Robust and Sensitive Workflow for Qualitative and Quantitative Analysis of Intact Monoclonal Antibodies Using Microflow LC and TripleTOF Mass Spectrometry

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Introduction

Protein biotherapeutics such as immunoglobulin G (IgG)-derived monoclonal antibodies are an attractive targeted therapy to treat an array of diseases like cancer, autoimmune disorders and infectious diseases. Understanding the primary structure, heterogeneity, and post-translational modifications of these biologics is essential to understanding function, developing novel therapeutics and ensuring product safety and quality. LC-MS analysis has become an essential tool for the identification, characterization and quantification of intact monoclonal antibodies (mAbs) and similar high-molecular-weight proteins[1,2]. Here, we describe a robust and sensitive workflow using a M3 MicroLC coupled to a TripleTOF® 6600 mass spectrometer for qualitative and quantitative analysis of intact mAbs. Analytical LC-MS methods often deliver insufficient sensitivity. Microflow LC-MS, in spite of its inherent sensitivity advantage, has not been used extensively because the necessary off-line desalting and sample clean up can result in sample loss, and long sample preparation cycles. The typical on-column desalting with a divert valve as used in analytical flow LC cannot be easily implemented in microflow LC, due to chromatographic dispersion at lower flow rates. The method we describe here takes advantage of a different on-column desalting approach to decrease sample preparation time and increase throughput and sensitivity.

Materials and Methods

Sample Preparation: Intact mAb Mass Check Standard (Waters, P/N 186006552), trastuzumab and adalimumab (Myoderm, NDC #50242-0134-68, NDC# 0074-3799-02 ABB) were serially diluted with 1 pM/µl of BSA (bovine serum albumin) in 0.1% FA (formic acid, Thermo Scientific P/N 28905). Adalimumab was desalted off-line using a 10K cut off Amicon Ultra Centrifugal filter (Sigma-Aldrich, P/N Z677108-96EA) with 10% acetonitrile in 0.1% FA , and recovered with 0.1% FA.

Microflow Liquid Chromatography: The on-column desalting and separation of all three mAbs was performed on an M3 MicroLC (SCIEX) using a Waters ACQUITY UPLC M-Class Protein BEH C4 column (300Å, 1.7 µm, 300 µm X 50 mm) at a 15 µl/min flowrate. All tests were performed in direct inject mode using the LC gradient in Table 1. The column temperature was maintained at 60° C. Five microliters of each serial dilution was loaded on column for each of the replicate analyses. Mobile phase A was 100% water with 0.1% formic acid and 0.01% trifluoroacetic acid (TFA, Thermo Scientific P/N 28904). Mobile phase B was 100% acetonitrile with 0.1% formic acid and 0.01%T FA. On-column desalting was performed using an isocratic step at 20%B with the electrospray voltage set to 0 to avoid salts entering the mass spectrometer during the on-column desalting process.

Table 1. Microflow LC Gradient Used for Intact Antibody Analysis.

Time(min)	% Solvent B
0	20
3	20
5	80
6	80
6.5	20
7	20
7.5	5
10	5

Table 2. MS and DuoSpray™ Source Parameters.

MS Parameters	Values
Electrode ID	25 um
Curtain Gas	25
Collision Energy	25
IonSpray Voltage	5500
Temperature (°C)	300
Ion Source Gas 1	35
Ion Source Gas 2	35
Declustering Potential	240
Polarity	Positive
Mass Range	1000-5000
Accumulation Time (sec)	1.0
Time Bins to Sum	80
Scan Type	TOF MS
Intact Protein Mode	On

Mass Spectrometry: MS analyses were performed using a TripleTOF 6600 equipped with a DuoSpray™ Source and 25 µm I.D. electrode (SCIEX). The MS method consisted of two periods: 3 min with ion spray voltage floating (ISVF) set to 0V during on-column desalting to avoid spraying salt into the MS, followed by 7 min with ISVF set to 5500V for sample analysis. The detailed MS and source parameters are shown in Table 2. A minimum of three replicate injections were performed for each serial dilution.

Data Processing: Analysis of intact mAbs, including spectral deconvolution, mass reconstruction and analysis of glycans and other post-translational modifications (PTMs), was performed using BioPharmaView™ (version 1.4.9170) software. mAbs quantitation analysis, including calibration curve, CV and accuracy, was performed using MultiQuant™ (version 3.0.2) software.

Data Reproducibility

The M3 MicroLC-MS method provided highly reproducible retention time, spectra, peak height and peak area for all three antibodies (Figure 1).

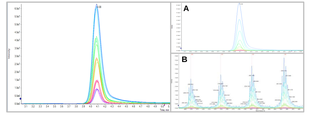

Figure 1. High reproducibility of retention time (RT) and sensitivity. XIC data for Waters Intact mAb Standard at 50-1000 ng load on column shows high reproducibility of RT (<0.1% RSD) and intensity (<3% RSD). Peak height corresponding to 0.1-1000 ng mAb load on column is shown in Figure 1a. The spectra for +52-55 charge species of Waters mAb in 0.1-1000 ng range are shown in Figure 1B.

Advantages of On-Column Desalting

Commercial mAbs require buffering salt for stabilization in solution. To achieve sensitivity and high resolution spectra it is critically important to remove any salts before LC-MS analysis. This is typically done either by off-line desalting or a trap-elute workflow. Here, we used on-column desalting to minimize sample preparation time and avoid sample loss. To confirm the viability of microflow on-column desalting, we compared this approach with on-membrane desalted adalimumab (50 ng load on column). We observed less sample loss and greater sensitivity using the on-column desalting method (Figure 2).

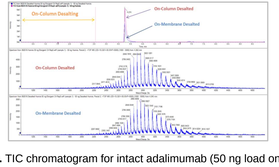

Figure 2. On-Column Desalting. TIC chromatogram for intact adalimumab (50 ng load on column) clearly shows the gain in sensitivity and efficient desalting using an on-column desalting approach.

Improvement in Signal to Noise Ratio

The data obtained for Waters Intact mAb Standard utilizing microflow LC provided improvement of s/n ratio, resulting in 5X improvement in sensitivity when compared to Analytical LC.

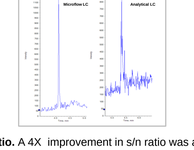

Figure 3 . Improved Signal to Noise Ratio. A 4X improvement in s/n ratio was achieved using microflow LC for 0.5ng of Waters Intact mAb Standard on column, which resulted in a 5X lower LLOQ.

Increased Dynamic Range

Quantitation of intact proteins was evaluated based on linear dynamic range of the method, the lower limit of detection (LLOD) and the quantitation reproducibility and accuracy. Three different intact antibodies, Waters Intact mAb Standard, adalimumab (Humira) and trastuzumab (Herceptin), were used to evaluate the analytical performance of the integrated M3 MicroLC coupled to the TripleTOF 6600. Quantitation was performed with MultiQuant using one or a sum of multiple protein charge states with linear regression and 1/x weighting (Figure 4).

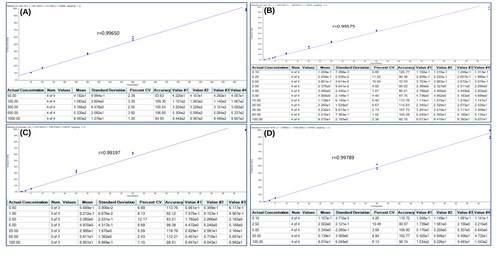

Figure 4. Linear Quantitation Curve for mAbs. Calibration curves for replicate analysis of different amount of Waters Intact mAb Standard (Figure 4A and 4B) with r =0.99 (charge state +48). Similar data and r values were obtained for quantitation of trastuzumab (charge state +55) (Figure 4C) and adalimumab (sum of charge states +46 to +55) (Figure 4D).

Intact mAbs Characterization and PTM Analysis

The sensitivity gained using the M3 MicroLC coupled with the high resolution and mass accuracy of TripleTOF 6600 enabled comprehensive analysis of intact mAbs at low ng level. We demonstrated successful characterization of Waters Intact mAb Standard in low nanogram amounts (2.5 ng), including N-terminal glutamate to pyroglutamate conversion and different glycosylations (M*+G0F+G0F, M*+G0F+G1F, M*+G1F+G1F, M*+G1F+G2F, M*+G2F+G2F). Characterization of mAbs glycoforms and other PTMs using BioPharmaView provided routine molecular weight determination and characterization of mAbs (Figure 5). Additional modifications such as oxidation, deamidation, pyroglutamic acid formation at the N-terminus and N-terminal lysine loss were identified on trastuzumab (Figure 6A) and adalimumab (Figure 6B).

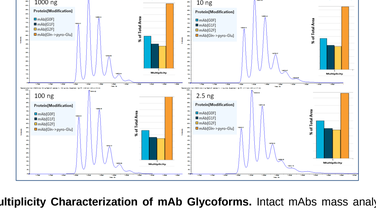

Figure 5. Quantitative Multiplicity Characterization of mAb Glycoforms. Intact mAbs mass analysis, including spectral deconvolution, mass reconstruction and analysis of glycans and other PTMs was performed.

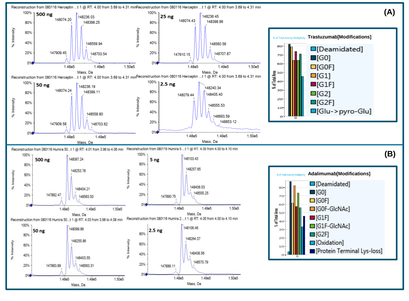

Figure 6. Characterization of Trastuzumab and Adalimumab. Intact trastuzumab (6A) and adalimumab mass analysis (6B) including spectral deconvolution, mass reconstruction and analysis of glycans and other PTMs was performed using BioPharmaView.

Conclusions

- Single-run characterization and quantitation of intact mAbs and similar biologics
- Robust quantitation of intact mAbs over a linear dynamic range of 3 orders of magnitude (0.1-100 ng on column)
- Quantitation at 5x lower concentration than can be achieved with standard-flow LC-MS
- Characterization of the major mAbs glyco-isoforms at the low nanogram levels
- Fewer sample preparation steps with on-column desalting; eliminating off-line desalting (~30 min process) and removal of carbohydrates (overnight digestion process)
- High throughput intact mass analysis of mAbs (~140 samples/day)
- Reduced solvent, desalting, enzyme and operation costs

References

1. Johansen, E., Shin, B-H., and C. Hunter. Simultaneous Quantitative and Qualitative Analysis of Proteolytic Digests of Therapeutic Monoclonal Antibodies Using a TripleTOF® System. SCIEX Technical Note, Document Number 7460213-01.
2. Eric Johansen, Jenny Albanese and Christie Hunter. Analysis of Intact and Reduced Therapeutic Monoclonal Antibodies using the TripleTOF 5600 System. SCIEX Technical Note, Document Number 4220211-01.

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Document number:[RUO-MKT-10-5375-A]