IMPROVED GLYCOPEPTIDE FINGERPRINTING AND MASS SPECTRA FOR CHARACTERIZATION OF RECOMBINANT AND ISOLATED GLYCOPROTEINS

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INTRODUCTION

Characterization of glycoproteins entails analysis of the primary structure of the protein and analysis of the attached oligosaccharides. Oligosaccharides are routinely characterizing by mapping after release from the protein. Peptide maps have the advantage of providing information about the site-specific micro heterogeneity of the glycans. The enhanced chromatographic resolution associated with Ultra Performance Liquid Chromatography™ has been demonstrated for peptide mapping.[1] The technique improves resolution by a factor of three or more. The Peptide Separation Technology Columns for UPLC™ include both 130Å and 300Å pore size materials. The separation of two protein digests on these pore sizes are compared here. One digested protein contains large highly sialylated N-linked and O-linked structures. A mouse monoclonal antibody, contains smaller non-sialylated glycans. Coupling UPLC™ chromatography to oa-Tof mass spectrometry allows high sensitivity identification of glycopeptides by deconvoluted exact mass measurement. Optimized chromatographic resolution of individual glycoforms of glycopeptides provides excellence in mass spectral quality, and may provide a simple UV method of glycoform quantitation.

METHODS

UPLC Conditions

Injection Volume:	10 µL of 3-5 pmol/µL digest solution
Columns:	Peptide Separation Technology ACQUITY UPLC™ BEH 130 C_{18} 1.7 µm 2.1 x 100mm ACQUITY UPLC™ BEH 300 C_{18} 1.7 µm 2.1 x 100mm
Temperature:	40°C (unless otherwise noted)
Flow Rate:	100 µL/min (unless otherwise noted)
Solvent A:	0.1% TFA in water
Solvent B:	0.08% TFA in acetonitrile
Mixer:	High Sensitivity Peptide Analysis mixer (P/N 205000403)

Gradient Table:

Time (min)	%A	%B
Init	100	0
2	100	0
118	50	50
120	25	75
122	25	75
125	100	0

MS Conditions

LCT Premier™	oa-Tof mass spectrometer
Capillary:	3000V
Cone:	35V
Source Temperature:	100°C
Source Pressure:	1.4 x e^0 Torr
Scan Range:	300-3000 *m/z*
Scan Rate:	2 scans/sec

RESULTS

Figure 1

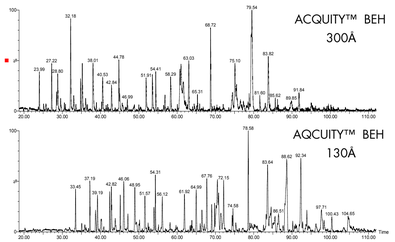


Figure 1. Comparison of the separation of tryptic peptides of bovine fetuin. Both the 300Å and 130Å pore size ACQUITY UPLC™ 1.7µm Peptide Separation Technology Columns provide excellent resolving power for peptides. For a more detailed discussion see Poster Th101.

Sialylated N– and O-linked Glycopeptides

Figure 2

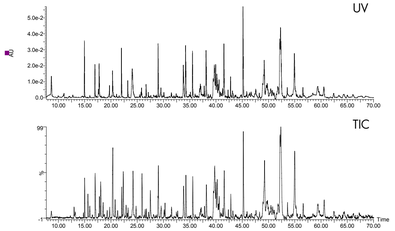


Figure 2. Tryptic digest of bovine fetuin analyzed with UPLC on a 300Å pore size C_{18} UPLC™ column with TFA mobile-phase modifier. Glycopeptide peak shapes are narrow, symmetrical and yield useful spectra.

MS Conditions for Glycan Detection

Glycopeptides were detected using the classic method of source fragmentation to create diagnostic glycan ions.[2] MS scans were alternated between normal source conditions (Aperture 1 = 15V) and fragmentation conditions (Aperture 1 = 50V). The fragment ion, *m/z* 657, identifies the presence of N- and O-linked sialylated glycans. Sialylated glycan termini, both N– and O-linked, produce *m/z* 292 (NeuAc) and *m/z* 657 (NeuAc-Gal-GlcNAc).

Figure 3

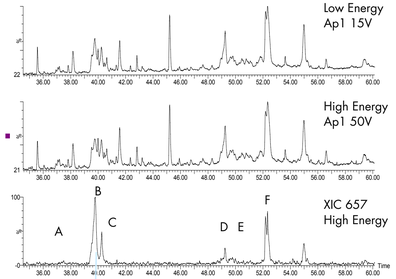


Figure 3. The intensity of the total ion chromatograms from the fetuin trypsin digest is comparable whether the source is tuned for molecular ions or fragments. Numerous sialylated glycopeptides were observed by fragment analysis. The flow rate in this case was 200 µL/min. Labeled peptides are described in Table I

Figure 4

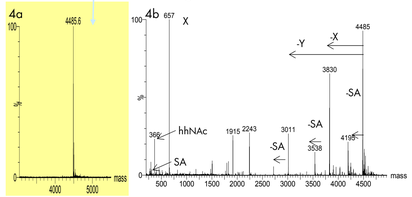


Figure 4. Deconvoluted mass spectra of the 3 sialic acid-containing region of Peak B. The spectra were collected in the same run at low (4a) or high (4b) aperture1 potentials. Under high energy conditions fragments are observed. With good chromatographic resolution useful glycan structural information is obtained in MS only mode.

Table I

Peak	Peptide	Major Glycoforms	Resolution
A	T10	O- 1 sialo-hex-hexNAc " +1 sialic acid	Overlapped
B	T11-12	N- Trisialo-Triantennary " +1 sialic acid	Resolved
C	T12	N- Trisialo-Triantennary " +1 sialic acid	Partially Resolved
D	T7	N- Trisialo-Triantennary " +1 sialic acid	Overlapped
E	T20	O- 3 sialo-hex-hexNAc 3 " +1 sialic acid 2 sialo-hex-hexNAc	Partially Resolved Resolved
F	T13	N- Trisialo-Triantennary " +1 sialic acid	Partially Resolved

Neutral N-linked Glycopeptides

Figure 5

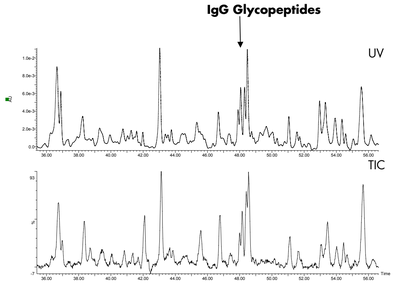


Figure 5. The glycopeptide containing portion of UPLC™ chromatograms are shown for a mouse IgG tryptic digest. The glycoforms have been significantly resolved.

Figure 6

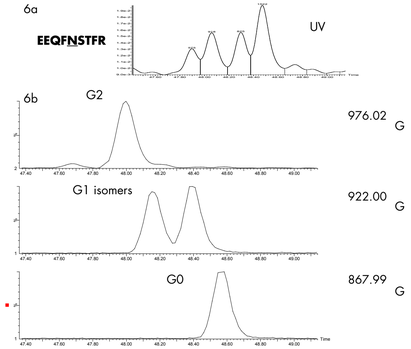
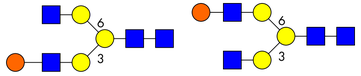


Figure 6. Mouse IgG glycoforms were detected by UV (6a) and extracted ion chromatograms (6b) of the triply-charged glycopeptides. Four peaks were observed for the three molecular weights indicating that the positional isomers of G1 have been separated by UPLC™.



G1 isomers

Optimizing Glycopeptide Chromatography

Figure 7

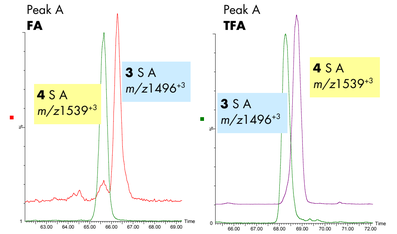


Figure 7. The fetuin peptide T11-12 has two major glycoforms. Both are complex triantennary structures with either 3 or 4 sialic acid termini. Elution order is reversed when formic acid is replaced with TFA.[3]

Figure 8

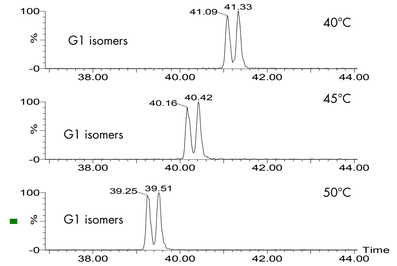


Figure 8. Increased temperature shortens IgG isomer run time without loss of resolution. In some cases the glycopeptide have been significantly shifted.

Figure 9

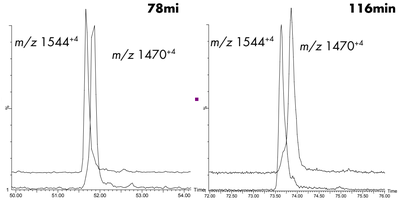


Figure 9. Shallow gradient slopes can improve resolution. Here are two glycoforms of T13, weighing 5876 and 6167. At 200µL/min in TFA buffer the decreased gradient slope increased the resolution.

Figure 10

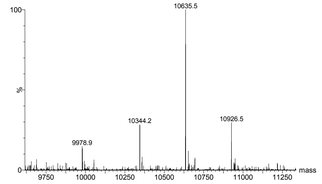


Figure 10. The largest glycopeptides observed in these experiments represents missed cleavages in the fetuin digest. The glycoforms have molecular weights approximately 10 kDa, and were separated on the 130Å pore size, 1.7 µm particle packing material.

DISCUSSION

Total ion chromatograms of bovine fetuin tryptic digest show many well resolved peaks on the two UPLC™ packing materials tested. The overall retention of the peptides and glycopeptides differ by approximately 4% acetonitrile, and narrow peak widths of 4 to 6 seconds are typical for both the 130Å and 300Å pore sizes. We undertook the study of glycopeptide chromatographic behavior on these materials.

The LCT Premier was adjusted to a source pressure of 1.4 x e^0. The aperture 1 voltage was toggled between 15V and 50V to obtain the molecular ions (15V) and glycan fragmentation (50V). Resolution of glycoforms, including isomers, has been achieved by UPLC™. In the monoclonal antibody example, the four glycopeptide peaks averaged 0.113 minutes in width. The complex glycoforms studied produced peak widths from 6 to 9 seconds. The glycopeptides detected by in-source fragmentation had strong UV absorbance consistent with UV quantitation.

CONCLUSION

- **Chromatographic resolution provides excellence in mass spectra of glycopeptides.**
- **High resolution separation of glycopeptides has been achieved on 300Å and 130Å pore size 1.7 µm particles.**
- **Good peak shape is obtained for sialylated and non-sialylated glycopeptides including positional isomers.**
- **Changing the gradient slope, temperature or mobile phase modifier can optimize separations.**
- **Data obtained in MS only mode produced spectra characterizing complex sialylated glycan structures.**

References

1. Mazzeo, J.R.; Wheat, T.A., Gillece-Castro, B.L. and Lu, Ziling *BioPharm International*, **2006**, *19:1*, 1-9
2. Huddleston; M.J., Bean; M.F. and Carr, S.A. *Anal. Chem.* **1993**, *65* 877-884.
3. Medzihradszky, K.F.; Besman, M.J. and Burlingame, A.L. *Anal Chem.* **1997,** *69*, 3986-3994.

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