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Targeted, Site-specific quantitation of N- and O-glycopeptides of Biologics using ^{18}O–labeling and product ion based mass spectrometry

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INTRODUCTION

It has been well established that glycosylation of therapeutic proteins have profound impact on their solubility, stability, pharmacokinetics and pharmacodynamics. Thus, establishing the comparability of glycosylation patterns between the innovator and biosimilar is an important critical attribute for the approval of biosimilars. Currently, quantitation of N-glycans from therapeutic IgG1 like antibodies is performed using fluorescence based labeling techniques. However, for some non-IgG therapeutic proteins such as Etanercept with multiple glycosylation sites, this method is less informative. Thus, there is an immediate need to develop an accurate and robust method for relative quantitation of glycopeptides in a site-specific manner. Here, we used glycopeptide product ions and 18O–labeling of C-terminal carboxyl group as a strategy to obtain quantitative information on glycopeptides. We used the peak areas of y and y0 ions, produced under higher energy CID fragmentation of N-, O-glycopeptides and a-glycopeptides, for the quantitation of glycopeptides and their occupancy. Furthermore, we used C-terminal 18O–labeled glycopeptides of innovator samples as reference standard for accurate determination of intensity fold change of glycopeptides in a site-specific manner. The accuracy, robustness and validation of this relative glycopeptide quantitation method were established using Rituximab. We have also demonstrated for the first time, that the utility of this methodology for establishing the relative quantitation and similarity of N- and O-glycopeptides between innovator and biosimilar samples of Etanercept.

MATERIALS AND METHODS

Sample Preparation:
Protein samples were reduced and carboxymethylated and then mixed with Tris/HCl (pH 8.5) buffer containing either trypsin or trypsin and Asp-N. The whole mixture was freeze-dried and then reconstituted with either normal water or 18O–labeled water to generate unlabeled and labeled samples, respectively, and then digested at 36^{0}C for 18 h.

LCMS Conditions:
The Eksigent Nano-LC system with a gradient of mobile phase A: water + 0.1 % formic acid and mobile phase B: acetonitrile + 0.1 % formic acid was used for the separation of peptides. The digested samples were loaded on to a ZORBAX 300SB-C18 (5 µm, 5x0.3mm, Agilent) cartridge and washed for 10 min at 20 µL per min flow rate using loading pump and were eluted from trap columns were eluted into the C18 analytical column (HALO Fused-Core C18, 90Å, 2.7 µm, 100 x 0.5 mm, Eksigent) at a flow rate of 15 µL per min. The mass spectrometric analysis of the eluted peptides was performed using MRMHR workflow with a TripleTOF® 5600+ system (Sciex, Framingham, MA, USA). For a typical MRM-HR experiment, only the precursor ion mass/charge, CE values are provided as a list and after the data acquisition the XICs of product ions ion are extracted using MultiQuant™ software.

RESULTS

For glycopeptide quantitation, the MRM-HR strategy was used where 18O–labeled glycopeptides were used as internal controls (Figure 1). The digested innovator and biosimilar samples were labeled with 18O–water and 16O–water, respectively. For relative quantitation , both the samples were mixed in 1:1 ratio , followed by LCMS analysis. The relative abundance and fold change was then calculated based on ratio of average area of unlabeled ions to the sum total area of both labeled and unlabeled y-ions.

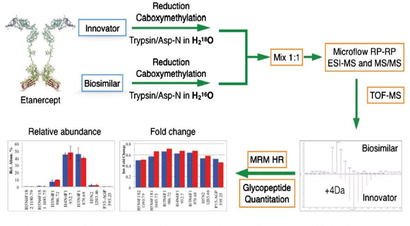

Figure 1: Overview of the strategy used for glycopeptide quantitation using MRM-HR.

In MRMHR strategy, looped MSMS spectra are acquired at high resolution and then fragment ions are extracted post-acquisition to generate MRM-like peaks for integration and quantification. The technique is sensitive and fast enough to enable performance similar to higher end triple quadrupole instruments. Here, fragment ions showing least interference were selected and in cases where the single fragment ion intensity was low, multiple sum ion were used for analysis. The Y-0 ion which is generally common to all glycoforms and the corresponding a-glycopeptides was mainly selected for quantitation.

Validation of the Methodology using Rituximab:

In order to validate our concept, commercially available Rituximab Innovator and Biosimilar were used for the relative quantitation of N-linked glycopeptides. The MRM-HR method was prepared for various precursors corresponding to corresponding to H5N4F1S2,H5N4F1S1, H4N4F1S1, H5N4F1, H4N4F1, H3N4F1, H5N2 glycopeptides and Fc-AGP, respectively. The collision energy for each precursor was ramped and optimized to get the best fragmentation pattern. Figure 2 shows a example MS and MSMS of H4N4F1 precursor ion 932.71 m/z (unlabeled) and 934.05 (labeled), where z is +3. The mass difference of 4Da was observed between the unlabeled and labeled precursor ion and y-ions of glycopeptides whereas as expected no mass difference was observed for the b-ions and oxonium ions between labeled and unlabeled product ions. Most of the glycopeptides showed ~~98.9 % labeling efficiency with minor variations.

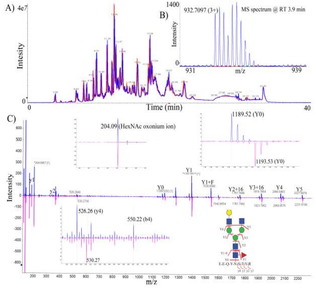

Figure 2: a) Overlay of TIC of Rituximab labeled and unlabeled digest in triplicate b) The TOFMS of precursor ions *m/z* 932.71 (unlabeled) and 934.05 (labeled) showing mass difference of 4Da, c) The mirror plot of MS/MS spectrum of labeled (blue) and unlabeled (pink). The inset shows the mirror plot of zoomed view for *m/z* 204.09 (oxonium ion), 526.26 (y4), 550.22 (b4) and 1189.52(Y0) ions.

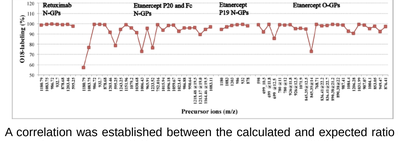

Figure 3: The labeling efficiency of the method calculated for Rituximab and Etanercept samples.

A correlation was established between the calculated and expected ratio of unlabeled vs labeled precursor and product ions based on XIC area. Excellent correlation ,~80 % average was observed for 18 different precursors in MS based quantitation whereas product ion based quantitation showed >95 % correlation (Fig. 4a). Further, in order to check the robustness of this method, results obtained were compared with the results obtained by conventional HILIC-HPLC analysis of 2-AB labeled glycans. The MSMS based quantitation showed similar trend with both the methods between the innovator and biosimilar Rituximab (Fig. 4c).

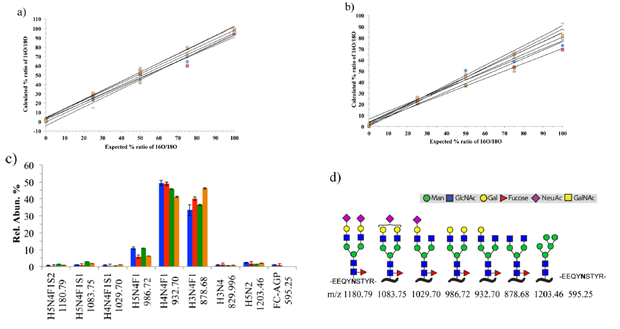

Figure 4: Validation 18O–labeling using Rituximab. Relative percentage intensity correlation between expected and observed labeling for product ion (a) and precursor ion (b) based quantitation of Rituximab glycopeptides. (c) Comparison of 16-O/18O-labeled glycopeptide quantitation (Red and Blue) with 2-AB labeled UHPLC analysis (Green and Orange) for Rituximab Innovator and Biosimilar samples respectively. The %CVs are represented as error bars. (d) The cartoon representation of glycans expressed on EEQYNSTYR peptide in Rituximab based on the CFG guidelines.

Demonstration of the method for N- and O-glycopeptide quantitation of Etanercept

Both MS and MSMS based quantitation was investigated for 92 precursor ions (46 unique GPs and AGPs) encompassing three orders of abundance (least abundant >0.05 %) and multiple charged species of N- and O-linked glycopeptides. Three N-linked glycopeptides corresponding to sequences DVVCKPCAPGTFSNTTSST (P19), DICRPHQICNVVAIPGNASM (P20) and EEQYNSTYR (P35) and four O-linked glycopeptide sequences LPAQVAFTPYAPEPGSTCR (P1), DAVCTSTSPTR (P21), SMAPGAVHLPQPVSTR (P22) and THTCPPCPAPELLGGP SVFFPPKPK (P27) were identified. The relative abundance of N-glycopeptides and AGP's in Innovator and Biosimilar of Etanercept are shown in Figure 5 and that of O-glycopeptides are shown in Figure 6

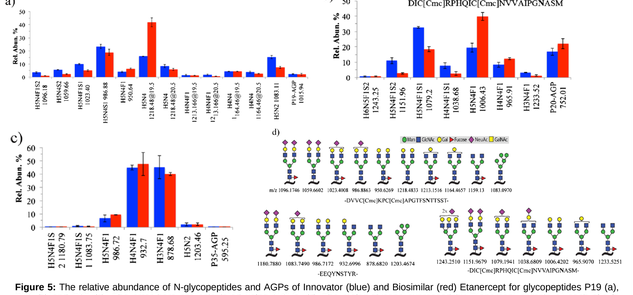

Figure 5: The relative abundance of N-glycopeptides and AGPs of Innovator (blue) and Biosimilar (red) Etanercept for glycopeptides P19 (a), P20 (b) and P35 (c). The error bars are calculated using %CVs. (d) The major glycans expressed by Etanercept sample at each site is represented in a cartoon form as per CFG guidelines

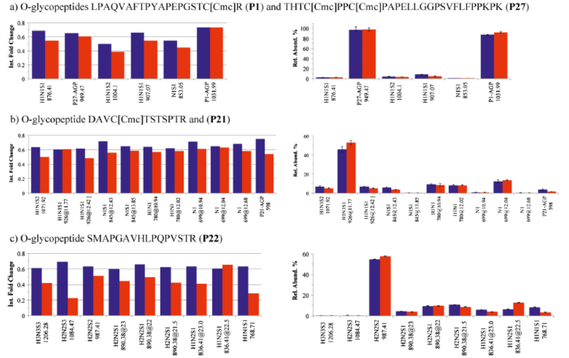

Figure 6: Etanercept Innovator (blue) and Biosimilar (red) Intensity fold-change (left) and relative abundance (right) of O-glycopeptides and AGPs for P1 and P27 (a), P21 (b) and P22 (c). The error bars on relative abundance data are %CVs

CONCLUSION:

1. The results showed a very similar site-specific expression of N- and O-glycopeptides between the samples but with minor variations.
2. The quantitative N-glycopeptide analysis of Etanercept Innovator and Biosimilar revealed that overall sialylation of N-glycans in Innovator sample is slightly higher compared to the commercially available biosimilar. Also, the fold-change in AGP's is low in Innovator as compared to Biosimilar Etanercept.
3. For the first time both N- and O-glycopeptides have been quantified for a glycopeptide using MRM-HR based strategy using 18-O labeled glycopeptides as internal controls.

REFERENCES:

1. J Srikanth et al. *Glycoconj J* 34 (1), 95-105, 2017.
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TRADEMARKS/LICENSING

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