Quantitation of Intact Therapeutic Protein in Plasma Matrix by LC/MS

Yihan Li[1], Ian Moore[2], Fan Zhang[1], Sahana Mollah[1]
[1]SCIEX, Redwood Shores, California, USA, [2]SCIEX Concord, Canada

ABSTRACT

A complete workflow to quantify intact therapeutic proteins in plasma matrix has been developed, covering three critical components in preclinical sample analysis: target protein enrichment/purification, LC/MS data acquisition and quantitative data analysis. Trastuzumab in rat plasma was enriched/purified with 1-hour immuno-capture workflow, to remove background matrix. The enriched/purified intact trastuzumab samples were analyzed by high-flow LC/MS in SCIEX X500B QTOF system. A novel data processing strategy with automated data reconstruction in PeakView® software was utilized to filter out interference from residual matrix proteins. Quantitation of trastuzumab in rat plasma at intact protein level was achieved with a linear dynamic range of 100 ng/mL to 50 000 ng/mL and a limit of detection of 50 ng/mL in 50 µL of rat plasma.

INTRODUCTION

With unprecedented growth of protein therapeutics in the pharmaceutical industry, therapeutic proteins have been an important component of medical practice and clinical research. This advancement is paralleled with development of analytical technologies and methods to detect, identify, monitor and quantify therapeutic proteins. LC/MS based quantitation of intact therapeutic proteins using high-resolution mass spectrometry (HRMS) is of great interest, as it offers a high throughput platform allowing accurate quantitation and differentiation of major modifications at the intact protein level.

An increasing number of quantitation methods directly analyzing intact therapeutic proteins have been published in the last five years, focusing on quantitation of clean intact monoclonal antibody standards or monoclonal antibodies in matrix after lengthy deglycosylation. However, there is a lack of literature on quantitation of intact therapeutic monoclonal antibodies in complex biological matrix, challenges of which include: 1) protein signal distributes over multiple charge states of multiple glycoforms, leading to low intensities from ions of interest; 2) interference from matrix proteins; 3) limited guidance on data analysis. Extracted ion chromatograms (XICs) from the non-deconvoluted full-scan mass spectrum are commonly used in data processing for quantitation purpose, as HRMS provides accurate mass spectra of multiply charged ions. This approach may not be feasible in the case of intact therapeutic monoclonal antibodies in biological matrix, due to interference from matrix proteins and low intensity of target ions at low concentrations.[1,2] Another approach is to select the peak of target intact therapeutic monoclonal antibody in LC chromatogram and deconvolute spectra underneath the selected peak, then use the deconvoluted data for quantitation.[1] However, this may lead to loss of original information during peak selection and impact the robustness of method especially for complex samples.[2] With the advancement of data analysis software, we developed a novel data processing strategy for quantitation purpose, utilizing the available protein deconvolution algorithm to reconstruct the entire data file by deconvoluting every full-scan mass spectrum recorded in the data file. XICs of major glycoforms of target intact therapeutic monoclonal antibody in the reconstructed data are used for quantitation.

This work aimed to present a complete generic workflow to quantify therapeutic proteins in plasma matrix at intact protein level, with fast and simple sample handling procedures to minimize sample manipulation, and straight-forward data processing strategy to enhance selectivity and sensitivity during quantitation.

MATERIALS AND METHODS

Sample Preparation:

Biotinylated anti-human IgG antibody was conjugated onto streptavidin coated magnetic beads. The conjugated beads were incubated with plasma samples, to capture trastuzumab by immuno-affinity. The beads were then isolated from plasma matrix by magnetic attraction. After plasma was removed, the beads were washed twice to clean up residual plasma proteins. 50 µL of 0.5% formic acid was added to elute trastuzumab from beads.

LC and MS Conditions:

SCIEX ExionLC™ AD system coupled to SCIEX X500B QTOF system was used with an Agilent PLRP-S reversed-phase, 50 x 2.1 mm, 300 Å, 5 µm column. A gradient of buffer A (99.9% water, 0.1% formic acid) and buffer B (99.9% acetonitrile, 0.1% formic acid) was used at a flow rate of 500 µL/min (buffer B concentration held at 25% for 3 min; ramped linearly from 25 to 45% from 3 to 4.5 min; ramped up again from 45 to 95% from 4.5 to 4.7 min; held at 95% from 4.7 to 7.5 min; then returned to 25% by 7.6 min and held at 25% until the end of the run at 10 min. The injection volume was set to 30 µL.

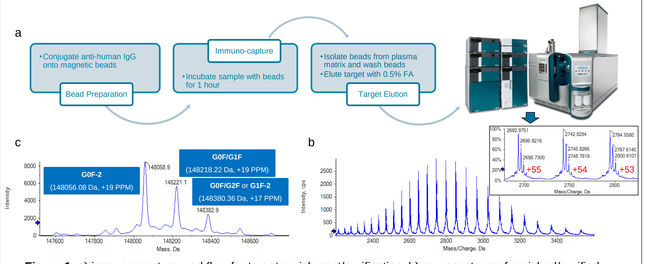

Figure 1. a) immuno-capture workflow for target enrichment/purification; b) raw spectrum of enriched/purified trastuzumab from rat plasma, generated in SCIEX X500B QTOF system; c) deconvoluted data of raw spectrum in fig.1b (Input m/z range: 2200-3600. Resolution: 5000. Output mass range: 130-170 kDa).

RESULTS

One of the challenges quantifying intact therapeutic proteins in biological matrix is interference from sample matrix. Though the majority of sample matrix was removed during sample preparation using immuno-capture workflow, interference from residual matrix proteins was still seen in the final data sets. The ions from residual matrix proteins (endogenous IgG, etc.) might overlap with target ions, as a result, generating XICs from non-deconvoluted spectra could not isolate trastuzumab signals from interference. (see Fig. 3b) The accuracy of quantitation suffered at low concentrations. Compared to the approach using XICs from non-deconvoluted mass spectra, data processing strategy utilizing deconvoluted data had clear advantages in case of complex samples:

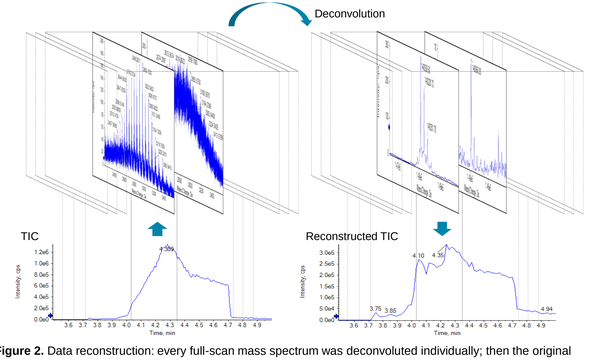

Figure 2. Data reconstruction: every full-scan mass spectrum was deconvoluted individually; then the original spectrum was replaced by the corresponding deconvoluted data, to generate a reconstructed data file.

both input m/z range and output protein mass range were defined in protein deconvolution algorithm, so that matrix proteins not in the selected output mass range were filtered out together with ions not in the selected input m/z range. With conventional data analysis software, deconvolution could only be done manually on selected raw mass spectra in individual data files. To overcome the concern of loss of original information and robustness of analysis, we developed an automated data reconstruction workflow in Research PeakView® 1.2.2.0 with Bio Tools (protein/peptide reconstruction) 1.0.0.0: every raw mass spectrum was deconvoluted, then the original raw mass spectrum was replaced by the corresponding deconvoluted data, to generate a reconstructed data file. (Fig.2) Automated batch analysis of multiple data files was also enabled in this process.

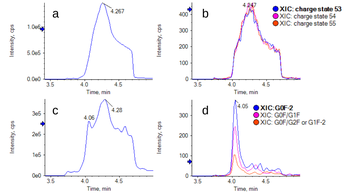

Figure 3. Chromatograms of 200 ng/mL trastuzumab in rat plasma. a) TIC, non-deconvoluted; b) XICs of the most abundant charge states (see Fig.1b) from non-deconvoluted data, isolation window ±0.1 Th; c) TIC after data reconstruction; d) XICs of major glycoforms (see Fig.1c) in deconvoluted data after data reconstruction, isolation window ±2 Da.

After data reconstruction, by generating XICs of major glycoforms of trastuzumab, interference from matrix proteins was filtered out thoroughly and clean trastuzumab peaks formed in LC chromatograms, as shown in Fig.3d. Peak selection and integration in resulting XICs were automatically done in Research PeakView®, with information of peak area and retention time provided. Quantitation was carried out using peak area of major glycoforms.

Glycoforms of G0F-2 and G0F/G1F were detected in 50 ng/mL of trastuzumab in rat plasma (Fig.5b), with S/N ratios of 4 and 2.25. Quantitation was achieved with a linear dynamic range of 100 to 50 000 ng/mL, using either peak area of glycoform G0F-2 or summed peak area of glycoforms G0F-2 and G0F/G1F (Fig. 6 and 7). By comparing data in Table 1 and 2, the observed consistency between calculated concentrations and accuracy confirmed that this data processing strategy was consistent in data deconvolution/reconstruction and XIC peak selection/integration. The ratio of glycoform G0F-2 peak area and glycoform G0F/G1F peak area could be used to estimate the concentration ratio of these two major glycoforms in the samples.

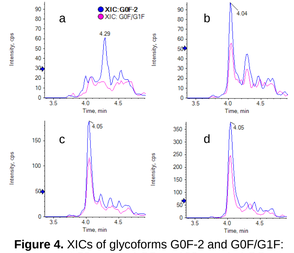

Figure 4. XICs of glycoforms G0F-2 and G0F/G1F: a) blank; b) 50 ng/mL, LOD; c) 100 ng/mL, LOQ; d) 200 ng/mL.

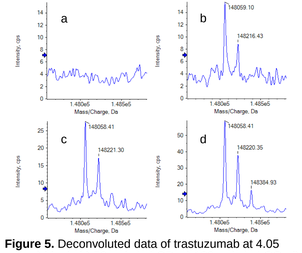

Figure 5. Deconvoluted data of trastuzumab at 4.05 min: a) blank; b) 50 ng/mL, LOD; c) 100 ng/mL, LOQ; d) 200 ng/mL.

Actual Conc. (ng/mL)	RT (min)	Peak Area	Calculate Conc. (ng/mL)	Accuracy %
100	4.05	1030.46	113.65	113.65
200	4.05	2366.06	178.36	89.18
500	4.05	7629.86	433.37	86.67
5000	4.05	92003.36	4521.04	90.42
10000	4.04	214493.22	10455.34	104.55
20000	4.05	418274.19	20327.97	101.64
50000	4.04	1027517.28	49844.13	99.69

Table 1. Data of calibration standards using peak area of glycoform G0F-2.

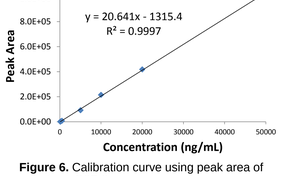

Figure 6. Calibration curve using peak area of glycoform G0F-2.

Actual Conc. (ng/mL)	RT (min)	Peak Area	Calculate Conc. (ng/mL)	Accuracy %
100	4.05	2087.54	115.97	115.97
200	4.05	4668.67	178.89	89.45
500	4.05	15363.03	439.58	87.92
5000	4.05	180089.79	4454.95	89.10
10000	4.04	425333.87	10433.02	104.33
20000	4.05	832089.10	20348.07	101.74
50000	4.04	2041509.55	49828.87	99.66

Table 2. Data of calibration standards using summed peak area of glycoforms G0F-2 and G0F/G1F.

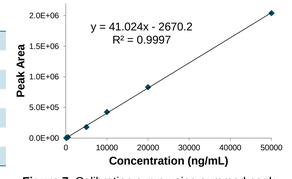

Figure 7. Calibration curve using summed peak area of glycoforms G0F-2 and G0F/G1F.

CONCLUSIONS

Here we report a complete workflow for quantitative analysis of therapeutic monoclonal antibody trastuzumab in rat plasma, achieving 2.7 orders of magnitude in linear dynamic range at intact level. This method minimizes sample manipulation, instead focuses on data processing strategy to improve selectivity and sensitivity. Interference from matrix is filtered out thoroughly XIC approach after data reconstruction, performed in batch processing mode. Validated in terms of selectivity, sensitivity, accuracy and consistency, this workflow could serve as a generic quantitation method for intact therapeutic proteins in complex biological matrix.

Acknowledgement

We would like to thank our colleague Lyle Burton for providing technical assistance and support.

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Document number: RUO-MKT-10-7788-A