Assessing Biosimilarity by Monitoring Multiple Critical Quality Attributes of an Intact Monoclonal Antibody Drug Using Orbitrap Native LC-MS

Aaron O. Bailey[1], Bobby Gamez[1], Terry Zhang[1], Stephane Houel[1], Shanhua Lin[2], Guanghui Han[3], Katie Southwick[1], Wendy Sandoval[3], Jonathan L. Josephs[1]. [1]Thermo Fisher Scientific, 355 River Oaks Parkway, San Jose, CA, USA, 95134; [2]Thermo Fisher Scientific, 1228 Titan Way, Sunnyvale, CA, USA, 94085; [3]Department of Microchemistry, Proteomics and Lipidomics, Genentech Inc., South San Francisco, CA, USA, 94080

ABSTRACT

Purpose: To demonstrate feasibility of workflow for monitoring multiple critical quality attributes of a therapeutic protein drug as analyzed in the intact form.

Methods: We analyzed proteins using pH-elution ion exchange coupled directly to native ESI-MS. Isoform identification was accomplished using Thermo Scientific™ BioPharma Finder™ software and XIC-based isoform relative abundance measurements were performed using Thermo Scientific™ Chromeleon™ software.

Results: We demonstrate that ion exchange native MS provides high precision intact mass measurement of Herceptin® and a trastuzumab biosimilar and that Chromeleon software may be used to measure the XIC-based relative abundances of individual CQA isoforms.

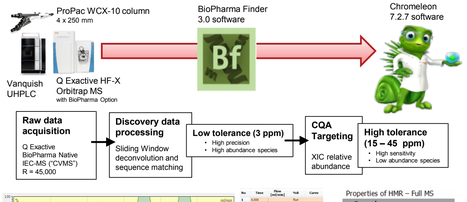
INTRODUCTION

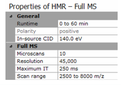
Manufacturing of innovator biologics can be successfully mimicked to produce generic “biosimilar” drug products. In order to satisfy safety and efficacy requirements, a biosimilar drug must be reasonably comparable to an innovator. Comparability is directly assessed by measuring a panel of critical quality attributes (CQAs). Recently, a multi-attribute method has been demonstrated to measure several CQAs simultaneously using LC-MS peptide mapping data. Additional CQAs, such as charge variants or size variants (i.e., aggregation/fragmentation), are conventionally monitored at the intact level using ion exchange (IEC) or size exclusion chromatography (SEC), respectively. Native LC-MS approaches, such as SEC-MS and IEC-MS, combine the traditional gold standard chromatographic assays with accurate mass measurement to allow isoform-specific monitoring of multiple CQAs of intact therapeutic proteins.

MATERIALS AND METHODS

Intact trastuzumab was separated by IEC (Thermo Scientific™ ProPac™ WCX-10 column) directly coupled to MS. Mobile phases consisted of aqueous 50 mM ammonium acetate, using a pH gradient from 6.8 to 10.1. LC-MS was accomplished using a Thermo Scientific™ Vanquish™ H-Class UHPLC system with a variable wavelength detector directly connected to a Thermo Scientific™ Q Exactive™ HF-X Orbitrap™ mass spectrometer. Native LC-MS raw data were analyzed using a time-resolved deconvolution approach utilizing Sliding Window and ReSpect algorithms in BioPharma Finder software. Chromeleon software was used to integrate XIC peak areas and relatively compare CQAs of innovator and biosimilar drug samples.

Figure 1. Platform for on-line native IEC-MS method. Essential instrumentation for our platform includes the ProPac WCX-10 weak cation exchange column, the Vanquish Horizon UHPLC, the Q Exactive HF-X Orbitrap mass spectrometer, and BioPharma Finder data analysis software.





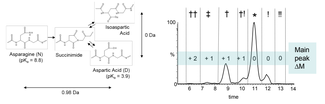
Modification	Example of Interfering Isoforms	ΔM (Da / ppm)	ReSpect Deconvolution and Sliding Window Mass / Merge Tolerances Needed for Method
Deamidation	G0F/G0F vs. G0F/G0F + Deamidation	1 / 6.6	3 / 3
Succinimide (from Aspartic Acid)	G0F/G0F vs. G0F/G0F + AspSucc	18 / 118	15 / 15
Lysine	G0F/G1F vs. G0F/G0F + Lysine	34 / 224	15 / 15
Sialic Acid	G2F/G2F vs. G0F/S1G1F	34 / 224	15 / 15
Hexose	G0F/G0F vs. G0F/G1F	162 / 1069	15 / 15

DEAMIDATION / ISOMERIZATION PATHWAY DOMINATES TRASTUZUMAB CHARGE VARIANT MS PROFILE

Deamidation and isomerization isoforms pose challenge of near-isobar heterogeneity

Trastuzumab includes several known residues in the light chain and heavy chain sequences, which may undergo deamidation (LC-N30) or isomerization (HC-D102) or both at once (HC-N55). These modified isoforms are considered to be ‘near-isobars’, which exhibit mass differences in the range of 0-2 Da, and pose an issue for accurate mass identification by MS. These deamidation and isomerization isoforms are observed in the charge variant profile and can be observed directly using pH-elution ion exchange coupled directly to Orbitrap native MS.

Figure 2. Asparagine deamidation and aspartic acid isomerization are observed as distinctly separated chromatographic peaks in the charge variant profile. These isoforms are near-isobars, ranging in theoretical mass differences of 0-2 Da, making independent intact mass analysis a challenging endeavor. Isoform identities were previously attained by Harris et al., using fractionation and peptide mapping of each LC peak (Reference 1).



Isoform Modification	ΔM (Da)
†† 2 x Deamidation LC-N30	+ 2
‡ IsoAspartate HC-N55	+ 1
† Deamidation LC-N30	+ 1
†† Deamidation LC-N30 IsoAspartate HC-D102	+ 1
* Relatively unmodified	0
! IsoAspartate HC-D102	0
!! 2 x IsoAspartate HC-D102	0

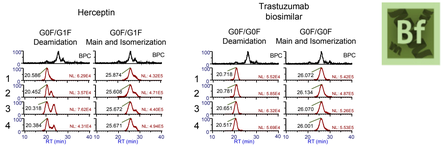
LOW TOLERANCE SLIDING WINDOW DECONVOLUTION (3 ppm) ALLOWS SUB-Da PRECISION FOR INTACT MASS MEASUREMENT

High precision of Orbitrap mass analyzer is demonstrated by reproducible results.

We injected 1 µL (20 µg) volumes of trastuzumab samples in formulation buffer and acquired native LC-MS data on a Q Exactive HF-X system. The results were analyzed using ReSpect and Sliding Window algorithms in BioPharma Finder software. We found a reproducible difference (4 replicates per sample) of ~1 Da between the abundant glycoforms corresponding to the acidic (deamidation) and main (relatively unmodified) LC peaks. Interestingly, we also observed a further ~1 Da difference between innovator Herceptin and a trastuzumab biosimilar, which we could not immediately explain.

Figure 3. High precision measurements show that an approximately 1 Da mass shift is reproducibly measured for abundant glycoforms at the main vs. acidic peaks, which is highly consistent with deamidation.

Sample	Modification (isoform)	Mean Average Mass (Da) *4 replicates	Avg Mass Standard Dev (Da /ppm)	Mean Mass Accuracy vs. Theoretical (Da /ppm)	Mean Relative Abundance (%)	Mean Apex RT (min)
Herceptin	2xA2G0F	148057.95	0.28 / 1.85	3.06 / 20.23	63.53	25.94
Herceptin	1xDeamidation (N),2xA2G0F	148059.36	0.83 / 5.46	3.50 / 23.09	4.76	21.34
Herceptin	1xA2G0F,1xA2G1F	148219.81	0.18 / 1.19	2.86 / 18.89	100.00	25.71
Herceptin	1xDeamidation (N),1xA2G0F,1xA2G1F	148221.33	0.43 / 2.85	3.41 / 22.48	10.63	20.44
Herceptin	2xA2G1F	148381.81	0.39 / 2.59	2.79 / 18.44	70.36	25.49
Herceptin	1xDeamidation (N),2xA2G1F	148383.05	0.99 / 6.55	3.05 / 20.15	5.08	20.58
Trastuzumab Biosimilar 1	2xA2G0F	148058.74	0.15 / 1.01	3.87 / 25.55	100.00	26.07
Trastuzumab Biosimilar 1	1xDeamidation (N),2xA2G0F	148060.26	0.30 / 1.95	4.42 / 29.16	7.87	20.67
Trastuzumab Biosimilar 1	1xA2G0F,1xA2G1F	148220.65	0.19 / 1.25	3.71 / 24.50	40.16	25.89
Trastuzumab Biosimilar 1	1xDeamidation (N),1xA2G0F,1xA2G1F	148222.84	0.65 / 4.26	4.95 / 32.67	4.44	20.57
Trastuzumab Biosimilar 1	2xA2G1F	148382.58	0.37 / 2.44	3.58 / 23.62	15.76	25.74
Trastuzumab Biosimilar 1	1xDeamidation (N),2xA2G1F	148383.60	0.91 / 6.00	3.62 / 23.87	0.90	20.32



MODERATE TOLERANCE SLIDING WINDOW DECONVOLUTION (15 ppm) ALLOWS SENSITIVE AND CONFIDENT DETECTION OF LOW LEVEL CQA ISOFORMS

Confident detection of low-level charge variant CQAs on intact mAb

Native IEC-MS analysis of intact trastuzumab resulted in a chromatographic profile consistent with previously published data. Time-resolved deconvolution analysis of the innovator drug resulted in identification of several specific isoforms comprised of differential N-glycosylation in combination with a variety of low level charge-imparting PTMs, such as deamidation, C-terminal lysine removal, or sialylation of N-glycans. We confirmed that the main peak consisted of several isoforms corresponding to variable N-glycosylations of the trastuzumab amino acid sequence. When comparing the innovator and biosimilar products, we found marked differences in glycoform profile measured at the main peak. Furthermore we found measureable differences in several charge variant isoforms when comparing innovator and biosimilar. These differences observed were consistent with a comparison of these same two samples via MAM HRAM peptide mapping analysis (data not shown).

Figure 4. Glycoform profiles are easily identified using ReSpect and Sliding Window algorithms in Biopharma Finder software. Our “moderate” mass tolerance of 15 ppm is wide enough to detect both deamidated and main peak forms of trastuzumab. We readily observed distinct glycoform profiles for Herceptin and biosimilar trastuzumab samples.

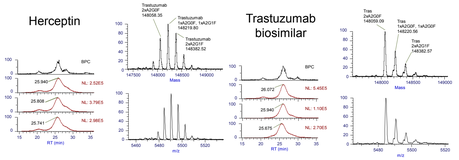
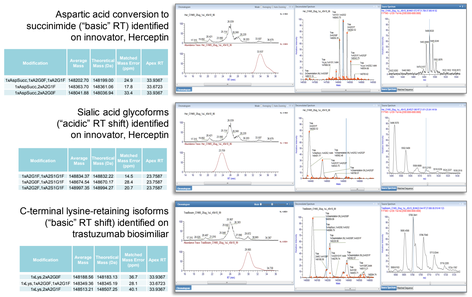


Figure 5. Screenshots from BioPharma Finder software show that lower-level CQA isoforms such as succinimide conversion from aspartic acid, sialic acid glycoforms, and C-terminal lysine-retaining isoforms are distinctly identified by mass and retention time shift relative to the main peak.



MONITORING CQA ISOFORMS IN CHROMELEON SOFTWARE

Large scale relative abundance measurements of isoforms identified by BioPharma Finder software

Quantitative monitoring of protein drugs can be easily accomplished using peptide mapping LC-MS using a so-called Multi-Attribute Method (MAM) (**Reference 2**). This type of approach allows CQAs to be become characterized and then monitored in a routine fashion. The combination of BioPharma Finder software and Chromeleon software serves as a powerful framework for MAM applications to enable deep protein characterization and further method streamlining for downstream process development.

We sought to develop a workflow that performs targeted quantitation of isoform charge states identified by Sliding Window deconvolution of native IEC-MS datasets. We manually exported the centroid *m/z* values of the charge states of isoforms identified by ReSpect / Sliding Window analysis in BioPharma Finder software. Centroid m/z values were averaged from the 4 replicates and these average values were targeted in Chromeleon.

Figure 6. BioPharma Finder intact protein analysis results can be curated to determine which species should be monitored as CQAs. Interesting isoforms can be selected (box checked) to mark for export in spreadsheet format. To export the constituent charge states’ centroid *m/z* values for the isoforms identified by BioPharma Finder software, right-click > “Export Checked” > “All Levels”.

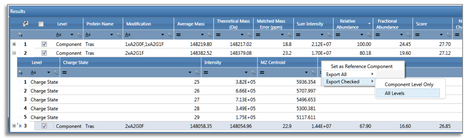


Figure 7. Isoform charge state *m/z* values and RT information can be used to create a processing method with flexible options for XIC integration. We quantified 3–4 charge states for each isoform, which is shown here in an overlay plot shaded for customized integration specific for the main peak and deamidated isoforms of G0F/G0F, extracted at 3, 15, 30, and 45 ppm tolerances.

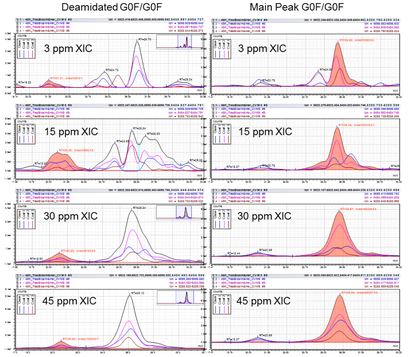
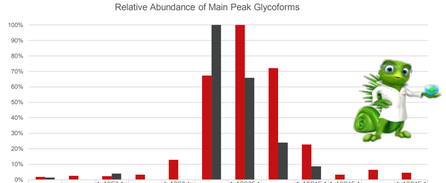
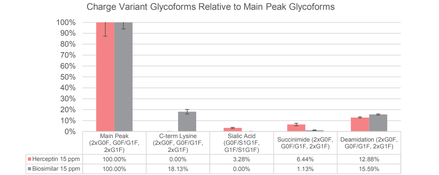


Figure 8. Intact glycoforms and charge variant CQAs can be simultaneously monitored. Charge variants CQAs are analyzed as groups of glycoforms, which may be useful for comparing innovator and biosimilar drugs. As shown below, we observed the biosimilar trastuzumab to contain decreased levels of succinimide isoforms compared to Herceptin, but to have markedly higher levels of C-terminal lysine retention.



	1xA2G0F	1xA2G1F	1xA2G0,1xA1G0F	2xA2G0	1xA2G0,1xA2G0F	2xA2G0F	1xA2G0F,1xA2G1F	2xA2G1F	1xA2G1F,1xA2G2F	1xA2G1F,1xA2S1G0F	2xA2G2F	1xA2G2F,1xA2S1G1F
Herceptin 30 ppm	1.85%	2.52%	2.37%	3.37%	12.94%	67.28%	100.00%	72.16%	22.84%	3.37%	6.39%	4.51%
Biosimilar 30 ppm	1.47%		4.01%			100.00%	65.86%	24.00%	8.64%			



	Main Peak (2xG0F, G0F/G1F, 2xG1F)	C-term Lysine (2xG0F, G0F/G1F, 2xG1F)	Sialic Acid (G0F/S1G1F, G1F/S1G1F)	Succinimide (2xG0F, G0F/G1F, 2xG1F)	Deamidation (2xG0F, G0F/G1F, 2xG1F)
Herceptin 15 ppm	100.00%	0.00%	3.28%	6.44%	12.88%
Biosimilar 15 ppm	100.00%	18.13%	0.00%	1.13%	15.59%

CONCLUSIONS

- Method for monitoring multiple CQAs of an intact biotherapeutic compound to allow direct comparison of innovator and biosimilar products.
  - Orbitrap mass analyzer is capable of mass measurements with high (sub-Dalton) precision
  - BioPharma Finder software can be used to manually export mass lists from native ion exchange MS experiments, and *m/z* values may be targeted and quantified in Chromeleon software.

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TRADEMARKS/LICENSING

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