In-depth O-glycosylation characterization and comparison of commercially available etanercept products using the ZenoTOF 7600 system

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ABSTRACT

In this study, an analysis is performed that compares a reference standard for etanercept with 6 commercially available biosimilars from different manufacturers. The samples are analyzed using high-quality MS/MS spectra on the ZenoTOF 7600 system and by combining protein subunit analysis and glycopeptide analysis. Electron activated dissociation (EAD) was used, which provides an excellent fragmentation of glycopeptides, leading to high confidence in sequence identification and efficient preservation of the glycan structures in the fragments. Biologics Explorer software provides powerful tools that enable fast and detailed comparisons of intact proteins and glycopeptides to increase confidence in sequence confirmation and accurate localization of O-glycosylation sites.

INTRODUCTION

Unlike N-linked glycans, there is no consensus site for O-linked glycosylation, so it is hard to predict the occupancy site from the sequence[1]. Traditional collision-based MS/MS approaches, such as collision-induced dissociation (CID), result in the loss of labile glycan moieties, which makes an accurate determination of glycosylation sites extremely challenging. EAD is superior in glycopeptide analysis, given its ability to preserve the glycan structures in the fragments. Etanercept is a biotechnological product that has a complex O-glycosylation profile. This study uses EAD to elucidate the differences between etanercept O-linked glycan profiles in the reference standard and in the biosimilars.

MATERIALS AND METHODS

Sample

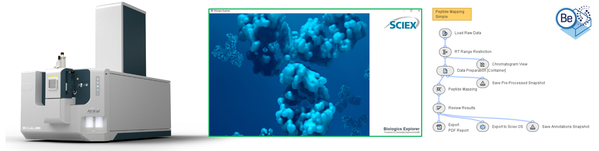
Etanercept was incubated with PNGase F overnight at 37℃ to remove N-glycans. Subsequently, the sample was treated with SialEXO at 37℃ for 4 hours to remove sialic acid. The samples were further treated with 7M guanidine-HCl in 50 mM Tris-HCl (pH 7.6) and dithiothreitol to reduce disulfide bonds. For the LC-MS analysis, 2–4 µL (1-2 µg) of etanercept subunits were injected. The treated sample was further processed for protein alkylation and enzymatic digestion using trypsin and Glu-C with an enzyme-to-protein ratio of 1:50. The alkylated sample was incubated overnight at 37℃. The peptides were acidified by formic acid and analyzed by the LC-MS system.

LC method

For the subunit analysis, the separation was achieved using a Biozen SEC column (150 x 4.6 mm, 1.8 µm, 200 Å) at a flow rate of 0.22 mL/min with 35% mobile phase B. The column was kept at 40°C in the column oven of a Shimadzu LC 40 system. For the O-glycosylation analysis of a glycopeptide, the peptides were chromatographically separated with previously described LC gradients.[2] The separation was achieved using a Biozen Peptide PS-C18 column (150 x 2.1mm, 1.6 µm) at a flow rate of 0.3 mL/min. The column was kept at 60°C in the column oven of a Shimadzu LC 40 system.

MS/MS

TOF MS, data-dependent acquisition (DDA) and MRM^{HR} experiments were all performed in SCIEX OS software using EAD mode on the ZenoTOF 7600 system. Data were analyzed in Biologics Explorer software.



ZenoTOF 7600 system | Biologics Explorer software

RESULTS

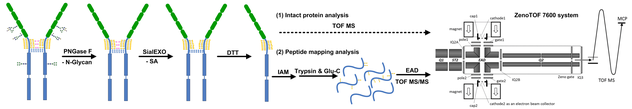


Figure 1. Overview of the O-glycosylation characterization by the ZenoTOF 7600 system. Etanercept is a fusion protein with 6 N-glycosylation and 26 O-glycosylation sites heavily sialylated. Structural elucidation of intact etanercept is challenging due to the complexity and heterogeneity induced by O-glycosylation and sialylation. To gain insight into the structure of etanercept, the O-glycans of etanercept were characterized on the reduced subunits and the glycopeptides with 2 separate sample preparations: de-N-glycosylation and de-sialylation. The ZenoTOF 7600 system has an EAD mode, which is superior in glycopeptide analysis given its ability to preserve the glycan structures in the fragments. EAD data are automatically analyzed and annotated using a streamlined, optimized workflow template offered by Biologics Explorer software.

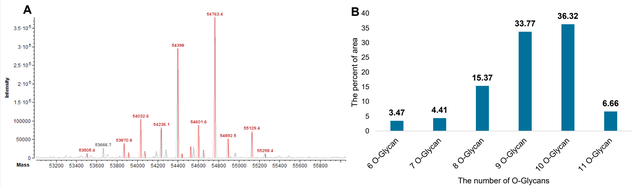


Figure 2. A representative deconvoluted spectrum of the etanercept reference subunits with de-N-glycosylation and de-sialylation (A) and the O-glycan distribution on the subunit level (B). By removing the of N-glycans and sialic acids, the O-glycosylation of the etanercept was accurately characterized on the reduced subunits level, and 11 O-glycosylation sites were successfully identified.

Peak #	Modifications	Glycan total number	Core1 number	Core2 number	Calc. Avg. Mass	Avg. Mass	Delta	Volume [%]	Core1 %	Core2 %
1	5*Core 1 + Core 2 + Lys-loss	6	5	1	53507.48	53505.43	-2.05	0.86	0.04	0.01
2	6*Core 1 + Core 2 + Lys-loss	7	6	1	53872.82	53870.79	-2.03	3.04	0.18	0.03
3	3*Core 1 + 3*Core 2 + Lys-loss	6	3	3	53913.87	53913.88	0.01	1.04	0.03	0.03
4	8*Core 1 + Lys-loss	8	8		54034.96	54032.64	-2.32	8.29	0.66	0.00
5	5*Core 1 + 2*Core 2 + Lys-loss	7	5	2	54076.01	54073.36	-2.65	1.37	0.07	0.03
6	7*Core 1 + Core 2 + Lys-loss	8	7	1	54238.15	54236.12	-2.03	6.40	0.45	0.06
7	9*Core 1 + Lys-loss	9	9		54400.29	54398.01	-2.28	24.59	2.21	0.00
8	6*Core 1 + 2*Core 2 + Lys-loss	8	6	2	54441.34	54439.69	-1.65	0.69	0.04	0.01
9	9*Core 1	9	9		54528.46	54526.45	-2.02	2.27	0.20	0.00
10	8*Core 1 + Core 2 + Lys-loss	9	8	1	54603.48	54601.61	-1.87	6.91	0.55	0.07
11	6*Core 2	6	0	6	54651.62	54650.52	-1.10	1.56	0.00	0.09
12	10*Core 1 + Lys-loss	10	10		54765.62	54763.38	-2.24	32.09	3.21	0.00
13	10*Core 1	10	10		54893.80	54892.49	-1.30	4.23	0.42	0.00
14	11*Core 1 + Lys-loss	11	11		55130.96	55129.45	-1.51	5.69	0.63	0.00
15	11*Core 1	11	11		55259.13	55258.37	-0.76	0.97	0.11	0.00
SUM									8.81	0.34

Table 1. The identification of the etanercept reference subunit using Biologics Explorer software. Two O-glycans were identified: Core1 and Core2. The total number of O-glycans was between 6 and 11. The results were very high credibility, and the MS tolerance of every identified peak in etanercept was under 3 Da. If the number of Core1 and Core2 O-glycans in every peak is multiplied by the corresponding peak area percentage, and the results are then added together, the sum could represent the average number of Core1 and Core2 O-glycans present in the etanercept samples (similar to calculating the antibody-drug conjugate drug-to-antibody ratio, or ADC DAR). In the etanercept reference, the Core1 and Core2 numbers were 8.81 and 0.34, respectively.

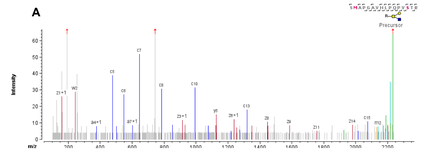


Figure 3. The comparison between the CID and EAD MS/MS spectra of the glycopeptide SMAPGAVHLPQPVSTR in the etanercept reference. The EAD-based glycopeptide analysis could get abundant MS/MS fragments of the peptide and preserve intact glycan structures in the fragments, which was superior for the identification and the relative content analysis of the O-glycosylation in the etanercept samples. In comparison, oxonium ions are the predominant fragmentations identified. No peptide backbone fragments were detected in CID MS/MS, which makes glycopeptide analysis challenging.

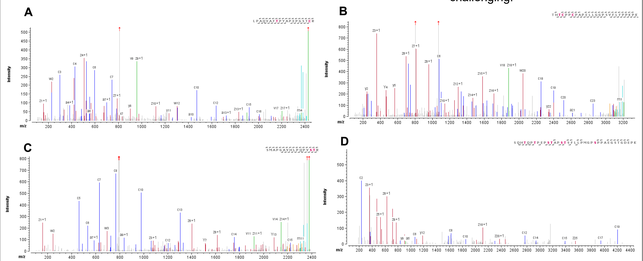


Figure 4. The glycosylation site identification of the etanercept reference on the glycopeptides leve lusing EAD mode. In a single-injection peptide mapping analysis, 11 O-glycosylation sites was identified, which was the same as the O-glycosylation results in the etanercept subunit level, including: A) T8; B) T245; C) S199, T200; D) T205, T208, S212, T213, S216, T217, S226.

O-glycan type	Occupancy (%)			
	T8	S199	T200	T245
Core 1	4.50	86.36	86.09	1.82
Core 2	N/A	0.18	10.24	N/A
Sum	4.50	86.54	96.34	1.82

Table 2. Comparison of O-glycan occupancy at different O-glycosylation sites using EAD mode. Three glycopeptides with only 1 or 2 O-glycosylation sites were analyzed, including LPAQVAFTPYAPEPGSTCR", "THTCPPCPAPELLGGPSVFLFPPKPK and SMAPGAVHLPQPVSTR (as shown A, B and C in Figure 4), respectively. For the selected four O-glycosylation sites, the S199 and T200 had the O-glycan occupancy of more than 86%, which could indicate that S199 and T200 were easier to glycosylate in the production process.

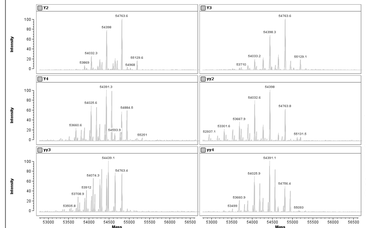


Figure 5. Comparative analysis of 6 commercially available subunits of etanercept biosimilars with de-N-glycosylation and de-sialylation. Biologics Explorer software provides powerful tools for comparative analysis of intact proteins to facilitate biotherapeutic development. The comparative results are shown in Table 3.

Sample #	O-glycan range	Core1 Avg.	Core2 Avg.	O-glycan Avg.
Y1	6-11	8.81	0.34	9.15
Y2	6-12	8.59	0.54	9.13
Y3	5-11	8.75	0.38	9.13
Y4	4-9	3.35	3.23	6.58
y2	5-11	7.20	0.62	7.82
y3	5-10	6.29	1.52	7.81
y4	4-8	3.33	3.21	6.54

Table 3. Comparison of the identified O-glycan number in the etanercept reference (Y1) and 6 commercially available biosimilars on the subunits level. The results indicated that the O-glycan average number of 6 biosimilars were very different. Y2 and Y3 were 9.13 (similar to the etanercept reference) and the others were 6.54–7.82 (lower than the reference).

Sample #	Occupancy (%)	
	S199	T200
Y1	86.54	96.34
Y2	85.54	95.68
Y3	88.34	98.89
Y4	80.95	85.80
y2	81.45	86.95
y3	81.88	87.88
y4	80.23	84.55

Table 4. Comparison of the O-glycan occupancy of S199 and T200 in the etanercept reference (Y1) and 6 commercially available biosimilars on the glycopeptide level by EAD mode.

CONCLUSIONS

- The etanercept subunit analysis achieved excellent data quality, mass accuracy and sensitivity, which could be used to quickly indicate the O-glycosylation difference of etanercept samples.
- Compared with CID, EAD mode provided an excellent fragmentation of glycopeptides, leading to high confidence in sequence identification. EAD efficiently preserved the glycan structures in the fragments and was superior in O-linked glycan localization.
- The intact mass and peptide mapping analysis demonstrated a different O-glycan distribution in the comparative O-glycosylation analysis using an etanercept reference and 6 commercially available biosimilars. The dominant species are Core1 and Core 2, while the relative abundance was different across biosimilars. For example, the O-glycan occupancy of S199 was about 80%–88% between the etanercept reference and the 6 biosimilars.
- Biologics Explorer software offers an easy-to-use intact protein and peptide mapping workflow. It provides automated data analysis with high accuracy and efficient results review and comparison for an improved user experience.
- The workflow combining intact protein and peptide mapping analysis for heavily glycosylated proteins was an effective method for biosimilar comparison using the high-quality MS/MS spectra of glycopeptides in the ZenoTOF 7600 system.

REFERENCES

1. Qingqiao Tan et al. Characterization and comparison of commercially available TNF receptor 2-Fc fusion protein products. mAbs, 2012, 4(6), 761-7741.
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