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Development of Biosimilars: Analysis of Etanercept Glycosylation as a Case Study

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Abstract

Glycosylation is a critical attribute of glycoprotein products as it has been shown that the type and degree of glycosylation can have a significant impact on the product efficacy and immunogenicity. In developing generic forms of glycoprotein based therapeutic products, it is necessary to characterize the glycosylation of these products to ensure that it conforms with the original product as well as natural form of the product. In this study, we have focused on the characterization of the N-linked glycans and, in less details, on the O-linked glycans found on Etanercept (Enbrel™). Using a series of methodologies, we mapped the N-linked glycosylation sites in Etanercept and also defined the types of glycan structures associated with each site. Separately, we also determined the extent of Etanercept O-glycosylation and the type of O-linked glycans.

Keywords: O-linked glycans: N-linked glycans; Biosimilars; HILIC-HPLC; Glycan release; Glycopeptide; Peptide mapping; Mass spectroscopy

Introduction

Biosimilarity, as defined in Section 351 of the PHS Act, means that "the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components," and that "there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product".

Glycosylation is an important feature of biopharmaceutical products as it has been shown that the type and degree of glycosylation affects product stability, activity, antigenicity, and pharmacodynamics [1]. For example, it has been shown that the degree of glycosylation and sialic content have a significant impact on the pharmacokinetics properties of erythropoietin (EPO) [2]. In fact, deglycosylated EPO has been shown to display elevated potency levels when compared *in vitro* to the full glycosylated form of EPO [3] however deglycosylated EPO fails to exhibit any activity when evaluated *in vivo* [4,5] Glycosylation has also been found to be necessary for IgG antibody interaction with the Fc receptor and, therefore, modulation of the FcR-mediated effector function, including antibody dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) [6]. In light of the large body of evidence linking glycosylation to glycoprotein efficacy and immunogenicity, a detailed characterization of the carbohydrate moieties in glycoproteins is an expected and necessary requirement in the development of biosimilar products.

Experimentally, the analysis of N- and O-linked glycosylation typically starts with the release of the glycans by enzymatic or chemical means, followed by derivatization and then chromatographic separation with in line mass spectroscopy for relative quantitation and structural characterization of the glycan species. Further product characterization involves identifying the sites of linkage for the glycan moieties and their relative occupancy. In this paper, we present strategies along with results for the glycosylation analysis of Etanercept (Enbrel™) in order to determine both the type of N- and O-linked glycan species found to be associated with this glycoprotein and the sites of linkage of the N-linked glycans.

Materials and Methods

Enbrel Deformulation

Approximately 2 mg of Enbrel were applied onto a Poros Capture Select Fc column (Life Technologies - Carlsberg, CA) that had been equilibrated with 50mM sodium phosphate/150mM sodium chloride pH 7.0. After loading the sample and washing the column with 10 column volumes of equilibration buffer, the captured protein was eluted with 12mM hydrochloric acid/150mM sodium chloride pH 1.9. The elution was monitored by absorbance at 280 nm. The eluted protein peak was collected as a single fraction which was then quickly neutralized to pH 7.0 by the addition 200mM monobasic/dibasic sodium phosphate pH 8.5.

N-linked glycan release and analysis procedure

Approximately 100mg of deformulated Enbrel (Etanercept) was treated with ca. 2mU of Peptide-N-glycosidase F (PNGase F) (Prozyme - Hayward, CA) overnight at 37°C [7]. The released glycans were harvested with ice cold ethanol. While the precipitated protein from the extraction was saved for intact mass analysis by ESI-ToF mass spectroscopy; the supernatant fraction containing the released N-linked glycans was brought to dryness by centrifugal evaporation (Thermo Fisher Scientific – Cambridge, MA). The dried glycans were derivatized with 2-aminobenzamide (2AB) (Sigma – St. Louis, MO) [8] in the presence of sodium cyanoborohydride (Acros Organics, NJ). After excess dye clean-up using cellulose based SPE cartridges (Prozyme – Hayward, CA), the N-linked glycans were analyzed by HILIC chromatography [9] using a NH2P-50 2D column (2.0×150 mm, 5µm) (Shodex – New York, NY). Resolved peaks were relatively

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quantified based on peak area and then identified by mass spectroscopy using an ion-trap mass spectrometer (Model 6220; Agilent – Santa Clara, CA).

Intact mass analysis

N-linked deglycosylated Etanercept was reduced with 50mM Dithiothreitol (Sigma - St. Louis, MO) at 37°C for 45 minutes. The sample containing ca. 5 mg of protein was loaded onto a C4 Micro-Trap (Bruker – Billerica, MA). The Micro-Trap was washed with 0.1% formic acid in water with the flow going to waste; the retained protein was then eluted with a step gradient of 0.1% formic acid in acetonitrile after redirecting the column flow into an electrospray time-of-flight mass spectrometer (Model 6220; Agilent – Santa Clara, CA) set-up to scan over a range from 300 to 3200 m/z.

Identification of N-linked Glycans with Ion-Trap Mass Spectroscopy

N-linked glycan peaks, collected from the HILIC chromatography, were dried, then resuspended into 10µL of 0.1% formic acid/water and injected onto a graphitized carbon nanocolumn (Agilent – Santa Clara, CA) that was mounted onto an ion-trap mass spectrometer (Model 6340; Agilent, Santa Clara, CA). The glycans were eluted off of the column using a 12 minute linear gradient of 0.1% formic acid in acetonitrile. The mass spectrometer was set-up to scan in standard mode over a range from 100 to 2000 m/z.

Identification of N-linkage Sites by Peptide Mapping with HILIC Glycopeptides Enrichment

Approximately 200 µg of Etanercept were subjected to reduction with 50mM DTT (Sigma – St. Louis, MO) for 1 hour at 37°C and then alkylated with 125 mM iodoacetamide (Sigma Aldrich – St. Louis, MO) for 45 minutes at room temperature in the dark. Excess reagents were removed by acetone precipitation. The resulting protein pellet was reconstituted in a100mM ammonium bicarbonate pH 8.0 and digested with trypsin (Promega – Madison, WI) at a ratio of 1:50 (protein:enzyme) at 37°C overnight. The digest was further treated with AspN (Promega – Medison, WI) also at a ratio of 1:50 and 37°C also overnight to generate shorter peptides. The trypsin/AspN digest was analyzed directly by injection an aliquot of the digest onto a Poroshell C18 column (2.1×75 mm, 5 µm) (Agilent, Santa Clara, CA) in-line with an ESI-Q-ToF mass spectrometer (Model 6538, Agilent – Santa Clara, CA) or partially purified by loading the digest onto a ZIC-HILIC tip (Protea Bioscience – Morgantown, WV) for glycopeptides enrichment. The enrichment step was performed using equilibration and elution reagents provided by the ZIC-HILIC manufacturer (Protea Bioscience – Morgantown, WV). The enriched peptides were then further analyzed using the Poroshell C18 column in line with an ESI-Q-ToF mass spectrometer.

O-linked glycan analysis

An aliquot of deformulated Etanercept, containing approximately 500 µg of protein, was dialyzed into 50mM Ammonium Acetate (pH 7.8), dried and then subjected to reductive beta-elimination using 0.05M NaOH with 1M sodium borohydride. The reaction was allowed to proceed overnight at 45°C. The released glycans were desalted over Dowex beads (H^+ form) (Sigma – St. Louis, MO), dried and then permethylated [10] using NaOH in DMSO, followed by the addition of iodomethane (Aldrich – St. Louis, MO). The permethylated glycans were extracted with chloroform, dried, then resuspended in methanol and analyzed by direct injection using an ESI-ToF MS (Model 6220, Agilent).

Results

Prior to any analysis, Enbrel was deformulated to remove any formulating excipients that might interfere with any of the subsequent analyses. The deformulation was conducted as described in the Materials and Methods section, utilizing a Poros Capture Select Fc column. A profile of the deformulation chromatography is shown in Figure 1, displaying the load peak, containing formulation components in Enbrel not retained by the column, and an elution protein peak, representing Etanercept.

An aliquot of the deformulated Enbrel product was treated with PNGase to release the N-linked glycan moieties. Once released, the glycans were labeled with 2AB and analyzed by HILIC chromatography. A chromatogram of the labeled glycans is provided as Figure 2. The order of elution off of this column, as indicated within Figure 2, is neutral glycans, followed by monosialylated species, and then diasyalylated ones.

The HILIC chromatography resolved glycans were collected and further analyzed by mass spectroscopy to confirm their structures. The established identity, provided graphically, and relative ratio of the various N-linked glycan species found in each of the collected peaks are summarized in Chart 1. As expected and summarized in Chart 1, approximately 50% of released N-linked glycans from etanercept are neutral with the remaining 50% are a combination of mono and dialylated species.

To map the N-linked sites of glycosylation, Etanercept was reduced and alkylated and then digested by the combination of trypsin and Asp-N enzymes or endoproteinase Asp-N alone, as described in Materials and Methods section. The digests were split into two aliquots, one of which was analyzed using a C18 column in-line with an ESI-Q-ToF mass spectrometer and the other was processed through a HILIC enrichment column and then similarly analyzed by chromatography using a C18 column also in-line with the ESI-Q-ToF

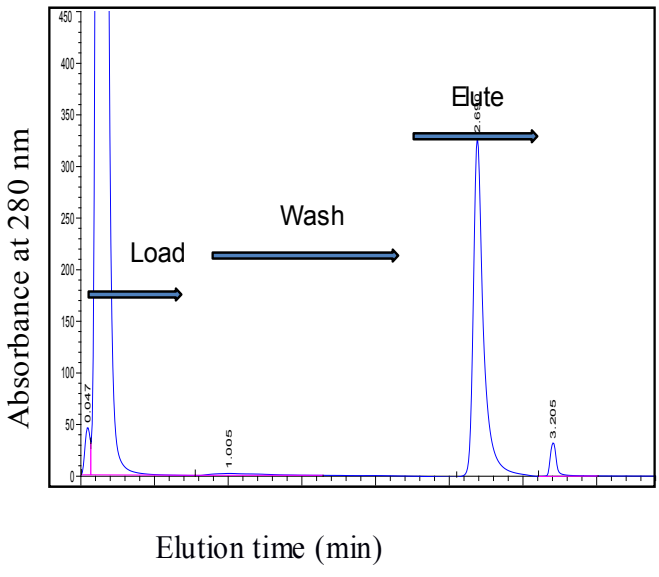

Figure 1: Enbrel deformulation chromatogram using a Poros Capture Select Fc column and monitoring of absorbance at 280 nm.

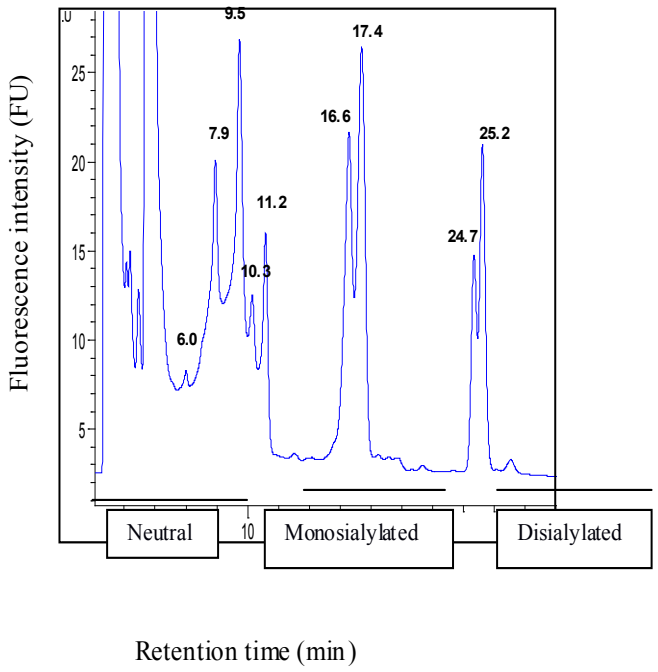

Figure 2: HILIC chromatography profile of N-linked glycans released from Etanercept with PNGase F and labeled with 2AB.

Peak retention time (min)	Relative abundance (%)	Glycan structures
6.0	1.1	
7.9	15.1	
9.5	22.4	
10.3	5.5	
11.2	6.7	
16.6, 17.4	32.9	
24.7, 25.2	16.3	

Chart 1: Summary of relative enrichment of the N-linked glycans from Etanercept and corresponding identity.

mass spectrometer. The obtained mapping results are shown in Figures 3 and 4. The observed mass spectroscopic results for the glycopeptides of interest, containing the N-linked consensus sites - NXS/T-, are displayed as Figures 5-7.

All three predicted N-linked glycosylation sites were found to be occupied. Of the three sites, the one at Asn149 was found to be most heterogeneous, based on the presence of neutral, mono and disialylated species. Similarly, the site at Asn171 also contained neutral, mono and disialylated glycan moieties, but with overall lower heterogeneity. The site at Asn317 was found to contain only neutral glycans. A summary of the type of glycans found at each of the three sites is provided in Chart 2.

To further characterized Etanercept, the N-linked deglycosylated protein was reduced to dissociate the dimeric form of the product and then analyzed by passage through a desalting column and into an ESI-ToF mass spectrometer. The deconvolution results of the mass spectrum are shown in Figure 8.

From the known etanercept sequence, the predicted molecular weight is 51239.7Da. However, the actual observed mass ranged from 57972.7 Da up to 59136.3 Da. The sizeable difference in mass implied that the polypeptide chain is significantly modified through O-linked glycosylation. Indeed by reductive beta-elimination, it was demonstrated that etanercept is O-linked modified primarily with mono and disialylated core-1 glycans (Figure 9).

Results from O-linked glycan analysis revealed that glycan species O-linked to etanercept are core-1 subtype structures with one or two moles of N-acetylneuraminic acid. Based on this type of O-linked structure and the observed mass shift between the N-linked deglycosylated etanercept and the predicted monomeric molecular mass of the polypeptide, it is concluded that each monomer contains 10 O-linked sites.

Conclusion

Many biosimilar products in development are complex glycoproteins, often containing multiple N- and O-linked glycosylation sites. The release, quantification and site mapping of N-linked glycans along with site occupancy for these glycoproteins is relatively routine and well defined based on the availability of an enzyme (PNGase F) that is highly specific for the release of N-linked glycans, along with well developed chromatographic and mass spectroscopic mapping procedures. In this study, using Etanercept as a case study, we were able to easily identify the N-linked glycans associated with this glycoprotein product, map the sites of linkage and establish the glycan structures and corresponding relative abundance present at each site, thus obtaining a full picture of the Etanercept N-linked glycosylation. Unfortunately, detailed characterization of O-linked glycosylation is not as routine as N–linked glycosylation. Firstly, there is no consensus site for O-linkage, thus even predicting the extent and sites of O-glycosylation is not possible. Secondly, the only commercially available enzyme is O-glyconase, which is only suitable for the release of unsubstituted core-1 disaccharides. For more general release of O-linked glycans,

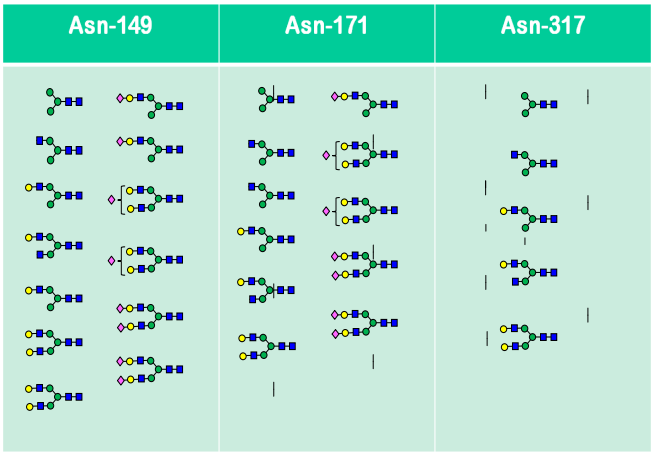

Chart 2: Summary of glycans found within each of the three N-linked glycosylation sites within Etanercept.

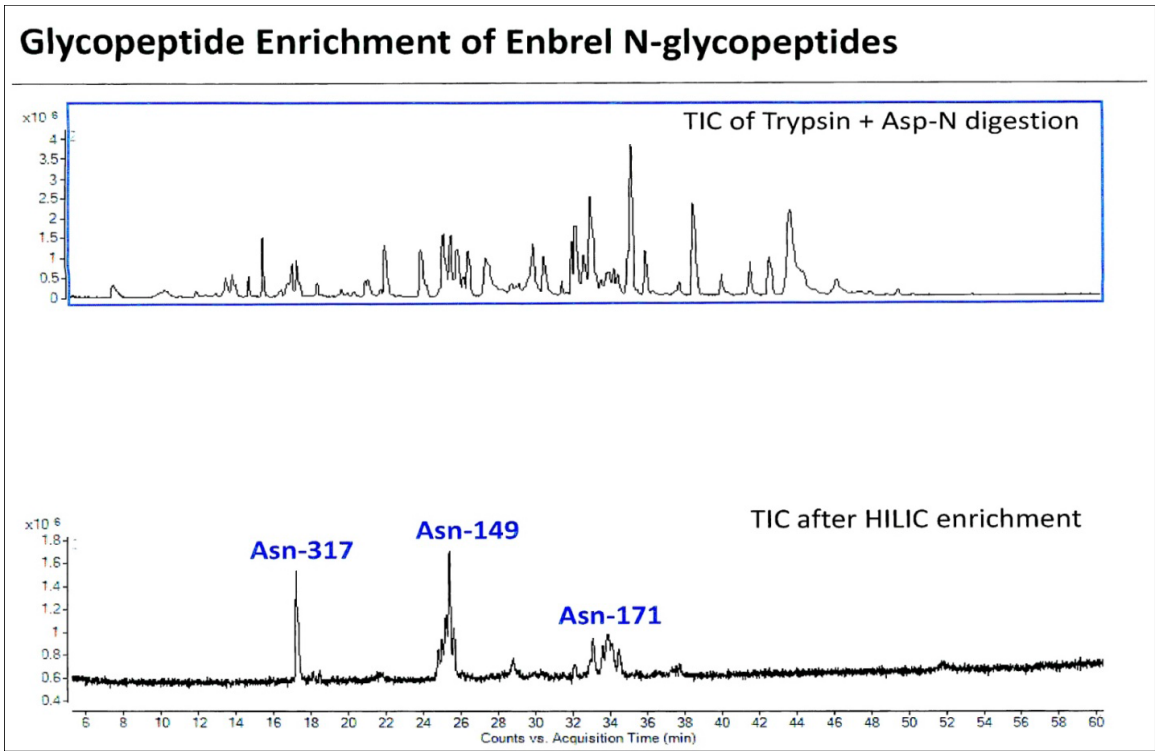

Figure 3: Peptide maps of Etanercept after digestion with trypsin followed by endoproteinase AspN. TIC profile displaying all peptides is shown in the upper panel. TIC profile of ZIC-HILIC enriched peptides is shown in the lower panel.

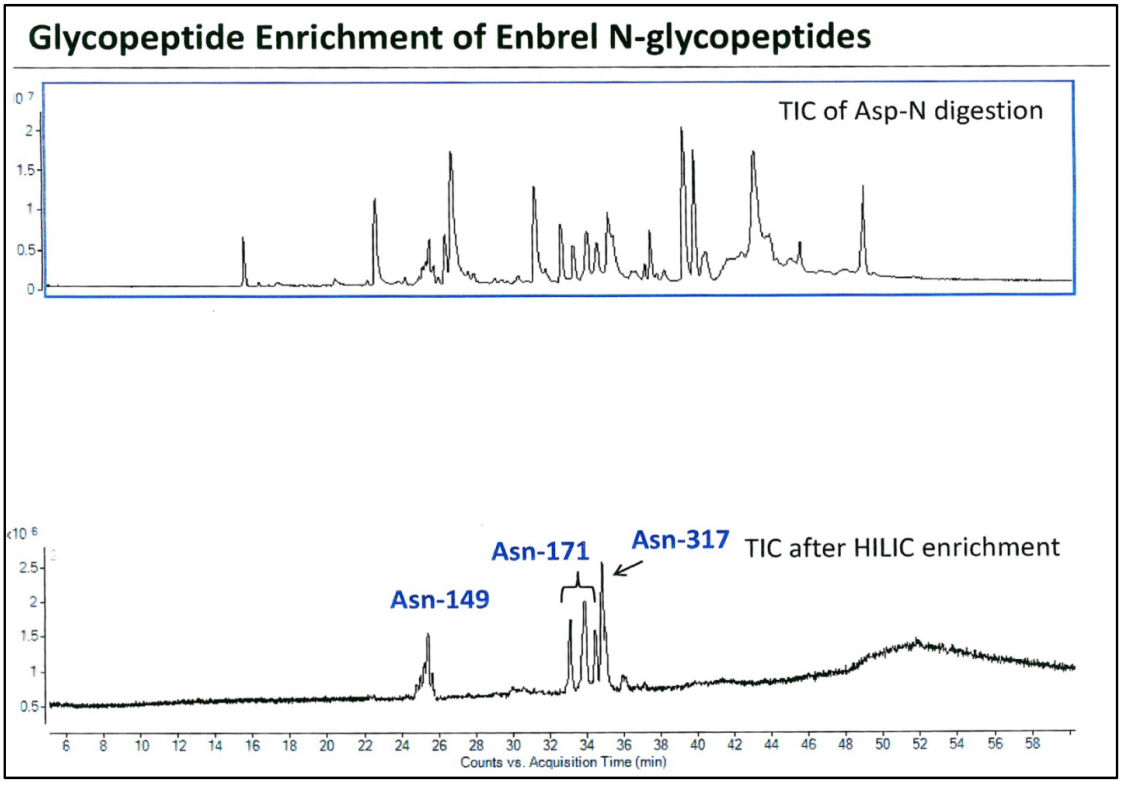

Figure 4: Peptide maps of Etanercept after digestion with endoproteinase AspN. TIC profile displaying all AspN cleavage peptides is shown in the upper panel. TIC profile of ZIC-HILIC enriched peptides is displayed in the lower panel.

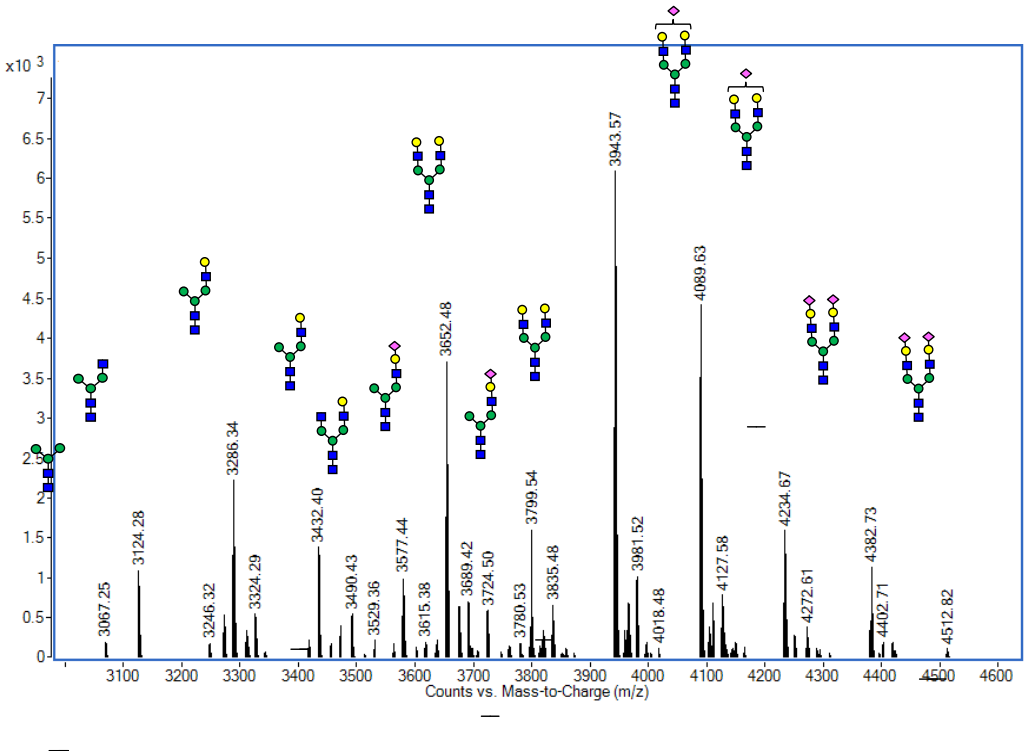

Figure 5: Mass spectrum of glycopeptide with N-linkage consensus site at Asn 149 and associated glycans with structural assignment based on measured masses.

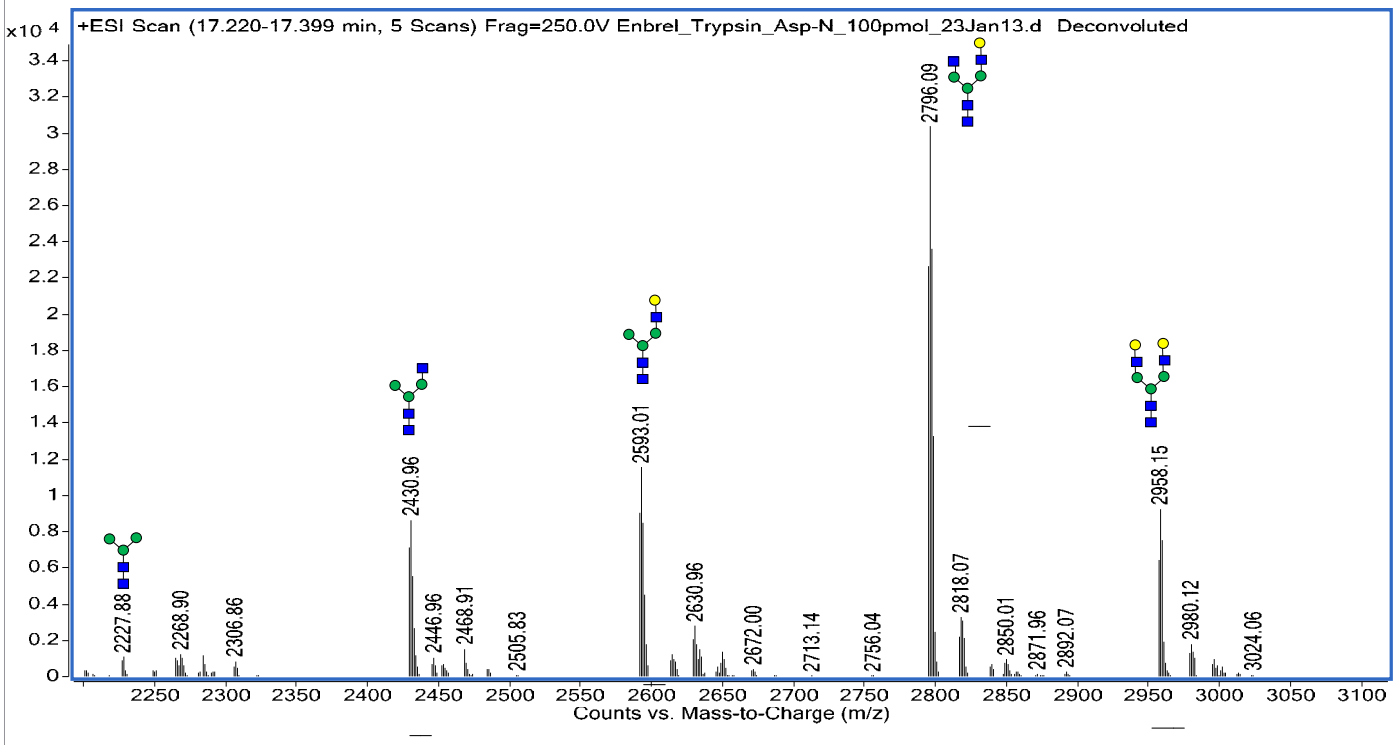

Figure 6: Mass spectrum of glycopeptide with N-linkage consensus site at Asn 171 and associated glycans with structural assignment based on measured masses.

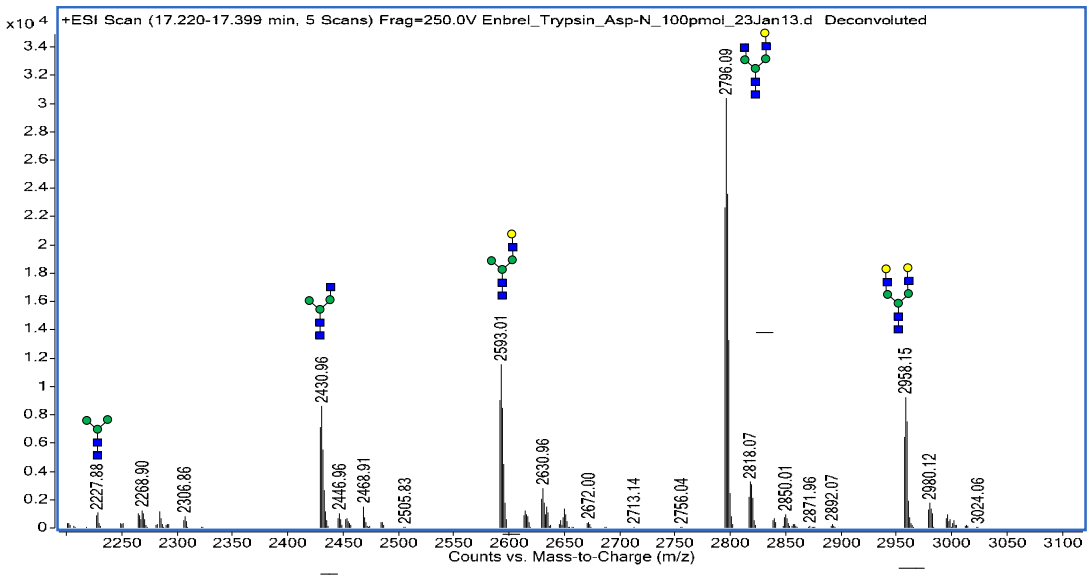

Figure 7: Mass spectrum of glycopeptide with N-linkage consensus site at Asn 317 and associated glycans with structural assignment based on measured masses.

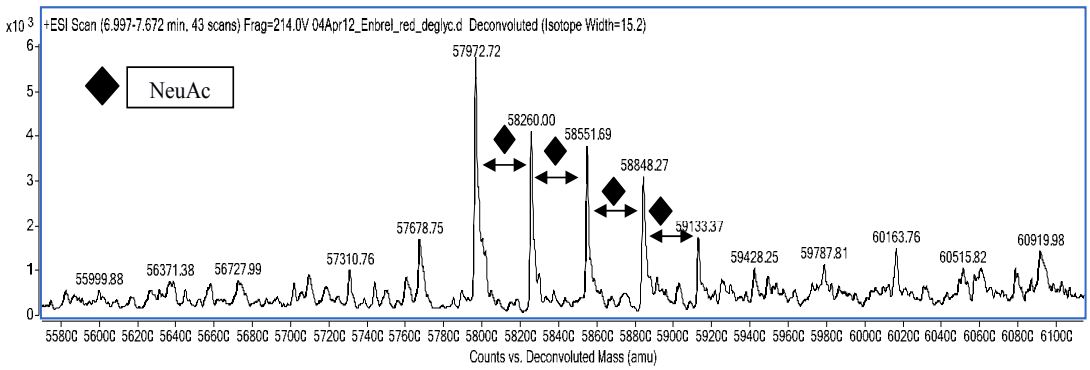

Figure 8: Molecular masses from the Mass spectrum deconvolution of N-linked deglycosylated and reduced etanercept.

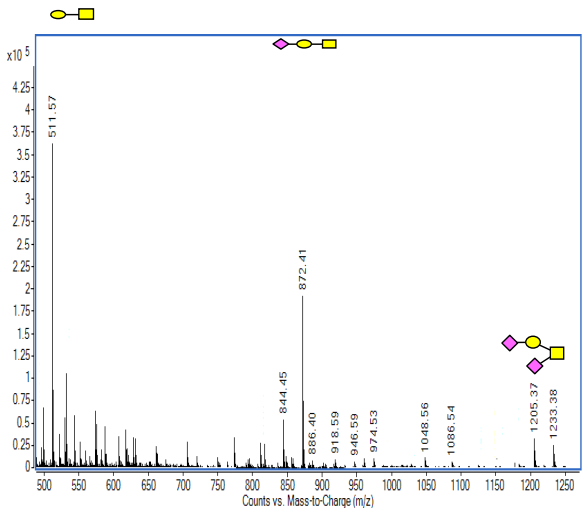

Figure 9: Mass spectral analysis of O-linked glycans released from etanercept by reductive beta elimination.

one must resort to chemical means which often result in partial degradation of the glycans though peeling or loss of reducing ends, thus eliminating the possibility of fluorescence labeling for ease of glycan detection and quantification. To overcome some of the challenges in the characterization of O-linkage glycosylation it will be important to develop more reliable, robust O-glycan release procedures, resulting in complete release of intact O-glycans that can be subjected to derivation for analysis and quantification by HPLC. Better mass spectroscopic methods, along with improvements in software and computing power will also be required for glycopeptide identification and mapping of O-glycosylation sites.

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