Separation of glycopeptides and their glycoforms using HILIC columns and UPLC/MS^E system

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OUTLINE

- **A failure to characterize the glycosylation of a therapeutic protein means that changes in their efficacy may be poorly understood. Comparison of HILIC and RP-LC results is shown indicating where RP methods alone would not be sufficient.**
- **A novel Ultra Performance Liquid Chromatography (UPLC) hydrophilic interaction chromatography (HILIC) columns method is presented here. HILIC mode enabled complete separation of glycopeptides from other non-glycosylated tryptic peptides as well as UV quantification of glycan micro-heterogeneity.**
- **Mass spectrometry detection (UPLC/MS^E) provided simultaneous qualitative and quantitative information (peptide mapping and glycosylation analysis).**

RESULTS

1. **Figure 1 illustrates that RP-UPLC is capable of partially resolving glycosylated peptides.**
2. **Figure 2 shows HILIC separation of an IgG peptide map. Note that glycosylated peptides are better retained than non-glycosylated ones.**
3. **Figure 3A shows that glycosylated peptides are well resolved in HILIC mode. Relative quantification is possible at 280 nm using peptide UV absorbance. In cases of incomplete resolution, the MS signal can be used for relative quantification (Figures 3B and 3C).**
4. **In Figures 3B and 3C the extracted ion chromatograms (XIC) confirm the presence of Man5, which coelutes with a G1Fa isomer of the peptide and is therefore invisible in the UV and TIC traces.**
5. **Quantitation by MS XIC or UV is comparable (see Tables in Fig.s 3A and 3B). The relative ratio of glycoform peaks is also in good agreement with LC-fluorescent analysis of 2AB labeled released glycans (data not shown).**
6. **The elevated energy MS trace contains peptide sequence information and can be used to investigate which peptides are glycosylated. Those peptides have characteristic fragments at m/z 204.10 and 366.15 originating from carbohydrate moieties (Figure 4).**

Figure 1: RP-LC peptide map of IgG. Glycopeptides with EEQFNSTFR are only partially resolved chromatographically due to micro-heterogeneity of glycans. (A) UV chromatogram, (B) extracted MS chromatograms. ACQUITY UPLC BEH130 PST 2.1 x 100 mm, 1.7 µm column, A: 0.1% TFA in water, B: 0.08% TFA in MeCN, grad. 0-50% B in 118 min.

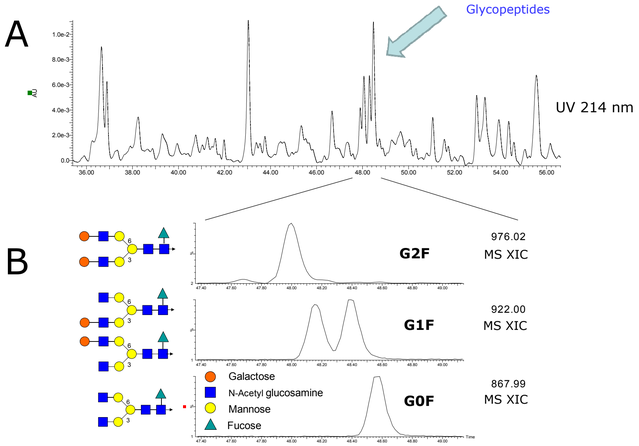


Figure 2: HILIC peptide map of IgG. Glycopeptides are resolved from non-glycopeptides; glycoforms are also resolved due to micro-heterogeneity of glycans. (A) Base peak ion (BPI) MS chromatogram, (B) UV 280 nm chromatogram. Glycopeptides are highlighted. ACQUITY UPLC BEH glycan column 2.1 x 150 mm, 1.7 µm , A: 10mM amm. formate, pH 4.5, B: 10 mM amm. formate in 90% MeCN, grad. 90-55 % B in 45 min. ACQUITY TUV and Synapt MS QTof instruments.

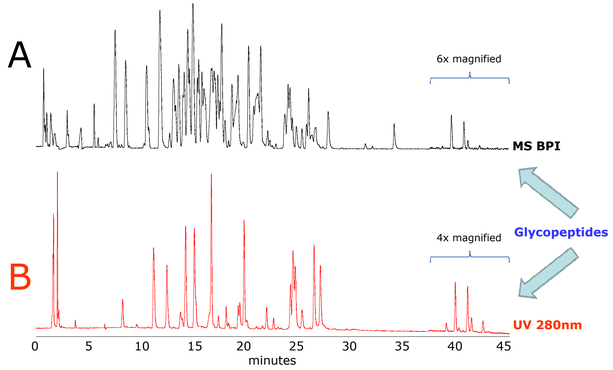
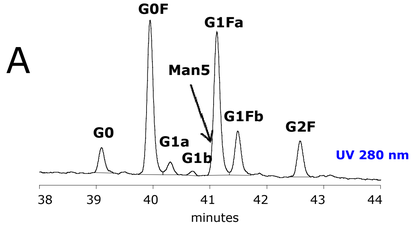
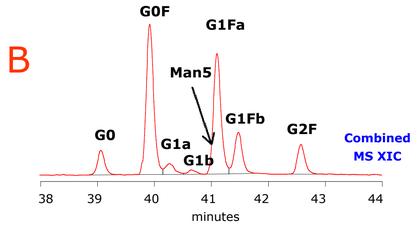


Figure 3: Detailed view of the separation of IgG glycopeptides in HILIC mode. The glycoforms of the peptide EEQYNSTYR are resolved reflecting various glycans. (A) UV chromatogram, (B) Combined XIC MS traces, (C) individual XIC chromatograms.



Rel. quantitation of glycopep. %	UV 280 nm			RSD %
G0	6.3	±	0.3	4.6
G0F	35.7	±	0.2	0.5
G1 a	3.1	±	0.1	4.4
G1 b	0.8	±	0.1	14.4
Man5	-		-	-
G1F a	34.4	±	0.1	0.2
G1F b	11.2	±	0.0	0.2
G2F	8.5	±	0.2	2.0



Rel. quantitation of glycopep. %	XIC MS			RSD %
G0	6.1	±	0.1	1.4
G0F	38.3	±	0.8	2.1
G1 a	2.4	±	0.2	10.3
G1 b	1.1	±	0.1	9.5
Man5	1.1	±	0.0	2.8
G1F a	31.2	±	0.6	1.9
G1F b	11.6	±	0.2	1.6
G2F	8.2	±	0.3	4.0

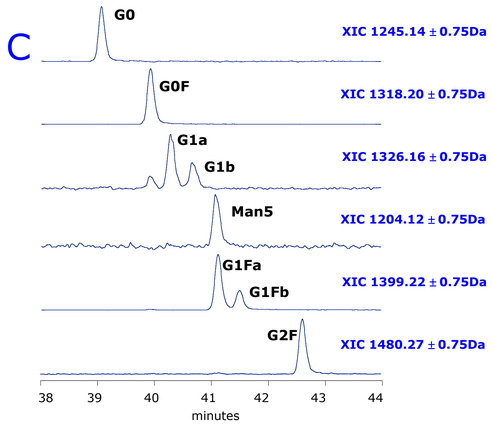
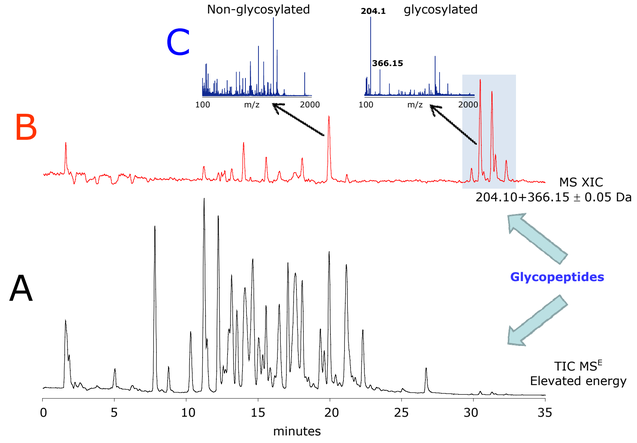


Figure 4: HILIC MS^E analysis of an IgG digest. MS^E data obtained at elevated collision energy are useful to confirm which peptides are glycosylated - characteristic glycan fragment ions are generated. (A) Total ion MS chromatogram (TIC), (B) XIC MS chromatogram of 204.10 and 366.15 Da sugar ions , (C) Examples of two MS^E spectra. HILIC grad. 90-40 % B in 45 min



FEATURES OF LC/MS^E

- **Two MS chromatograms are acquired in parallel (precursor-MS and fragment-MS^E data)**
- **MS chromatogram provides quantitative information.**
- **Combination of MS and MS at elevated energy data provide for sequence information.**
- **Improved detection sensitivity for low abundance ions in a complex mixture.**
- **Run-to-run reproducibility for MS^E is higher than data-dependent methods**
- **Glycopeptides can be assigned by characteristic 204.10 and 366.15 Da sugar ions.**

CONCLUSIONS

1. **Novel Glycan HLIC columns packed with sub 2 µm amide sorbent developed for separation of 2-AB labeled glycans are also useful for analysis of peptide maps and glycosylated peptides in particular.**
2. **UPLC/MS^E analysis can tentatively assign glycosylated peptides via their retention and MS ion pattern (fragments with m/z 204.10 and 366.15 Da).**
3. **Quantitative analysis of glycosylation can be performed by UV (detecting peptide absorbance).**
4. **Incompletely resolved glycoforms can be quantified using MS signal.**

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