss of ammonium is considered irreversible, but the succimidyl-, aspartyl- and iso-aspartyl- peptides are interconvertible in aqueous solution, with mass shifts that can permit differentiation of these sequences. Relevant mass shifts from asparagine are shown, and for many practical purposes, mass analysis alone is insufficient to resolve aspartate from iso-aspartate sequences. Reliable chromatographic resolution permits ready use of MS measurements of distinct, but isobaric structures.



Reversed-phase (RP) separations often result in limited chromatographic selectivity for resolution of deamidated peptides from their unmodified counterparts. As previously shown by Hao et al. (J. Proteome Res. 11, 1804 (2012)) using ERLIC-MS/MS, deamidated products can separate based on pI difference, allowing identification. The change in hydrophilicity of amino acid side chains resulting from these modifications change the HILIC selectivity of the peptides to allow for chromatographic separation. Below is an example comparing the separation of the Asn-, Asp-, and iso-Asp- variants of synthetic IgG peptides that differ at a single site (N^{380}, see table right for sequence details). The same mobile phase conditions and temperature were employed, reversing the direct of the acetonitrile/buffer gradient to effect elution on RP and HILIC columns. *Note the greater selectivity difference for resolving these peptides in HILIC, compared to RP.*

Comparison of HILIC and RP for Resolving Deamidated and Isomerized Asn Peptides

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Temp: 60 °C; Detection: Shimadzu MS-2020 Single Quad MS
Mobile Phase A: water/50 mM Ammonium Formate, pH4.4; Mobile Phase B: acetonitrile/0.1% Formic acid; Gradient:
HILIC – 80%-46.2% in 60 min.; RP - 10-70% B in 60 min; Injection Volume: 4 µL (0.1 µg);



A peptide retention model based on amino acid composition was created using a HALO® penta-HILIC column with gradient elution. Coefficients for each amino acid were derived using linear regression analysis and these coefficients can be summed to predict the retention of peptides, with the separation calibrated using a reducing terminus labeled oligosaccharide standard (glucose units, GU). This model was shown to have a high correlation coefficient (0.946), on par with previously reported RP and HILIC models. It also includes optimized coefficients for hydrophobic residues at the N-terminus and hydrophobic residues one residue over from the N-terminus. The use of dextran as a retention time calibrant was essential for making this model capable of being used on any LC-MS system, switching from capillary scale separations to analytical separations, etc. More details are available in Badgett, et al., *Chromatogr. Today* 8: 39 (2015) and Badgett, et al. *Am. Soc. Mass Spectrom.* 28: 818 (2017). The most recent iteration includes correction for N-terminal hydrophobic residue effects, which we have found to be significant.

This model has recent been extended to consider modifications, including deamidation, isomerization, glycosylation, and oxidations of peptides. For the current work, we employ coefficients shown in the table, and supplemented by the value of 1.409 for iso-aspartate retention coefficient.

Amino Acid	Coefficient
Alanine (A)	0.164
Cysteine (C)*	0.293
Aspartic Acid (D)	0.800
Glutamic Acid (E)	0.719
Phenylalanine (F)	-0.967
Glycine (G)	0.233
Histidine (H)	1.564
Isoleucine (I)	-0.615
Lysine (K)	2.121
Leucine (L)	-0.799
Methionine (M)	-0.337
Asparagine (N)	0.610
Proline (P)	0.129
Glutamine (Q)	0.703
Arginine (R)	1.828
Serine (S)	0.334
Threonine (T)	0.357
Valine (V)	-0.306
Tryptophan (W)	-1.138
Tyrosine (Y)	-0.430
Intercept	1.535
R-Squared Value	0.94553

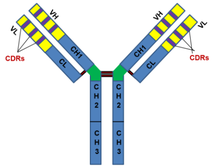
Asn Deamidation and Isomer Formation in IgG Sequences

There is a broad interest in the frequency of occurrence and environmental conditions that determine the presence and rates of deamidation and isomerization of Asp and Gln residues in both peptides and proteins. In addition to the acquisition of a negatively charged amino acyl side chain, subsequent isomerization of the Asp and Glu containing sequences is a backbone rearrangement, with implications on short and long range conformation of peptides and proteins, and the potential to alter biological recognition, including potential therapeutic features of a peptide or protein.

Of particular interest are sequences in human IgG or humanized monoclonal antibodies. For this study we selected the sequences shown below, many of which are common across the mAb spectrum, and some of which are unique to the humanized IgG1 mAb employed for the current work, trastuzumab. The sequences shown are in reference to the cartoon representation of the H-chain and L-chain subunits of IgG1 shown below. Various details are omitted for clarity.

IgG Peptides Studied

Trastuzumab Light Chain			
25	ASQDV**N**TAVAWYQQKPGK	42	$N^{30}T$
Trastuzumab Heavy Chain			
76	NTAYLQM**N**SLR	86	$N^{83}S$
99	WGG**D**GFYAMDYWGQGTLVTVSSASTK	124	$D^{102}G$
279	FNWYV**D**GVEVHNAK	292	$D^{284}G$
306	VVSVLTVLHQDWL**N**GK	321	$N^{315}G$
375	GFYPSDIAVEWES**N**GQPE**N**NYK	396	$N^{388}G$--$NN^{393}N^{394}Y$
421	WQQG**N**VFSCSVMHEALH**N**HYTQK	443	$N^{425}V$--$N^{438}H$

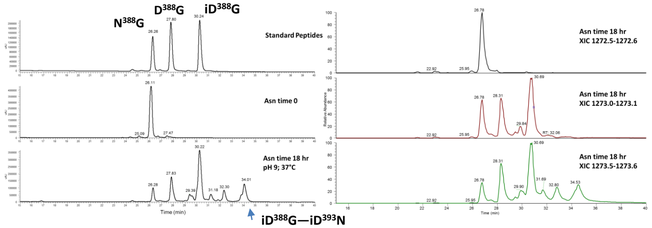


IgG Peptide with Multiple Asn as a Model System: GFYPSDIAVEWESNGQPENNYK

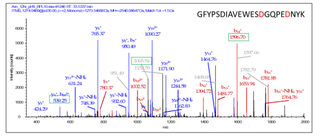
The bespoke peptides of interest were obtained by synthesis, with the N388 position substituted as well by Asp (D388) and isoAsp (iD388). LC/MS analysis confirmed the authenticities of the peptides by high accuracy MS, by CID fragment identification, and by ETD fragment identification, including the presence of relevant ***c*** and ***z*** ions, diagnostic for the 57 mass unit shift on the peptide ion fragments obtained for the isoAsp sequence.

HILIC with online high resolution MS and data dependent MS/MS (CID) was conducted for mixtures of the 3 synthetic peptides, as well as for samples of the Asn variant that was incubated for various times at 37°C at pH 9 in Ammonium Bicarbonate or Tris-HCl solutions (100 mM Buffer, 2 µg/mL peptide). Shown below are capillary LC HILIC separations, monitored by absorbance (220 nm) and MS detection with extracted ions (0.1 m/z, 30,000 resolution), in this case for a sample rated with Ammonium Bicarbonate. Note that the MS and Absorbance detection are shifted by 0.52 min. Retention times below refer to those at the MS detector.

Columns: 0.5 x 150 mm Halo PentaHILIC; Flow rate: 12 µL/min; Temp: 60 °C; Detection: Abs (220 nm) or Orbitrap Velos Pro MS Mobile Phase A: water/50 mM Ammonium Formate, pH4.4; Mobile Phase B: acetonitrile/0.1% Formic acid; Gradient: Hold 80%B for 4 min.; 80%-48% in 64 min.



Mass analysis and CID fragmentation identify the retention of the $N^{388}G$—(D/iD)^{393}N peptide as eluting at 29.4 min. Degradation of $iD^{388}G$—$N^{393}N$ rapidly formed two peptides with 1 Da shift at 31.69 and 32.80 min, confirmed by CID as deamidations to D or iD at position 393, and eventually all peptides degraded to predominantly 34.53 minutes, which is proposed to be the $iD^{388}G$—$iD^{393}N$, as supported by mass analysis and CID fragmentation. An example of a CID MS/MS spectra for the degradation of $iD^{388}G$—$N^{393}N$ to -(Di/D)^{393}N is shown at right. No evidence of the formation of a triple deamidation was obtained (N^{394}).

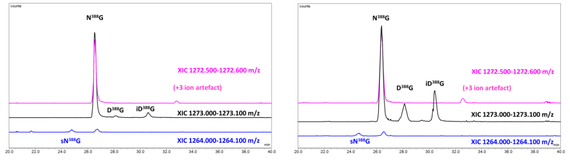


Deamidation and Isomerization in IgG Tryptic Digests

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.

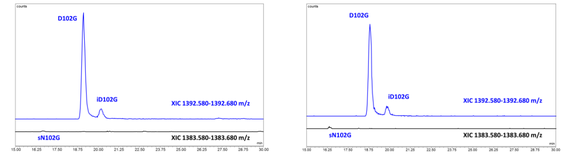
GFYPSDIAVEWESNGQPENNYK in Trastuzumab Digest

Native ***Stressed***



WGGDGFYAMDYWGQGTLVTVSSASTK in Trastuzumab Digest

Native ***Stressed***



Agreement of Predicted and Observed Retention and Resolution for Fragments

Retention positions for all confidently identified peptides and modifications are noted, with comparison to retention position predicted by the current model. Although the deviations of absolute retention showed moderate variability, the average of deviation was small (-0.16), suggesting that the model is accurately predicting retention. Resolution of isobaric peptides is crucial for the method to be useful and it is noted that separation resolution values show overall good agreement in prediction and observed.

Trastuzumab Light Chain			Rt Obser	Rt Pred	ΔRt (O-P)	Res Obser	Res Pred
25 ASQDV^{N}TAVAWYQQKPGK	42	$N^{30}T$	29.14	31.62	-2.48		
		$D^{30}T$	30.64	33.49	-2.85	1.50	1.87
		$iD^{30}T$	33.91	34.82	-0.91	3.27	1.33
Trastuzumab Heavy Chain							
76 NTAYLQM^{N}SLR	86	$N^{83}S$	15.03	16.5	-1.47		
		$D^{83}S$	not det	n/a	n/a		
99 WGG^{D}GFYAMDYWGQGTLVTVSSASTK	124	$D^{102}G$	19.12	16.1	3.03		
		$iD^{102}G$	20.05	18.61	1.44	0.92	2.51
279 FNWYV^{D}GVEVHNAK	292	$D^{284}G$	22.07	20.89	1.18		
		$iD^{284}G$	23.99	22.95	1.04	1.92	2.06
306 VVSVLTVLHQDWL^{N}GK	321	$N^{315}G$	16.05	15.29	0.76		
		$D^{315}G$	16.5	18.68	-2.18	0.45	3.39
		$iD^{315}G$	19.43	20.9	-1.47	2.93	2.22
375 GFYPSDIAVEWES^{N}GQPE^{N}NYK	396	$N^{388}G$--$N^{393}N^{394}Y$	26.57	26.84	-0.27	4.03	1.92
		$D^{388}G$--$N^{393}N^{394}Y$	28.14	28.99	-0.85	1.57	2.15
		$iD^{388}G$--$N^{393}N^{394}Y$	30.55	30.53	0.02	2.41	1.54
		$iD^{388}G$--$iD^{393}N^{394}Y$	34.58	32.45	2.13	4.03	1.92
		$iD^{388}G$--$iD^{393}iD^{394}Y$	36.57	34.84	1.73	1.98	2.39
397 TTPPVLDSDGSFFLYSK	413	$D^{408}G$	18.18	13.86	4.32		
		$iD^{408}G$	20.66	16.67	4.02	2.51	2.81
421 WQQGNVFSCSVMHEALHNHYTQK	443	$N^{425}V$--$N^{438}H$	28.85	32.31	-3.46		
		$D^{425}V$--$N^{438}H$	31.06	36.05	-4.99	2.21	3.74
		$iD^{425}V$--$N^{438}H$	37.51	37.72	-0.21	6.45	1.67
				Mean	-0.16	2.51	2.27
				SD	2.46		

Conclusions and Future Directions

- 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. This extends other work we have recently described using an SRM QQQ approach.
- A recently described HILIC separation peptide retention model is further supported, predicting the retention effects of deamidation and isomerization with reasonable accuracy.

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