Development of a Glycan Database For Waters Ultra Performance Liquid Chromatography (UPLC) Systems

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Abstract

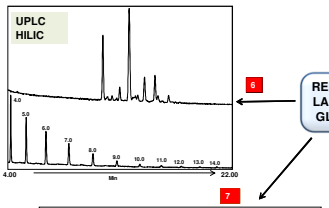
Glycosylation is the most complex post-translational protein modification and influences protein structure and function. Over half of all proteins are glycosylated and in order to elucidate their function we also need to understand their glycan composition and structure. The two major classes of glycans are *N*-and *O*-linked glycans. *N*-glycans derive their name from the nitrogen in the side chain of asparagine with in the sequence Asn-Xaa-Ser/Thr. Manufacturing of protein life sciences products can be very difficult and a number of factors can have a major effect upon their glycosylation, such as dissolved oxygen, pH, carbon source and temperature. Such fluctuations in the process can put product integrity at risk and thus the ability to correctly monitor glycosylation is required. In collaboration with Waters, NIBRT has developed an *N*- glycan database for the ACQUITY/H-class UPLC. GlycoBase 3+ is based upon reproducible separation technology (HILIC-UPLC) with tight standard deviation, coupled with a bioinformatics/web-enabled database solution. Glycobase 3+ is currently populated with over 300 2-AB-labeled *N*-linked Glycan structures common to the biopharmaceutical industry. This novel database will assist in the identification of *N*-linked glycans based on a variety of data attributes and will facilitate the rigorous characterization of biopharmaceutical protein products.

Materials & Methods

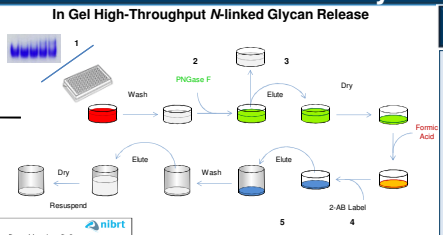
Herceptin® monoclonal antibody glycans were released via in-gel blocks. Ribonuclease B and yeast Invertase were separated first by 15% SDS-PAGE. *N*-glycans were released with peptide-*N*-glycosidase F and were labelled with 2-aminobenzamide (2AB). All labelled glycans were analysed on a Waters ACQUITY UPLC with a BEH (Bridged Ethyl Hybrid particles) glycan chromatography column (2.1 mm X 150 mm 1.7 µm) and fluorescent detection was achieved using a Waters FLR Fluorescence Detector (Waters Corporation, Millford, MA, USA). Instrument control and data acquisition was done under the control of Empower chromatography workstation software. A linear gradient of 50 mM ammonium formate buffer, pH 4.4 and acetonitrile was used for glycan separation. The detection wavelengths were λ_{ex} = 330 nm and λ_{em} = 420 nm. For MALDI analysis (Waters MALDI Micro MX.22) dried glycan samples were dissolved in 1-2 µl of 0.1% TFA. 10mg/ml DHB (2,5-dihydroxybenzoic acid) matrix containing 1 mM ammonium citrate was added and samples were spotted onto the target plate. A Quadrupole-TOF Ultima Global (Waters MS-Technologies) was also used in negative-ion mode. Dried sample was re-suspended in 25% MeOH and directly infused via a proxeon borosilicate capillary.

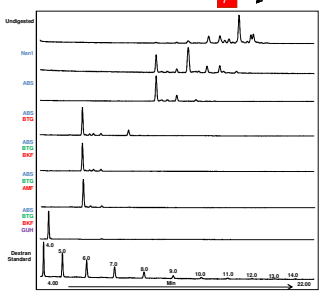
High-Throughput UPLC based Method for *N*-linked Glycan Analysis

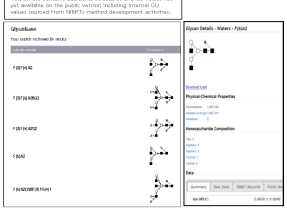
1. Glycoprotein Immobilisation
2. Glycan Release
3. Glycan Elution
4. 2-AB Labelling
5. 2-AB Glycan Elution
6. HILIC UPLC Pooled Glycoprofiling
7. Exoglycosidase Digestions
8. Preliminary Assignments
9. Structure Assignment

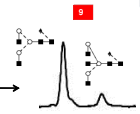


RELEASED LABELLED GLYCANS









Nomenclature

- Specific symbols and abbreviations are used for each identified residue
- The linkage and anomericity between the monosaccharide's is clearly shown
- Linkage type is characterized by either a solid line (β-linkage) or a dashed line (α-linkage), where linkages have not been determined, a waved-line is used
- Linkage positions are indicated by the angle linking two sugar residues

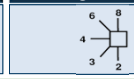
Glycan Symbols

GalNAc	Glc
Fuc (deoxy galactose)	GlcNAc
Man	NeuNAc
Xylose	Gal

Glycan Linkages

- β-linkage
- α-linkage
- unknown β-linkage
- unknown α-linkage

Linkage Positions



Results

Figure 1: Herceptin® (trastuzumab) Monoclonal antibody

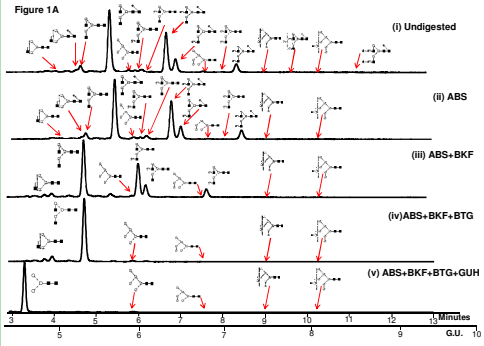


Figure 1A: UPLC analysis of total Herceptin® (trastuzumab) monoclonal antibody glycans and exoglycosidase array by HILIC-fluorescence (20% of the released *N*-glycan pool)(10 replicates), **(i)** Whole *N*-glycan pool released by PNGase F, **(ii)** ABS releases α2-3 /6/8 sialic acids. **(iii)** Fucosidase from bovine kidney (BFK) releases α1-2,1-6 fucose. **(iv)** Bovine testes ß-galactosidase (*BTG*) releases galactose β1-3,1-4 linkages and **(v)** GUH (hexosaminidase) release β GlcNAc but not GlcNAc linked to β 1-4 Man

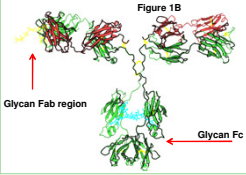


Figure 1B: Molecular model of IgG1 depicting the two conserved glycan site one in C_H2 domain (Asn 297) within the antibody. Glycans in the Fc region are critical to antibody binding to the Fcγ receptors.

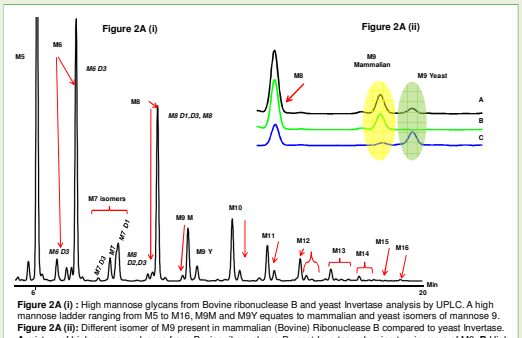


Figure 2A (i) : High mannose glycans from Bovine ribonuclease B and yeast Invertase analysis by UPLC. A high mannose ladder ranging from M5 to M16, M9M and M9Y equates to mammalian and yeast isomers of mannose 9. **Figure 2A (ii):** Different isomer of M9 present in mammalian (Bovine) Ribonuclease B compared to yeast Invertase. **A** mixture of high mannose glycans from Bovine ribonuclease B yeast Invertase showing two isomers of M9. **B** High mannose glycans from ribonuclease B and **C** High mannose glycans from yeast Invertase, confirming two distinct M9 isomers present in mammals and yeast.

Figure 2: Mammalian (Bovine) Ribonuclease B and yeast Invertase

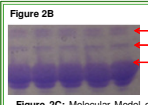


Figure 2B: One dimensional SDS PAGE separation of Ribonuclease B, two contamination bands where observed **(ii, iii)** and confirmed by LC-MS/MS analysis to be transferrin and serotransferrin thereby confirming the need to check the purity of Glycoproteins

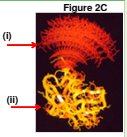


Figure 2C: Molecular Model of Ribonuclease B **(i)** indicates the position of the major Glycan structure (High Mannose) on this protein. **(ii)** Ribonuclease B protein. **Woods RJ, Edge CJ, Dwek RA. Nat Struct Biol. 1994 Aug;1(8):499-501.**

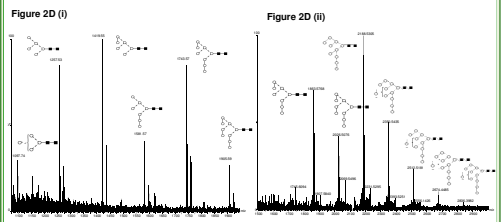


Figure 2D (i): Bovine ribonuclease B total *N*-glycan pool analysed using a Waters MALDI Micro MX, preliminary results suggest that in this mass range, Man4 to Man9 glycans are present (2AB labelled Na^+ adducts). **Figure 2D (ii):** Yeast Invertase total *N*-glycan pool, preliminary results suggest that in this mass range, Man9 to Man14 glycans are present (non 2AB labelled K^+ adducts).

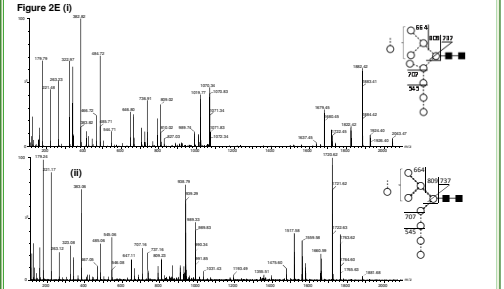
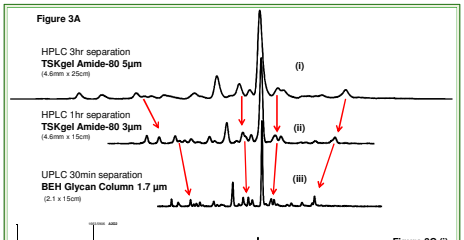
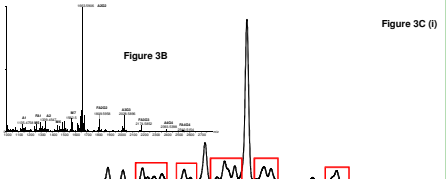


Figure 2E: Negative ion MS/MS spectrum of Invertase Man9 **(i)** and Man 10 **(ii)** (Waters-Micromass QTOF Ultima Global)**.** The diagnostic ions illustrating the Man_9 isomer, which is distinctive to Invertase, are the D- and ^{0,4}A-type ions 809 and 737 of the core mannose residue. The Man_{10} isomer from yeast Invertase displays a similar fragmentation to the Man_9 isomer. The additional hexose is on the 3-arm with the likely linkage shown above. It is important to note this is further supported with the absence of ions: 826 (664+162), 971 (809+162) and 899 (737+162).

Figure 3:UPLC resolution





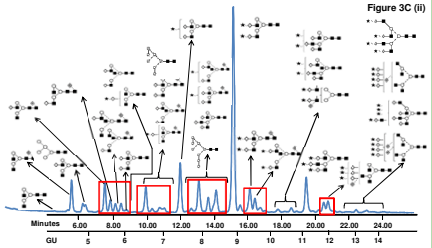


Figure 3A: Comparison of The 3 hour method **(i)** to our current 1 hour HPLC separation of complex human serum glycans on a TSK-GEL 3 µm Amide-80 columns **(ii)** to a WATER's UPLC method with BEH Glycan column 1.7µm, 2.1mmx15cm **(iii)**. **Figure 3B:** ABS exoglycosidase digest analysed by Waters MALDI Micro MX of human serum glycans confirmed the non sialylated structures present. **Figure 3C** The same serum glycans analysed on a Water's ACQUITY UPLC **(ii)**, it can be observed that there is dramatic decrease in run times and improved separation of glycans compared with the current HPLC methods **(i)**. The most abundant glycans in each peak are shown.

Conclusions

- ACQUITY UPLC with BEH glycan Columns (1.7 µm) can rapidly resolve Herceptin *N*-linked glycan structures in 12 minutes
- UPLC is faster than the traditional glycan normal-phase HPLC runs, that can take from 1 to 3 hours to complete.
- The ACQUITY UPLC brings the benefits of shorter run times, better resolution and higher sensitivity, compared with standard HPLC applications.

Future work

- Development of UPLC-FLR-MS/MS analysis fro glycans and UPLC-MS/MS analysis for glycopeptides

Acknowledgements & References

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