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Application News

Liquid Chromatograph Mass Spectrometer LCMS-9030

Released Glycan Analysis of Trastuzumab Biosimilar using LCMS-9030

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User Benefits

- The methodology for released glycan analysis of trastuzumab biosimilar using LCMS-9030 for sample analysis; and Protein Metrics and SimGlycan software for data processing is described.
- Excellent and stable mass accuracy, comprehensive fragmentation pattern and high sensitivity offered by LCMS-9030 helped in identification of N-glycan profile of trastuzumab biosimilar.

■ Introduction

Monoclonal antibodies (mAbs) are a major class of biopharmaceuticals with indications now covering a large panel of diseases, from cancer to asthma, including central nervous system disorders, infectious diseases and cardiovascular diseases. Glycosylation is a very commonly occurring post-translational modification (PTM) in mAbs, that plays a vital role in the safety and efficacy of many therapeutic mAbs. Throughout therapeutic antibody development, glycosylation analysis is frequently performed not only to profile their biochemical characteristics, but also to assess the stability of expression cell lines and robustness of downstream processes. Furthermore, as an important structural and functional attribute of antibodies and related proteins, glycosylation is a critical aspect in comparing the biosimilar monoclonal antibodies with the innovator's molecule. This application note describes the methodology for glycosylation profiling of trastuzumab biosimilar at released glycan level using LCMS-9030 for sample analysis and Protein Metrics and SimGlycan software for data processing.

■ Experimental

Trastuzumab biosimilar samples were buffer exchanged by using Amicon centrifugal ultrafilters having molecular weight cut-off of 10 KDa as per manufacturer's instruction. The rapid in-solution enzymatic deglycosylation of 250 µg of buffer exchanged trastuzumab biosimilar was carried out according to the manufacturer's instruction of a commercially available sample preparation kit.

The collected N-glycan solutions were analyzed immediately without the need for further concentration using LCMS-9030, a quadrupole time-of-flight (Q-TOF) mass spectrometer from Shimadzu Corporation, Japan (Fig. 1).

- **UFaccumulation**™
- **UFgrating**™
- **UFflighttube**™
- *iRef***TOF**™

Fig. 1 LCMS-9030 Quadrupole Time of Flight Mass Spectrometer

Analysis was performed in Data Dependent Acquisition (DDA) mode in positive polarity using ElectroSpray Ionization (ESI) interface. DDA data acquisition was controlled by the LabSolutions™ LCMS software. Mass range of 500-2500 m/z was used for MS1 TOF survey scan. Base peak chromatogram intensity threshold of more than 1000 was used to trigger the MS/MS fragmentation with collision energy spread of 25-75 V. Use of collision energy spread allowed acquisition of comprehensive fragmentation pattern for any given precursor ion.

Five dependent (MS/MS) events were set to allow sufficient MS/MS data collection. Ion exclusion and inclusion settings are available in the LabSolutions LCMS software to automatically exclude background ions and include ions of interest, respectively. All data acquisition was performed with single external TOF calibration. No intermediate TOF calibration/lock masses were used during the data acquisition/processing. Details of analytical conditions are given in Table 1.

Table 1 Details of analytical conditions for released glycan analysis

HPLC system	Nexera™ X2
Column	Amide HILIC column (2.1 × 150mm; 1.8µm)
Column oven	45 °C
Mobile phases	A- 20 mM Ammonium formate (pH: 4.5) B- Acetonitrile
Flow rate	0.4 mL/min
Injection Volume	1 µL
LCMS system	LCMS-9030
Interface	Heated ESI
Polarity	Positive
Temperatures	Interface: 300 °C Desolvation line: 200 °C Heater block: 400 °C
Gas flow rates	Heating gas: 15 L/min Nebulizing gas: 3 L/min Drying gas: 15 L/min
Gradient program ((%B concentration)	0.01-43.5 min 80 to 54 %B; 43.5-45 min 54-0 B%; 45-50 min 0 B%; 50-52 min 80 B%; 60 min stop
Acquisition mode	DDA
Mass range for TOF survey scan	500-2500 m/z
Mass range for precursor ion	500-2000 m/z
Mass range for MS/MS scan	100-2800 m/z
Collision energy spread	25-75 V

The LCMS-9030 quadrupole time-of-flight (Q-TOF) mass spectrometer is a powerful instrument that integrates the world's fastest and most sensitive quadrupole technology with TOF capabilities for accurate mass measurement. Patented technologies of LCMS-9030, UFflighttube™ and iRefTOF™, ensure excellent Mass Measurement Accuracy (MMA) with stability which helps in identification of different glycans present in the sample. UFaccumulation™ and UFgrating™ offer superior sensitivity which helps in detecting low abundant glycans present in the samples.

LC-MS profiling of the released glycans was carried out by using Protein Metrics software suite. All parameters except those mentioned in Table 2, were kept in default mode. The same samples were also analyzed using SimGlycan v.5.94 software. We created a glycan template consisting of 14 unique compositional glycan structures, previously reported from the Trastuzumab samples and stored their structure-specific in-silico fragments generated based on Domon and Costello Nomenclature. The two technical replicates of the Trastuzumab sample were imported in the software (in .lcd file format) and subjected to the LC-MS peak processing using an optimized set of peak picking parameters shown in Table 3.

Table 2 Protein Metrics parameters used for N-glycan analysis

Workflow used	Released glycans IgG
Label used	Instant PC (261.148 Da)
Charge range	1 to 5
Mass tolerance	10 ppm
Ionization mode	Positive
Minimum detectable m/z	250
Reference glycan library	N-Glycan 309 mammalian no sodium

Table 3 Peak picking parameters for SimGlycan analysis

MS1 peak threshold intensity	0.1%
Peak width	0.1-2.0 min
Minimum peak height	1000
Charge state range	1-3
[M+i] peak >=	40% of [M+j] peak
Isotopic peak	[M+0], [M+1], [M+2]

The peak-lists of the technical replicates are aligned using an m/z and retention time tolerance to find out the unique and universal LC compounds. The MS/MS scans were later clustered to the appropriate compounds using a proprietary algorithm. The scans clustered under the detected compounds were subjected to database search using a precursor and product ion error tolerance of 10 ppm and 20 ppm, respectively. The experimental MS/MS fragment ions were matched against in-silico fragment ions from the target database. Based on the nature of the fragmentation technique used, only the protonated glycosidic ion species, predominantly b and y ions were considered for fragment matching. The software was tuned to report only those candidate glycan structures, which have 80% of their monosaccharide residues explained by structure specific characteristic ions in the MS/MS spectra.

■ Results and Discussion

The analysis of LC-MS data obtained using Protein Metrics software suite as well as SimGlycan software confirmed the presence of twelve unique glycoforms in trastuzumab biosimilar. Out of the twelve glycoforms observed during released glycan analysis, MS profiling confirmed all twelve glycoforms whereas MS/MS analysis confirmed nine glycoforms. The total ion chromatogram (TIC) for released glycan analysis of trastuzumab biosimilar is shown in Fig. 2, while the summary of all unique glycoforms identified by Protein Metrics is given in Table 4.

The alignment of peak-lists and subsequent database search using SimGlycan software yielded a list of glycoforms. Fig. 3 shows the alignment of peak-lists obtained from MS data, comparison of an obtained fragment spectra to the in-silico fragments generated based on Domon and Costello Nomenclature, and the predicted structure of that glycoform.

The nine glycoforms confirmed by analysis of MS/MS data using SimGlycan software are given in Table 5. MS/MS level analysis of the released glycans from trastuzumab biosimilar could not detect 3 glycoforms, namely HexNAc(2)Hex(3), HexNAc(4)NeuAc(1)Fuc(1)Hex(5) and HexNAc(4)NeuAc(2)-Fuc(1)Hex(5).

Fig. 2 TIC for released glycan analysis of trastuzumab biosimilar

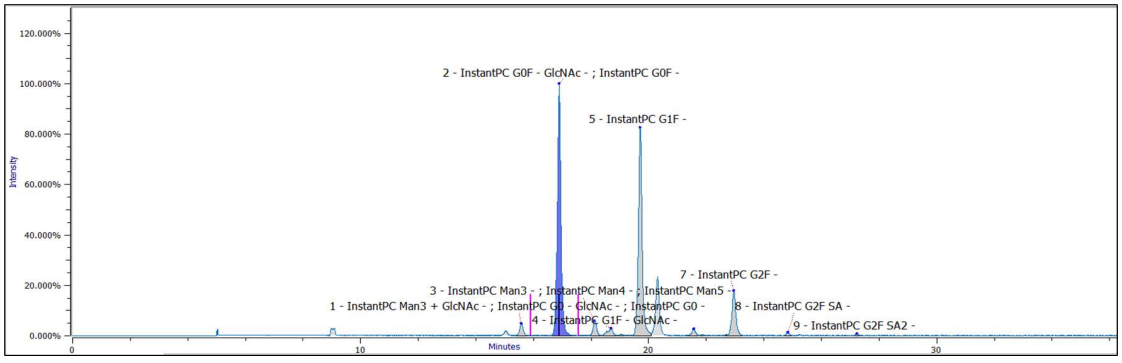

Table 4 Glycoforms identified in trastuzumab biosimilar using Protein Metrics software

							Samples Id ←	1
							Sample name	TMAB RELEASED GLYCAN_002 (%)
Peak #	Apex time ↑	[Glycan Name]	Glycans	Obs.M ↑	Calc.M ↑	[ppm] ↑		
13	15.0311	G0F - GlcNAc	InstantPC HexNAc(3)Fuc(1)Hex(3)	1520.62	1520.61	1.46		1.08
14	15.5745	G0	InstantPC HexNAc(4)Hex(3)	1577.64	1577.63	1.64		2.00
		G0 - GlcNAc	InstantPC HexNAc(3)Hex(3)	1374.56	1374.55	1.60		2.00
		Man3 + GlcNAc	InstantPC HexNAc(3)Hex(3)	1374.56	1374.55	1.60		2.00
15	16.8903	G0F	InstantPC HexNAc(4)Fuc(1)Hex(3)	1723.7	1723.69	2.01		41.04
16	18.1272	Man4	InstantPC HexNAc(2)Hex(4)	1333.54	1333.53	5.33		2.47
		Man5	InstantPC HexNAc(2)Hex(5)	1495.59	1495.58	3.00		2.47
17	18.6937	G1F - GlcNAc	InstantPC HexNAc(3)Fuc(1)Hex(4)	1682.67	1682.67	0.92		1.83
18	19.7193	G1F	InstantPC HexNAc(4)Fuc(1)Hex(4)	1885.75	1885.74	1.66		35.74
22	22.9982	G2F	InstantPC HexNAc(4)Fuc(1)Hex(5)	2047.8	2047.8	2.60		8.28
24	24.8892	G2F SA	InstantPC HexNAc(4)NeuAc(1)Fuc(1)Hex(5)	2338.9	2338.89	1.25		0.61
27	27.3009	G2F SA2	InstantPC HexNAc(4)NeuAc(2)Fuc(1)Hex(5)	2630	2629.99	6.07		0.48

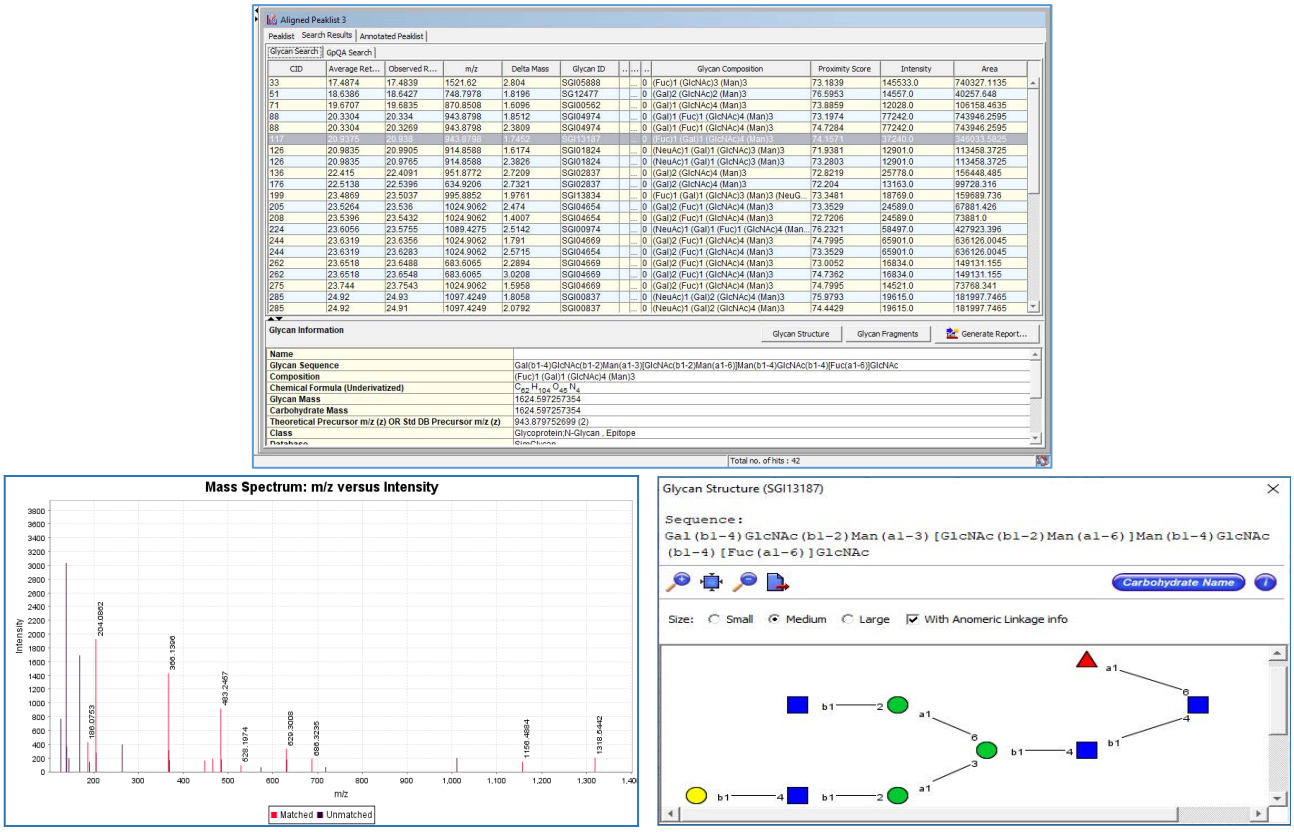

Fig. 3 Top: List of glycoforms identified in trastuzumab biosimilar; Bottom left: Comparison of obtained MS/MS spectrum to Domon-Costello Plot Bottom right: Structure of G1F glycoform as predicted by SimGlycan

Table 5. Glycoforms confirmed by MS/MS analysis using SimGlycan software

No.	Compound ID	Average Retention Time	Average m/z	Observed m/z	Glycan ID	Polarity	Adduct	Charge	Area	Composition
1	123	19.7	842.3401	842.3401	G1FGlcNAc	Positive	H	2	44598.615	(Fuc)1 (Gal)1 (GlcNAc)3 (Man)3
2	102	19.7	943.8798	943.8798	G1F	Positive	H	2	606441.801	(Fuc)1 (Gal)1 (GlcNAc)4 (Man)3
3	170	22.9	683.6065	683.6065	G2F	Positive	H	3	24557.3235	(Fuc)1 (Gal)2 (GlcNAc)4 (Man)3
4	46	16.9	1318.5407	1318.5407	Man3F	Positive	H	1	18931.878	(Fuc)1 (GlcNAc)2 (Man)3
5	1	15.0	761.3137	761.3137	G0FGlcNAc	Positive	H	2	19424.883	(Fuc)1 (GlcNAc)3 (Man)3
6	36	16.9	862.8534	862.8534	G0F	Positive	H	2	801166.2015	(Fuc)1 (GlcNAc)4 (Man)3
7	70	18.1	667.7714	667.7714	Man4	Positive	H	2	12069.5985	(GlcNAc)2 (Man)4
8	61	18.1	748.7979	748.7979	Man5	Positive	H	2	46398.093	(GlcNAc)2 (Man)5
9	8	15.5	789.8244	789.8244	G0	Positive	H	2	47342.9115	(GlcNAc)4 (Man)3

The neuraminic acid containing glycans such as HexNAc(4)NeuAc(1)Fuc(1)Hex(5) and HexNAc(4)-NeuAc(2)Fuc(1)Hex(5) are inherently prone to fragmentation and ionization. This may have contributed to the failure to confirm their presence via MS/MS analysis. LCMS 9030 offered excellent mass accuracies for the identified glycoforms, the representative data demonstrating the mass accuracy (less than 7 ppm) obtained for the twelve identified glycoforms. (Table 3) Glycoforms are usually analyzed in MS-scanning mode. However, MS scan does not provide information on branching pattern and isomeric and/or isobaric structures. Thus, tandem mass spectrometry can be used to obtain fragmentation pattern of targeted glycan precursors to determine their structures. This can provide significant information to enhance the confidence and precision in glyco-profiling. Collision energy spread function of LCMS-9030 helps acquire MS/MS fragmentation pattern over a range of collision energy (25-75 V) instead of obtaining MS/MS spectra at single or few selected collision energies The collision energy spread helps to obtain a comprehensive MS/MS fragmentation pattern required to confirm glycoforms structure.

■ Conclusion

- Complete released glycan analysis workflow using Protein Metrics and SimGlycan data analysis softwares for trastuzumab biosimilar is described.
- Excellent mass accuracy with good stability, comprehensive fragmentation pattern and sensitivity offered by LCMS-9030 helps to identify the additional glycan structures that may otherwise get missed during conventional LC-MS mode.
- Software solutions such as Protein Metrics and SimGlycan softwares play a crucial role in mass spectrometry-based glycan analysis workflows.

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