Structural Characterization and Differentiation of Biosimilar and Reference Products Using Modern Analytical Technologies

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Abstract

Regulatory authorities have identified post-translational modifications (PTMs), three-dimensional structures and protein aggregation as being important to development strategies for biosimilars. High resolution mass spectrometry provides accurate and high resolution characterization of various protein properties including primary structure, type and location of PTMs, low abundant sequence post-PTMs, and variants or impurities. This poster describes detailed examples to demonstrate the advantages of combining orthogonal analytical methods in PTM analysis of N-linked glycosylation, glycation, oxidation, deamidation, isomerization, C-terminal variants and N-terminal variants. At the process development stage, modern analytical technology is used to identify, monitor and/or control specific PTMs in order to improve the product similarity profile with respect to defined critical quality attributes. Traditional chromatographic and/or electrophoretic peak groups are utilized as a surrogate for controlling the production processes.

Introduction

PTMs can regulate protein functions by modulating protein activity, turnover, cellular location and protein-protein interaction. In addition, The efficacy and immunogenicity can be highly dependent on the protein sequence and the presence or absence of specific PTMs. Each PTM of a mAb is of a great concerns for regulatory agencies. Advanced and sensitive analytical methodologies are needed to confirm as unambiguously as possible PTMs in a protein sequence, structure. Mass spectrometry has emerged as routine and essential analytical tools for the quality control and structure characterization of therapeutic protein, assuring their batch-to-batch consistency, safety, potency and stability.

At Oncobiologics, orthogonal methodologies, using a range of state-of-the-art techniques, have been employed to demonstrate analytical similarity through comprehensive assessment of primary structure, secondary and higher order structure, size variants, charge heterogeneity, glycosylation, and PTMs.

Methods

- Peptide map for relative quantification (%) of PTMs is performed by specific ion current chromatogram analysis of tryptic peptide map using UPLC-Orbitrap mass spec and the quantification software Pinpoint. Sum of ion intensities from four mono/isotopic ions at two change states is used for relative quantification. PTMs are listed in Table 1.
- N-glycosylation is released using PNGase F and monitored by hydrophilic interaction chromatography.
- Charge heterogeneity is monitored by cation-exchange chromatography (CEX) without carboxypeptidase B treatment.
- Formation of aggregates and fragments are monitored by size exclusion chromatography (SE-UPLC) and capillary electrophoresis (nr-CE-SDS).

Simultaneous Assessment of PTMs Using Mass Spectrometry

Table 1. List of PTMs using Pinpoint Software

Annotation	
C-terminal variants	C-terminal Lys
	C-terminal Proline
	C-terminal Proline Amidation
	C-terminal Gly
N-terminal variants-HC	N-terminal Pyro-E or Pyro-Q
N-terminal variants-LC	LC-Signal peptide
Misincorporation	Misincorporation N-S
Mannosylation	Mannosylation SSSx0
	Mannosylation SSSx1
	Mannosylation SSSx2
	Mannosylation SSSx3
N-glycosylation	EEQYNSTYR
	EEQYN[N-glycosylation-G0F-GlcNac-Man]STYR
	EEQYN[N-glycosylation-G0-GlcNac]STYR
	EEQYN[N-glysosylation-man 5]STYR
	EEQYN[N-glycosylation-G0F-GlcNac]STYR
	EEQYN[N-glycosylation-G0]STYR
	EEQYN[N-glycosylation-G1F-GlcNac]STYR
	EEQYN[N-glycosylation-G0F]STYR
	EEQYN[N-glycosylation-G1]STYR
	EEQYN[N-glycosylation-G2F-GlcNac]STYR
	EEQYN[N-glycosylation-G1F]STYR
	EEQYN[N-glycosylation-G2]STYR
	EEQYN[N-glycosylation-G2F]STYR
Oxidation	Five Methionine Sites
Deamidation & Succimidation	Ten Asparagine Sites indicated by stress treatment
Isomerization	Eight Aspartate Sites indicated by stress treatment
Glycation	Glycation K98

Results

Sequence Heterogeneity

Identification/Quantitation of an elongated N-terminus due to alternate cleavage sites of the signal peptides.

Figure 1: CEX profile of a mAb showing the cell-expressed signal peptide

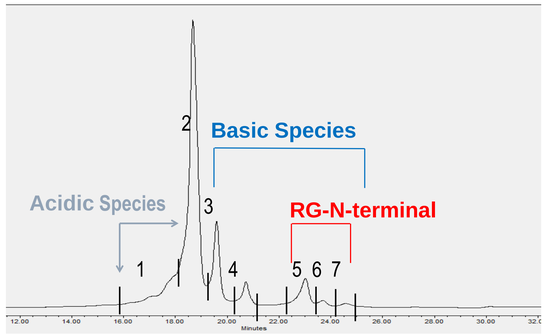


Table 2. Peptide map of the mAb showing the Light Chain N-terminal peptide and improperly cleaved signal peptide

Fraction	1	2	3	4	5	6	7
Correct N-terminus (%)	99.6	100	99.5	97.0	61.5	62.8	82.4
Addition of RG at N-terminus (%)	0.4	0	0.5	3.0	38.8	37.2	17.6

N-glycosylation Site and Quantitation

Figure 2. Correlation of glycans analysis using orthogonal methods

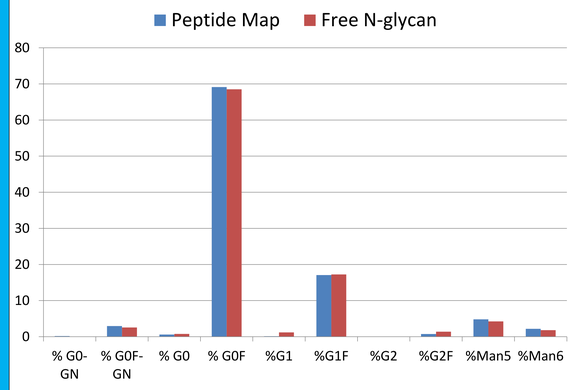


Table 3. N-glycosylation analysis by peptide map and free N-glycan methods

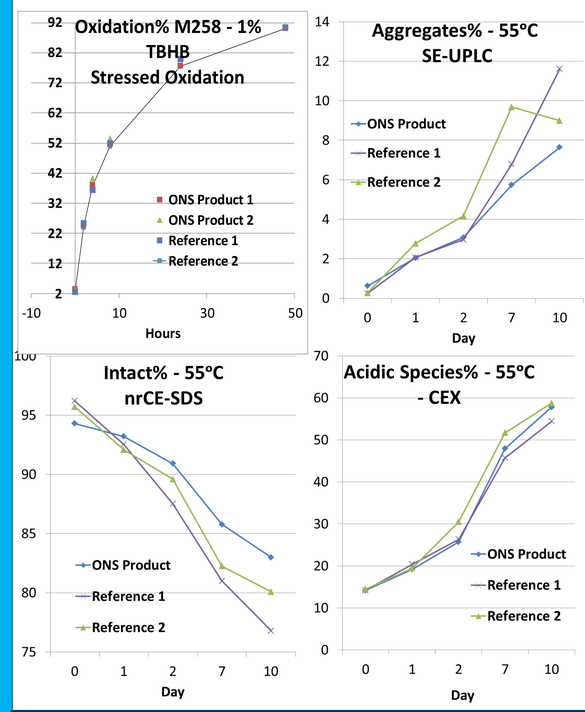
Method		G0-GN	G0F-GN	G0	G0F	G1	G1F	G2	G2F	Man5	Man6
Peptide Map	Mean (%)	0.2	3.0	0.6	69.1	0.1	17.1	0.0	0.8	4.8	2.2
	SD	0.04	0.27	0.10	1.07	0.04	0.84	0.00	0.10	0.43	0.19
Free N-glycan	Mean (%)	0.0	2.5	0.8	68.5	1.2	17.2	0.0	1.4	4.2	1.8
	SD	0.00	0.28	0.05	1.06	0.09	0.77	0.00	0.13	0.36	0.20

* Data is from 10 lots of reference product analyzed on orthogonal glycan methods

Results

Similarity Assessment in Forced Degradation Study

Figure 3: Examples of Oxidation and Temperature Treatment



Discussion & Conclusion

1. The application of various separation chromatography and mass spec technologies has provided analytical support for clone selection, process optimization, and formulation development during biosimlar development.
2. Efforts have been made to develop a fast gradient peptide map method for PTMs quantitation. Inaccurate quantitation of PTMs has been observed due to ion suppression from co-eluted peaks. A stable isotopic internal standard allows a normalized response and resolves the issue.
3. Various mAbs showed specific degradation pathways under stressed oxidation conditions. In one case, the FcRn-methionine was found to be far more susceptible to oxidation than the CDR-methionine. For another mAb, both CDR- and FcRn-methonine were sensitive to oxidation. Implementation of CQA and CQA criteria are defined based on structure-function data.