Fragmentation-free characterization of glycopeptides with ion mobility

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Abstract

Haptoglobin is a protein that is produced in the liver, can be found in blood serum, and has four possible sites for glycosylation. Increased haptoglobin levels are a potential indicator of maladies such as anemia and various liver diseases[2]. The glycosylation profiles of haptoglobin may also alter its function, but the biological effect of this is still largely unknown. Therefore, understanding the glycosylation patterns of diseased versus healthy haptoglobin samples may lead to more accurate predictive diagnoses.

Current methods of glycopeptide analysis of haptoglobin are insufficient to address the identification of all possible glycoforms. These conventional methods usually require tandem mass spectrometry (MS/MS) for glycan identification, which can lead to limited glycoform identifications due to similar fragmentation patterns and long reverse-phase liquid chromatography (RPLC) methods as well as co-elution problems.

Herein, we discuss how to leverage high-resolution ion mobility (HRIM) as an orthogonal separation technique for the analysis of haptoglobin glycopeptides ranging from 5-11 AA in length. RPLC-HRIM-MS data and MS/MS fragmentation data were analyzed via Protein Metrics Byos Software (PMI). This study demonstrates that RPLC-HRIM-MS analysis of haptoglobin glycopeptides allows for improved identifications of the glycosylation patterns of glycopeptides than MS/MS fragmentation spectra alone.

Methods

Run Type	MSMS	HRIM
Method Time	70 min	20 min
Column	AdvanceBio Peptide Map	AdvanceBio Peptide Map
Mobile Phase A Mobile Phase B	H_2O + 0.1% FA ACN + 0.1% FA	H_2O + 0.1% FA ACN + 0.1% FA
Flow Rate	0.2 mL/min	0.4 mL/min
Gradient	0 min: 1% B 50 min: 41% B 55 min: 90% B 60 min: 95% B 70 min: 1% B	0 min: 1% B 15 min: 41% B 16 min: 90% B 18 min: 95% B 20 min: 1% B
Column Temp	50 °C	50 °C

References: (1) Haptoglobin Test: Purpose, Procedure, and Results (healthline.com); (2) **Glycopeptide Biomarkers in Serum Haptoglobin for Hepatocellular Carcinoma Detection in Patients with Nonalcoholic Steatohepatitis**; (3) **Quantitative Liquid Chromatography-Mass Spectrometry-Multiple Reaction Monitoring (LC-MS-MRM) Analysis of Site-specific Glycoforms of Haptoglobin in Liver Disease**; (4) **Haptoglobin polymorphism affects its N-glycosylation pattern in serum**

Glycopeptide Method Data Comparison

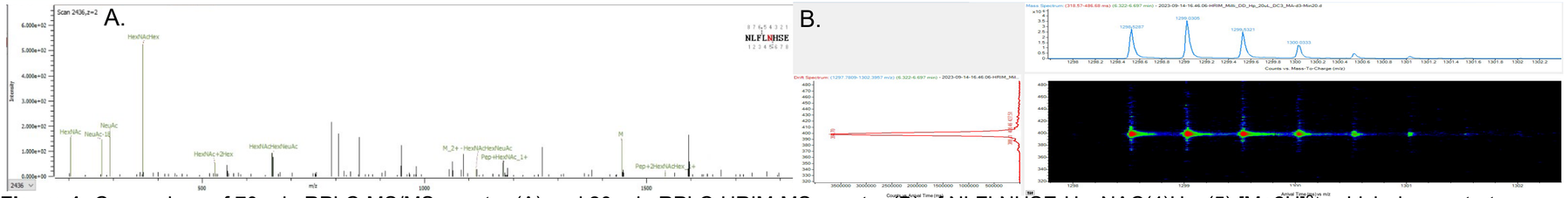

Figure 1: Comparison of 70-min RPLC-MS/MS spectra (A) and 20-min RPLC-HRIM-MS spectra (B) of NLFLNHSE-HexNAC(4)Hex(5) $[M+2H]^{2+}$which demonstrates the three-dimensional separation of peaks using the MOBIE platform.

LC-HRIM-MS Overview for Glycopeptides

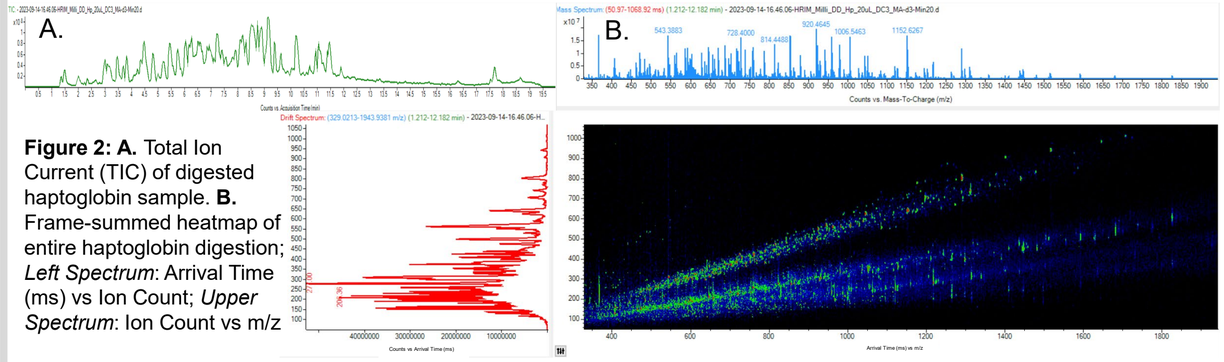

Figure 2: A. Total Ion Current (TIC) of digested haptoglobin sample. **B.** Frame-summed heatmap of entire haptoglobin digestion; *Left Spectrum*: Arrival Time (ms) vs Ion Count; *Upper Spectrum*: Ion Count vs m/z

Each LC-HRIM-MS feature within the haptoglobin digestion was analyzed semi-automatically. Coeluting glycopeptide isomer candidates were identified using PMI's HRIM Peptide software package and manually verified. Each feature was defined by starting and ending m/z values and starting and ending arrival time (ms) values. These boundaries were used to extract ion chromatograms (extracted ion chromatograms, EICs) for each feature.

Potential Isomers of NATAK-HexNAc(5)Hex(6) $[M+2H]^{2+}$

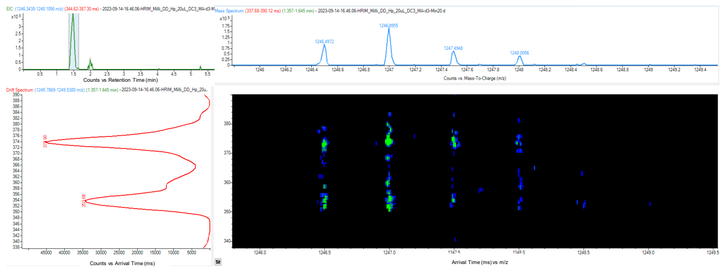

Figure 3: HRIM-MS heatmap of suspected NATAK-HexNAc(5)Hex(6) $[M+2H]^{2+}$ glycopeptide, *m/z* = 1246.5, RT = 1.41 min; *Upper Left Spectrum*: EIC of this feature; *Lower Left Spectrum:* Ion Arrival Time (ms) vs. Ion Count; *Upper Middle Spectrum:* Ion Count vs. *m/z.*

Glycopeptide NATAK-HexNAc(5)Hex(6)$[M+2H]^{2+}$ was identified as eluting at 1.41 min (same glycopeptide was identified at 2.26 min in 70 min MSMS run). Two potential glycoforms of this peptide with the same retention time and m/z were resolved with ion mobility.

Potential Isomers NLFLNHSE-HexNAc(5)Hex(6) $[M+2H]^{2+}$

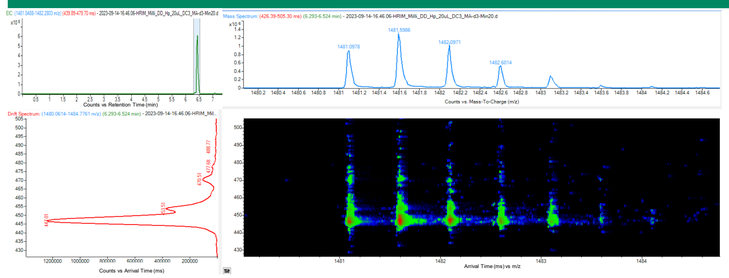

Figure 4: HRIM-MS heatmap of suspected NLFLNHSE-HexNAc(5)Hex(6) $[M+2H]^{2+}$ glycopeptide, *m/z* = 1481.1, RT = 6.45 min; *Upper Left Spectrum*: EIC of this feature; *Lower Left Spectrum:* Ion Arrival Time (ms) vs. Ion Count; *Upper Middle Spectrum:* Ion Count vs. *m/z.*

Glycopeptide NLFLNHSE-HexNAc(5)Hex(6) $[M+2H]^{2+}$ was identified as eluting at 6.45 min. Three potential glycoforms of this peptide with the same retention time were partially-to-fully resolved with ion mobility.

Possible Glycan Isomer Structures

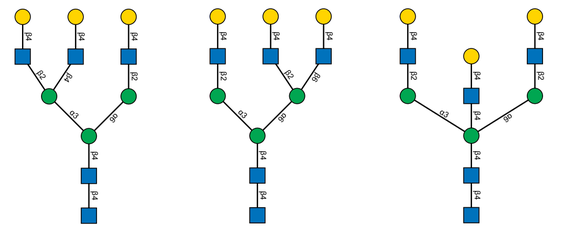

Figure 5: Comparison of possible HexNAc(5)Hex(6) N-linked glycan isomeric structures.

Analysis of LC-MS/MS data (not shown) appeared to have several coeluting ions with no unique fragments to allude to specific glycopeptide structures; conversely, analysis of LC-HRIM-MS data revealed ion features with unique arrival times indicative of possible isomeric glycoforms (Figures 3 and 4).

Conclusions

- High-resolution ion mobility offers an orthogonal separation technique for identification of glycopeptides and potential isomers in a complex mixture.
- Glycopeptide separation using the MOBIE platform allows shortening of chromatographic runs, thereby increasing sample processing throughput.