Evaluating an Isotopically Labeled IgG for the Relative and Absolute Quantitation of N-linked Glycans

Ron Orlando,[1,2] Shujuan Tao,[1] Yining Huang,[1] Emily Betchy,[1] Barry Boyes,[1,2,3] Alex Harvey[2]

[1]Complex Carbohydrate Research Center & Departments of Biochemistry & Molecular Biology and Chemistry, University of Georgia, Athens, GA30602

[2]GlycoScientific, LLC, Athens, GA 30602

[3]Advanced Materials Technology Inc, Wilmington, DE

Overview

Evaluate a mouse monoclonal IgG (Mab) possessing isotopically labeled glycans (iGlycoMab) for use as an internal standard for both the relative and absolute quantitation of N-linked glycans.

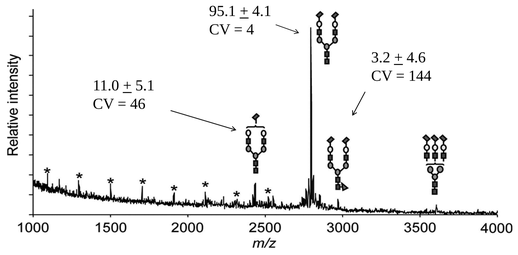
Introduction

The ability to accurately quantitate the glycan chains attached to glycoproteins has wide-ranging implications. Numerous studies over the past 40 years have demonstrated that abnormal glycosylation occurs in virtually all types of human cancers, and demonstrate the potential of using glycan markers in either a diagnostic or a prognostic manner. The glycosylation on recombinant protein therapeutics is also known to have profound effects, with one of the better known examples being the increased serum half-lives of Erythropoietin (EPO) resulting from glycoengineering. Hence, the quantification of glycoprotein glycans play important roles from the discovery of new diagnostic/prognostic markers to the development of various therapeutic agents.

A current impediment for performing quantitative glycomics is the shortage of widely available standard glycoproteins and isotopically labeled reagents to enable accurate quantitation. The issue with glycan quantitation was highlighted by inter-laboratory studies conducted by the Human Proteome Organization (HUPO) and the Association of Biomolecular Resource Facilities (ABRF). Both of these studies demonstrated errors greater than several hundred percent in the analysis of mid-to-low level glycans were compared across participating laboratories.

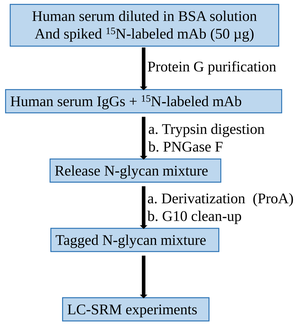
HUPO 2007 – Glycan Quantitation[1]

MALDI-MS of permethylated glycans from sample transferrin



The inability to accurately quantitate low level glycans is particularly worrisome since it is often glycans of low abundance that have the largest impact, as is seen with the therapeutic human intravenous immunoglobulin G (IVIg).

Experimental



Samples were prepared by performing a serial dilution of human serum with a solution of 80 mg/mL bovine serum albumin. Each sample was spiked with the same amount of the ^{15}N labeled mAb, to give a concentration of 0.2 µg/µL in each sample. The ^{15}N mouse mAB (iGlycoMab) was produced by Glycoscientific with a patent pending process. Serum IgGs and the ^{15}N mAb were purified from the other proteins using a protein G column. Glycans were prepared by a general glycomics workflow.[2] Briefly, N-glycans were released with PNGase F, then derivatized by reductive amination with Procainamide (ProA). After clean-up, the labeled N-glycans were separated on a 2.1 mm ID Penta-HILIC column operated at 0.4mL/min, 60°C on a Nexera UFLC system, which was coupled in series to a UV detector then a Q-Trap 4000 MS analyzer. The relative ratios of the glycans were obtained by SRM detection of the analyte glycans, and the isotopically labeled glycans were used as internal standard for quantitation.

Acknowledgement

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Numbers R41GM113666-01 (RO) and R44GM093747-02 (BB).

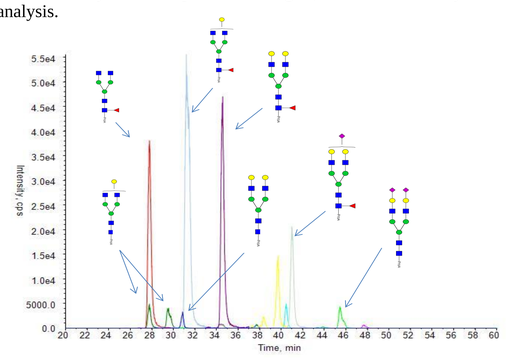
References

1. Wada Y et al. Glycobiology 2007;17:411-422
2. Tao, SJ et el, Anal. Chem. 2014:86 (21), 10584-10590.
3. Markey SP. Quantitative mass spectrometry. Biological Mass Spectrometry. 1981;8(9):426-30.

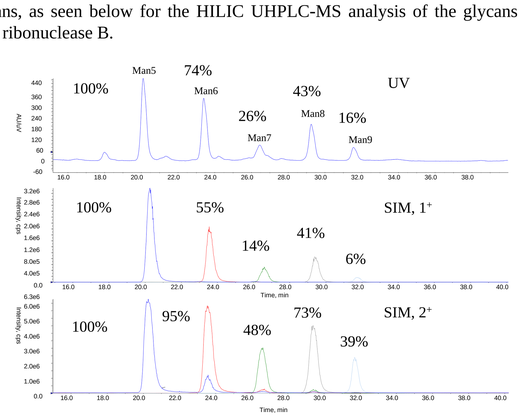
The Quantitation Quagmire

Typical glycan analytical workflows involve labeling the reducing terminus of the released glycans with a chromo/fluorophore, such as 2-Aminobenzamide (2-AB) or procainamide. This labeling enables the direct and accurate quantitation of glycans using spectrophotometric detection after a separation technique.

The issue with UV/florescence quantitation is that even with relatively simple mixtures, such as the glycans released from human serum IgGs, many of the glycans are not resolved by current separation strategies, as shown below by HILIC UHPLC-MS analysis.



The use of mass spectrometry (MS) allows the identification of these co-eluting components (as seen above), however the MS "detection efficiency" differs between glycans, as seen below for the HILIC UHPLC-MS analysis of the glycans released from ribonuclease B.



Use of an IgG with Isotopically Labeled Glycans as an Internal Standard

The large number of steps typically involved in the glycan workflow causes issues with the Robustness, Repeatability, and Reproducibility, which can be readily seen by comparing results obtained by different researchers analyzing the same samples. To investigate this aspect, three researchers analyzed two samples, one contained 10x the amount of human serum IgGs of the other.

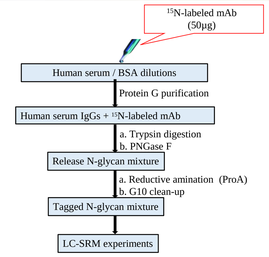
	Average	SD	CV	% Error
Researcher 1	5.3	0.8	16.1	-47.1
Researcher 2	6.8	0.5	7.3	-32.1
Researcher 3	9.7	0.8	8.4	-3.3
Combined	7.3	2.2	30.4	-27.4

These results demonstrate that each researcher is capable of obtaining consistent results, however these results differ from the other researchers and the actual value. Presumably, the large spread in deviations was associated with systematic errors, which would accumulating during the large number of parallel sample handling procedures.

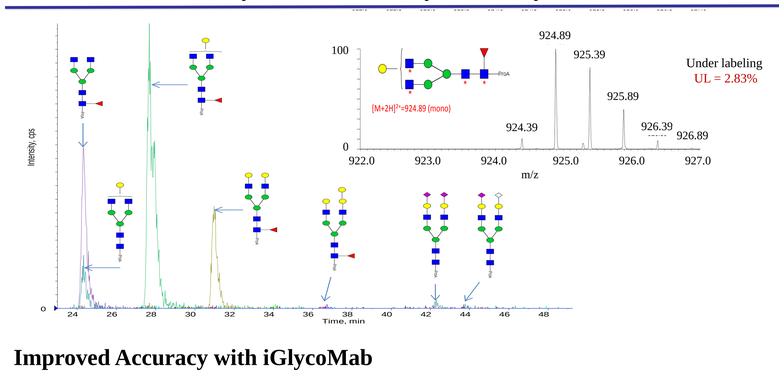
Combining the results of these researchers lead to a larger standard deviation (SD)/coefficient of variation (CV) without a significant improvement in the accuracy, once again indicative of systematic errors.

Use of an IgG with Isotopically Labeled Glycans as an Internal Standard

The use of internal standards is the accepted strategy to facilitate quantitation via MS.[3] The closer the chemical properties of the internal standard to its analyte, the better it compensates for the various sources of error, and thus the most desirable internal standard is typically an isotopically labeled version of the analyte itself.[3] Consequently, the optimal internal standard for glycoproteins is a labeled glycoprotein, which has led GlycoScientific to develop monoclonal antibodies with ^{15}N labeled glycans (iGlycoMabs). This enables the addition of the internal standard directly into the sample prior to processing and thus over comes systematic errors associated with parallel sample handling. The current work shows results with mouse iGlycoMab.

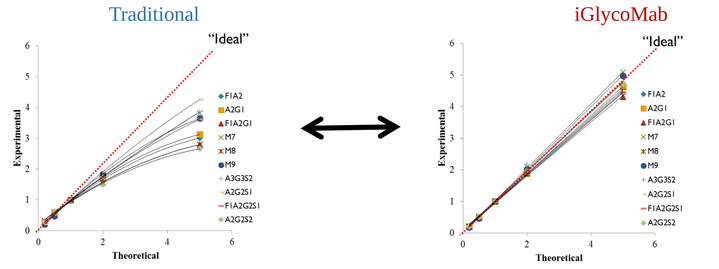


LC-SRM and MS Analysis of released iGlycoMab Glycans



Improved Accuracy with iGlycoMab

The use of iGlycoMab improves accuracy and overcomes systematic errors associated with parallel sample handling.



$$\text{Relative ratio} = \frac{I_x}{I_{50}}$$

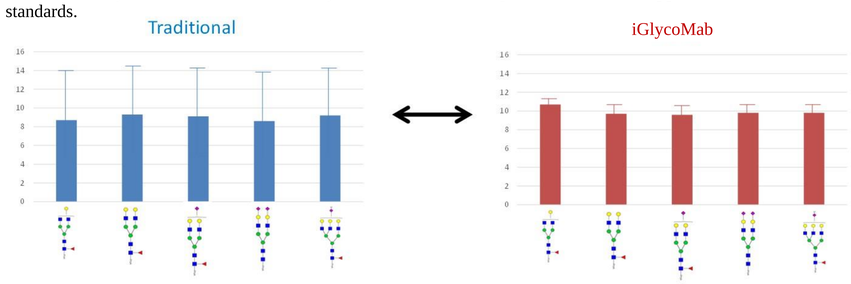
I_x is the signal intensity for N-glycan from x µL human serum IgG;
I_{50} is the signal intensity for N-glycan from 50 µL human serum IgG.

$$\text{Relative ratio} = \frac{I_x/M'_{50}}{I_{50}/M_{50}}$$

I_x is the signal intensity for N-glycan from x µL human serum IgG;
I_{50} is the signal intensity for N-glycan from 50 µL human serum IgG;
M'_{50} is the signal of 50 µg ^{15}N-labeled mAb in x µL human serum IgG;
M_{50} is the signal of 50 µg ^{15}N-labeled mAb in 50 µL human serum IgG.

Improved Precision with iGlycoMab

The use of iGlycoMab reduces the %CV by ~ a factor of 10 compared to traditional approaches without internal standards.



Conclusions:

The use of iGlycoMab improves both the accuracy and precision because it overcomes systematic errors including those associated with parallel sample handling.

iGlycoMab can be added directly to any glycoprotein sample before any sample processing.

iGlycoMab can be used with any glycan/glycomics work-up/analysis (not limited to mAb)