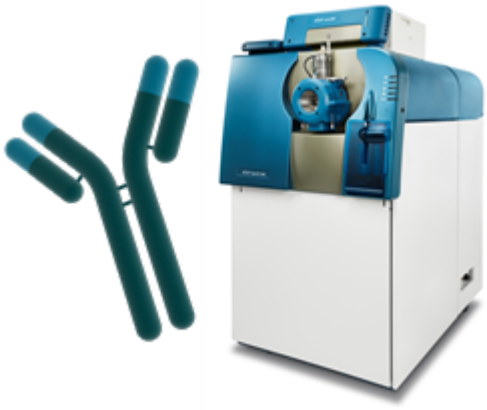
SCIEX Answers for Science. Knowledge for Life.™

Protein Quantitation Workflows using the TripleTOF 6600: A Case Study for Rituximab

Posted June 28, 2016 at 10:17 AM
By Jeff Plomley



Although the triple-stage quadrupole (QQQ) mass spectrometer remains the pillar for quantitative LC-MS/MS bioanalytical assays, due in part to the platforms' high duty cycle when operated in multiple-reaction monitoring (MRM) mode, the applicability of high-resolution mass spectrometry (HRMS) has become of increasing importance for protein quantitation given the complexity of proteolytically digested samples in the surrogate peptide approach. While the QQQ demonstrates high sensitivity and specificity, the relatively low-resolution measurement of *m/z* may fail to differentiate analyte response from nominally isobaric background interference.

In contrast, HRMS with accurate mass assignment of product ion allows interference to be resolved through judicious selection of a post-acquisition mass extraction window whose tolerance is largely dictated by the effective resolution and stability of mass calibration.

In this editorial, we examine how researchers at Algorithme Pharma, an Altasciences Clinical Research company, have leveraged the high resolution/accurate mass (HRAM) capabilities of the TripleTOF 6600 (/products/mass-spectrometers/qtof-systems/tripletof-systems/tripletof-6600-system) in comparison to the QQQ platform for their protein quantitation challenges, exemplified in the development of an assay for the monoclonal antibody (mAb) Rituximab using a dual surrogate peptide approach.

TripleTOF 6600 Features Ideal for Quantitation
Since mass resolving power is independent of acquisition speed in time-of-flight measurements, the TripleTOF 6600 system has a distinct advantage for quantitative applications compared to trap-based platforms in that a sufficient and constant number of data points can be generated across chromatographic peaks, independent of analyte concentration. Furthermore, although the TripleTOF 6600 system uses an

ADC detector, it operates in a “TDC mode”, thus generating less background noise than a traditional ADC, thereby improving S/N. Detection in “TDC mode” also allows extension of dynamic range in the direction of high signals while maintaining low-level signal detection. A maximal time resolution of 25 ps translates to achievable mass resolutions ($m/\Delta m_{FWHM}$) of 35,000 in TOF MS mode, 30,000 in High-Resolution TOF-MS/MS mode, and 20,000 in High Sensitivity TOF-MS/MS mode. This mass resolving power is significant in allowing the differentiation of analyte *m/z* from potentially close eluting interference.

In order to achieve product ion chromatogram selectivity in a TOF-MS/MS experiment and simultaneously avoid missing data points in the analyte XIC, the mass extraction window requires careful titration within the tolerable limits for a given mass resolution. Predictably, analyte S/N increases as the mass extraction window decreases, with reproducibility of the extraction window reliant upon mass stability being maintained throughout the analysis. Mass assignment in TOF depends on the arrival time of the ion to the detector, and changes in accelerating voltage and temperature create flight time shifts in each scan. Since the TOF mass spectrum is generated from the sum of many transients, these factors randomly combine to widen spectral peak widths and deteriorate mass accuracy. However, in the case of the TripleTOF 6600, Algorithme Pharma researchers noted the remarkable stability of mass calibration, with reproducibilities for mass assignment < 3 ppm when measured over a 12-hour period using only a single external calibration event (Figure 1).

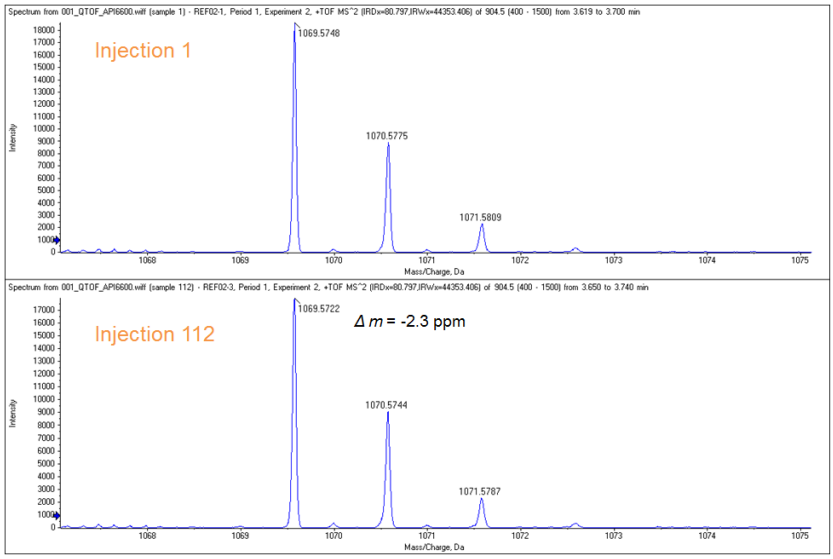


Figure 1. Reproducibility of mass assignment for the *m/z* 1069 product ion from the light chain of Rituximab after > 100 sample injections (~12 hr) with external mass calibration performed once prior to batch injection. A mass drift of -2.3 ppm demonstrates excellent stability.

Accurate Mass Filtering: Leveraging Gas Phase Selectivity to reduce Sample Preparation Complexity and Increase Sample Throughput

The application of high resolution with accurate mass product ion XICs in a TOF-MS/MS experiment oftentimes eliminates the requirement for advanced sample preparation techniques, such as immunoenrichment of target analyte or immunodepletion of endogenous immunoglobulins; the latter approach to minimize sequence homology with the surrogate peptide of interest. Following the peptide mapping workflow outlined in Figure 2, researchers identified surrogate peptides representing the light chain (LC) and heavy chain (HC) of the variable regions of the Fab fragments of Rituximab. Sample preparation involving simple pellet digestion with subsequent analysis on an API 5000 operated in MRM mode revealed significant interference for the LC at the transition required for optimal sensitivity (Figure 3); in marked contrast, the selectivity with a 25 mDa product ion extraction window from the TripleTOF 6600 data acquired in Enhanced MS/MS mode allowed the targeted LLOQ of 1 µg/mL to be achieved; notably, the ^{12}C and $^{13}C_1$ of the two most predominant product ions could be summed for improved sensitivity without loss in selectivity resulting in a three-fold S/N gain.

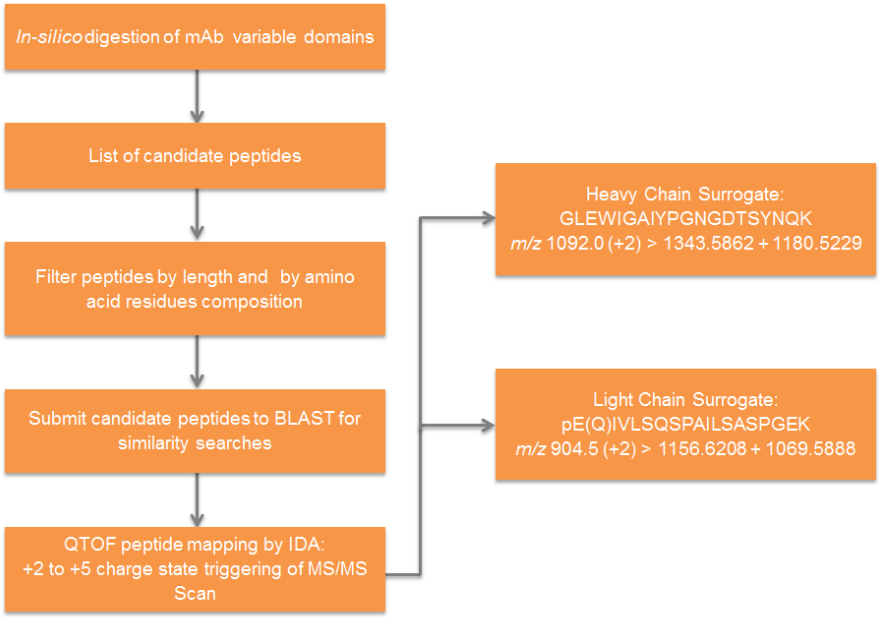


Figure 2. Workflow for the selection of surrogate peptides from the variable regions of the Fab fragments of Rituximab, a chimeric mAb consisting of two light chains (LC) with 213 amino acids and two heavy chains (HC) with 451 amino acids.

For the HC, the targeted LLOQ of 1 µg/mL could be realized using the API 5000. However, the abundance of co-extracted endogenous components closely eluting chromatographically were of concern to researchers (Figure 4). In an approach analogous to that for the LC, accurate mass product ion filtering and summation of the isotopes of the two most predominant fragment ions using the TripleTOF 6600 simplified the background matrix signal for the HC, instilling a greater level of confidence in assay selectivity.

An advantage of high-resolution accurate mass data is the ability to titrate a number of mass extraction windows post-acquisition. As illustrated in Figure 5, while absolute response decreases as the extraction window is narrowed, S/N increases, providing an overall lower level of detection.

Should the highest resolution of the TripleTOF 6600 not be required to differentiate analyte response from interference, sensitivity can be increased using the Enhanced MS/MS scan function, wherein ions are trapped in the collision cell, accumulated, then released to the TOF pulser. While researchers noted a small loss in resolution, having no impact on assay selectivity, response gains were approximately five-fold (Figure 6).

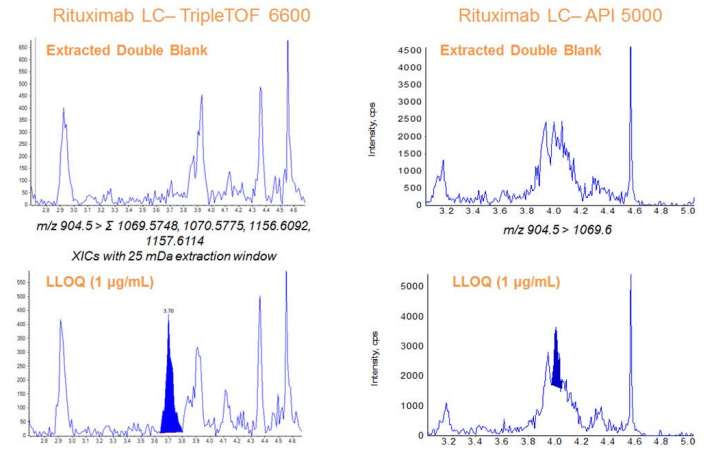


Figure 3. Comparison of TripleTOF 6600 and API 5000 double-blank and LLOQ response for the Light Chain of Rituximab following pellet digestion. TripleTOF data were acquired in Enhanced MS/MS mode with Q1 set to open resolution and QQQ data in MRM mode.

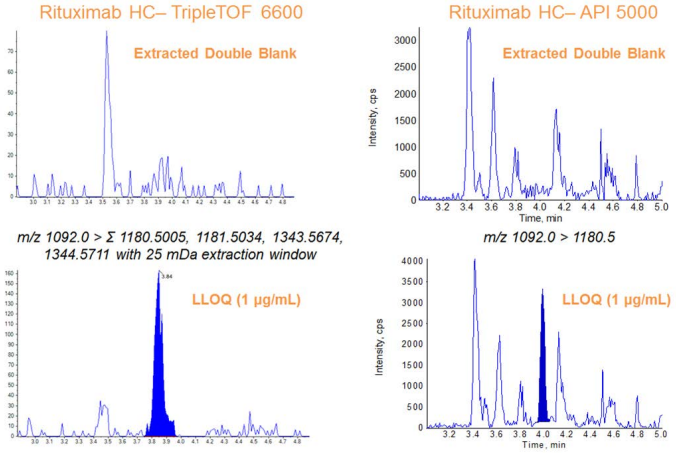


Figure 4. Comparison of TripleTOF 6600 and API 5000 double-blank and LLOQ response for the Heavy Chain of Rituximab following pellet digestion. TripleTOF data were acquired in Enhanced MS/MS mode with Q1 set to open resolution and QQQ data in MRM mode.

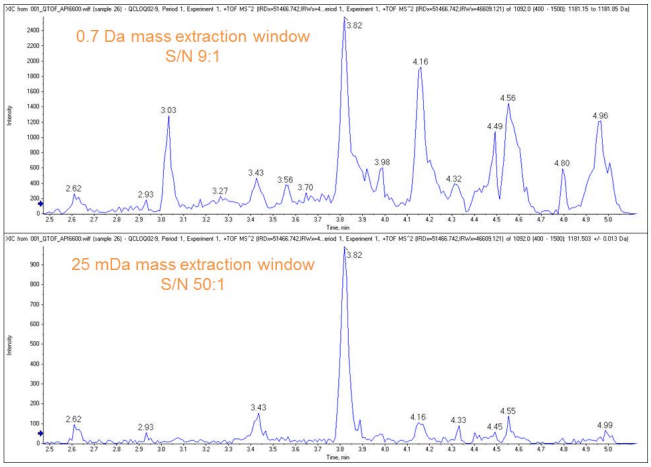


Figure 5. Signal-to-noise comparison for product ion mass extraction windows of 0.7 Da (equivalent to nominal resolution in the QQQ) and 25 mDa for the HC of Rituximab acquired on the TripleTOF 6600.

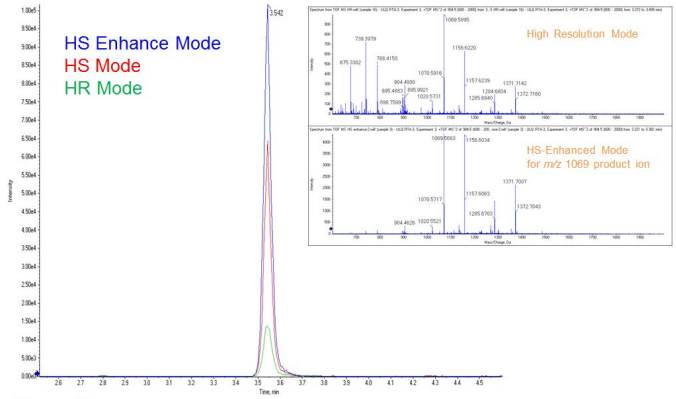


Figure 6. Sensitivity comparison of the High Resolution (HR), High Sensitivity (HS), and HS-Enhanced Mode of the TripleTOF 6600 for the measurement of the LC of Rituximab *(m/z* 904.5 > Σ 1069.5748, 1070.5775, 1156.6092, 1157.6114 with 25 mDa extraction window) with product ion spectra demonstrating an abundance bias ≥ the mass specified for enhancement.

Quantitation

An advantage of the pellet digestion approach used for the Rituximab assay is the dissociation of any ADA (due to the initial methanol precipitation step) and thus the quantitation, unlike an immunopurification approach, is for total analyte (free and ADA bound). Calibration curves for both the LC and HC were linear from 1.0 – 400 µg/mL sampling 50 µL of human plasma. The use of stable-isotope labeled internal standard (flanked peptides) for both the LC and HC provided reproducible and efficient compensation for tryptic digestion, as indicated by precision (% CV) and accuracy (% nominal) data within acceptance criteria. Since two surrogate peptides were used to quantify one protein over identical calibration ranges, acceptance criteria was applied which dictated the percent difference between surrogate concentrations be ≤ 20% for all individual calibrants and QCs.

Conclusion

By leveraging the high resolution and accurate mass capabilities of the TripleTOF 6600, researchers at Algorithme Pharma were able to achieve their specificity/selectivity goals for Rituximab using simple pellet digestion, thereby avoiding rate-limiting immunoaffinity approaches. In addition, the selectivity gains of the TOF-MS/MS experiment allowed a quantitation approach using two surrogate peptides to achieve an increased confidence in the structural integrity of the mAb and improved assay reliability. Due to significant interference at the QQQ MRM transition for the light chain of Rituximab, the dual peptide quantitation approach would not have been feasible at the necessary LLOQ of 1.0 µg/mL.

The author gratefully acknowledges both the provision of data and insightful discussions with the Large Molecule LC-MS Method Development team at Algorithme Pharma.

RUO-MKT-18-3977-A