Quantitative analysis of protein-biotherapeutics:
Recent advances in LC-MS/MS technology for improved selectivity and sensitivity

With an ever increasing flow of protein-based biopharmaceutical candidates entering the drug development funnel, reliable bioanalytical techniques are required to support quantitation in various biological fluids in order to generate toxicokinetic, pharmacokinetic, and bioavailability data. Trends in the scientific literature clearly indicate that the coupling of liquid chromatography with tandem mass spectrometry (LC-MS/MS) is replacing traditional ligand binding assays due to improved selectivity and linear dynamic range, and that the mainstream MS approach for quantitation relies upon targeted proteomics techniques involving internal standardization, proteolytic digestion, surrogate peptide selection, peptide and/or protein purification, LC separation and MS/MS detection. This review focuses primarily on recently reported advancements in MS workflows that have resulted in improved sensitivity through the effective elimination of interference from matrix components. Further, case studies are presented illustrating advantages of an immunocapture pre-digestion approach when LC-MS/MS alone fails to provide suitable required assay performance.

Keywords

Time-of-Flight, differential mobility, MRM3, high-resolution, accurate Mass, immunocapture

INTRODUCTION

The biologics market continues to experience rapid rates of growth, with > 50% of approved drugs expected to be protein-based biotherapeutics by 2019 (1). Further, patents for several existing biologic blockbusters are set to expire within the next two years, which predictably shall lead to a surge in biosimilars. Therefore, the increased importance of protein therapeutics necessitates performance improvements in bioanalytical techniques, with LC-MS/MS emerging as the most attractive and flexible technology able to cope with the high demands of throughput and complexity of analysis. While ligand-binding assays (LBAs) have historically been the most popular technique for protein quantitation, they demonstrate lower selectivity, are often unable to distinguish catabolites from parent drug, provide limited dynamic range, and are unsuitable for high-throughput early development work due to the time-consuming task of raising antibodies (2). In contrast, the increasingly mainstream workflow to quantify protein therapeutics using a surrogate peptide following proteolytic digestion with subsequent LC-MS/MS detection offers rapid method development, a wide dynamic range, and significant resistance to interference from matrix components such as antidrug antibodies (3-6). However, one drawback of LC-MS/MS when compared to LBA can be less sensitivity.

In this article, we survey several LC-MS/MS strategies which improve assay selectivity whilst lowering achievable detection limits, including high-resolution accurate mass measurements by quadrupole time-of-flight (QTOF), differential mobility mass spectrometry,

and MRM³ analysis using hybrid quadrupole/linear ion traps (QTrap). Further, we examine how coupling LBA and LC-MS/MS through a pre-digestion immunocapture (IC) enrichment step can remove interfering matrix components, resulting in detection limits lower than LC-MS/MS alone.

High-Resolution Accurate Mass Time-of-Flight Measurements

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. Using QTOF mass spectrometers operated with mass resolutions ($m/\Delta m$) of ca. 30,000 and mass accuracies < 5 ppm, several research groups have demonstrated the selectivity and sensitivity superiority of this platform in differentiating targeted surrogate peptides from co-extracted endogenous interferences (7-10).

The advantages of QTOF HRMS were made most apparent in a simple pellet digestion assay for the monoclonal antibody (mAb) Rituximab, which leveraged surrogate peptides from both the light-chain (LC) and heavy-chain (HC). Analysis of extracts by QQQ in MRM mode revealed significant interference for the LC at the transition required for optimal sensitivity; in marked contrast, the selectivity obtained from the QTOF using a product ion mass extraction window of 25 mDa 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 gain in S/N (Figure 1).

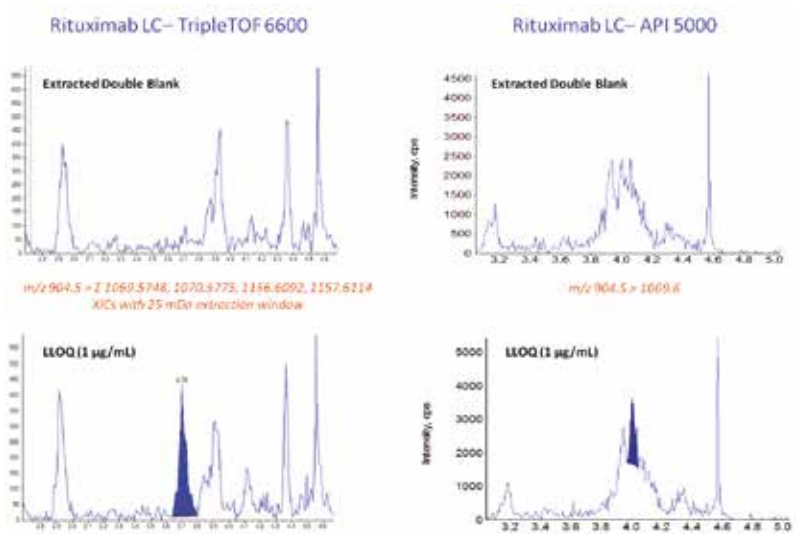

Figure 1. Comparison of the Sciex TripleTOF 6600 and Sciex API 5000 for extracted blank and LLOQ response for the Light Chain of the monoclonal antibody (mAb) Rituximab following pellet digestion.

TripleTOF data were acquired in enhanced product ion scan MS/MS mode for the precursor ion *m/z* 904.5, with summation of *m/z* 1069.5748, 1070.5775, 1156.6092 and 1157.6114 product ions. High-resolution accurate mass chromatograms were generated using a 25 mDa mass extraction window for the summed product ion current. Triple-stage quadrupole data from the Sciex API 5000 was acquired in multiple-reaction monitoring (MRM) mode for the transition *m/z* 904.5 > *m/z* 1069.6.

Hybrid Quadrupole/Linear Ion Traps and MRM³

When MRM transitions for a surrogate peptide fail to discriminate against interference, the third quadrupole in a QTrap can be operated as a linear ion trap to potentially generate lower detection limits by dissociating primary fragment ions into secondary fragment ions, a process referred to as MRM³ (**Figure 2**). Several researchers have recently demonstrated the implementation of such an MRM³ strategy to achieve lower detection limits whilst simplifying sample preparation workflow (11- 14). For example, Fortin et al. describe an MRM³ procedure for targeting PSA in the low ng/mL range in non-depleted human serum, with results correlating well against established ELISA tests (11). Previously, an MRM method was attempted monitoring the surrogate peptide LSEPAELTDAVK, however the use of HAS depletion proved a difficult step to automate and detection was limited to 50 ng/mL due to background interference. In contrast, a clinically relevant 4 ng/mL detection limit was achieved using MRM³ (Figure 3) and the immunodepletion step could be eliminated from the sample preparation strategy, thus improving sample throughput. Further, in studying trypsin hydrolyzed bacterial protein models TP171, TP574, TP435 and core NS4, it was observed that in moving from MRM to MRM³, detection limits improved three-to-six fold (Table 1).

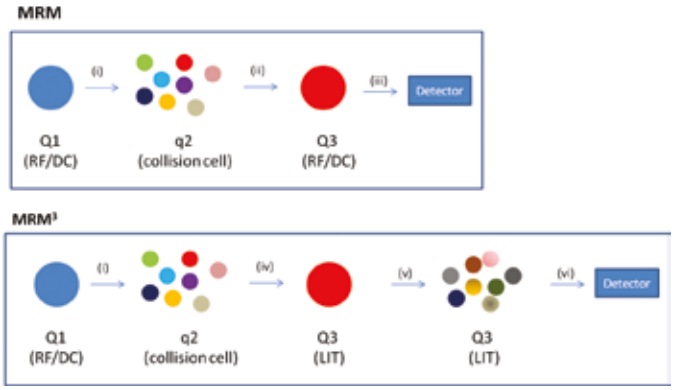

Figure 2. Comparison of events involved in the MRM and MRM³ experiment on the Sciex QTrap, a hybrid quadrupole/linear ion trap.

Precursor ions generated in the ion source are selectively filtered through the first resolving quadrupole (Q1) and fragmented by axial acceleration into the collision cell (q2) at translational energies up to 100 eV (i). In the MRM scan function, the third resolving quadrupole (Q3) rf/dc ratio is set to filter just one fragment m/z (ii) to the detector (iii). In the MRM³ scan function, the entire progeny ion ensemble is transferred from q2 to Q3 now operating as a linear ion trap (LIT). Once fragment ions originating from q2 are trapped in Q3, a single m/z is isolated (iv) then dissociated by single-frequency resonance excitation in the presence of nitrogen, with internal energy deposition controlled by a combination of the waveform amplitude and its duration of application (v). Second generation progeny ions are then rapidly scanned to the detector at rates between 10 and 20 kDa/s (vi).

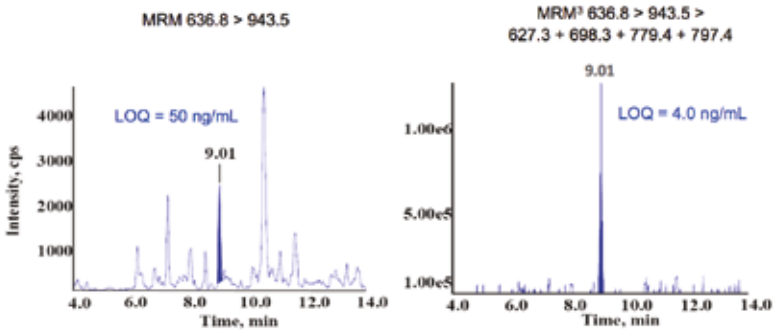

Figure 3. Reduction in background interference using MRM³ in the LIT for the analysis of PSA in non-depleted human serum.

The sensitivity of the MRM³ analysis was increased by summing several key secondary fragment ions, thereby furnishing a clinically relevant detection limit of 4 ng/mL.

		LOQ (ng/mL)	
Protein	Matrix	MRM	MRM³
PSA	Human serum	50	4
TP171	Human serum	40	10
TP574	Human serum	100	50
TP435	Human serum	500	80
coreNS4	Human serum	50	10

Table 1. Summary of the sensitivity gains achieved using MRM³ for a number of proteins quantitated using the surrogate peptide approach with trypsin digestion.

Differential Mobility Mass Spectrometry

Differential Mobility Mass Spectrometry (DMS) can be used as an orthogonal gas-phase separation method interfaced between the LC and mass spectrometer (i.e. LC-DMS-MS) in order to isolate analyte from isobaric interference based upon molecular cross-section. The application of DMS is most reported for cyclic peptides which have poor fragmentation efficiency in order to discriminate against high interference when acquiring data in parent-to-parent pseudo-MRM (SRM) mode (i.e. precursor *m/z* is used for both Q1 and Q3). An LC-DMS-SRM approach was implemented by Fu et al. for the cyclic peptide Pasireotide, achieving a 10 pg/mL detection limit in human plasma, three-fold lower than previous RIA methods and five-fold lower than LC-MS/MS (15). Researchers at Ironwood Pharmaceuticals noted similar performance for their cyclic peptide PN1944 (16), where DMS eliminated significant interference in SRM mode (Figure 4).

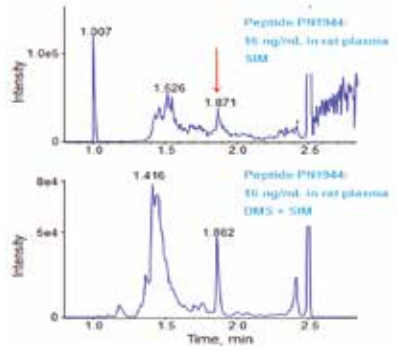

Figure 4. Improved signal-to-noise ratio (s/n) for the cyclic peptide PN1944 using DMS.

Analysis of the peptide in SRM mode was necessary due to poor fragmentation which resulted in a high level of background interference due to poor selectivity (top panel). In contrast, separation of the peptide using DMS prior to MS detection demonstrated a marked improvement in S/N (bottom trace).

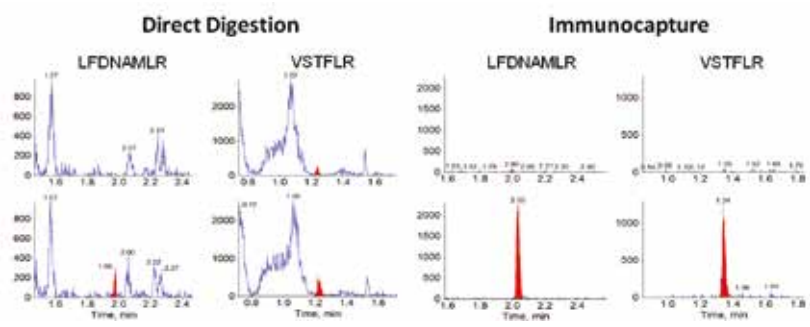

Figure 5. Blank and LLOQ (25 ng/mL) chromatograms of the two surrogate peptides used to quantitate Pegvisomant derived from rat plasma extracts using direct digestion (left) and pre-digestion anti-PEG immunocapture (right).

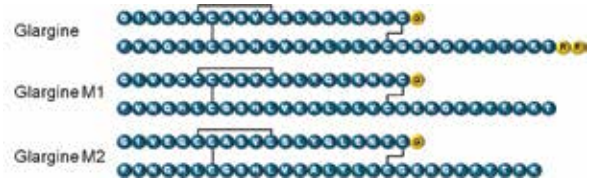

Figure 6. Amino acid sequence for insulin glargine and M1/M2 catabolites.

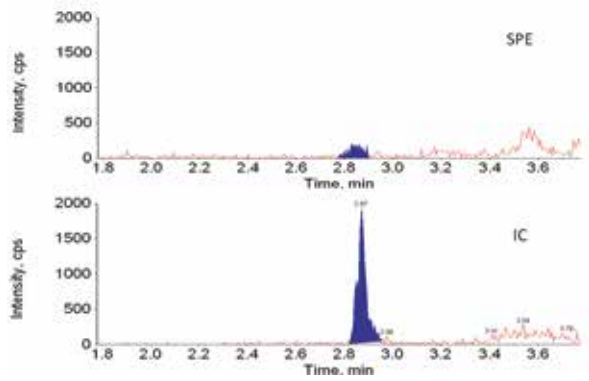

Figure 7. Comparison of insulin glargine response at 300 pg/mL in human plasma using SPE (top) versus IC (bottom).

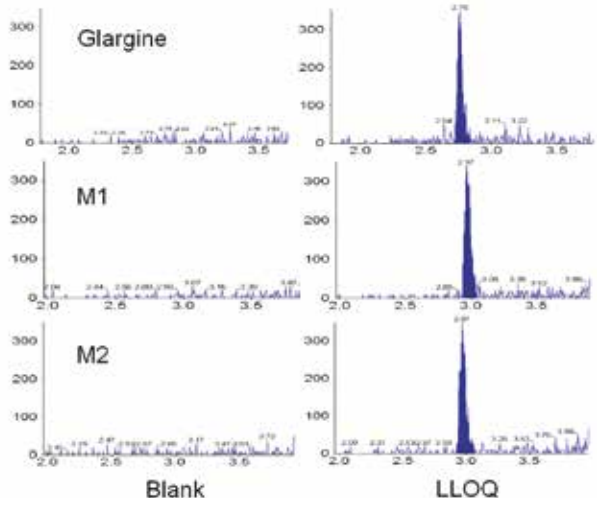

Figure 8. Chromatograms of extracted blank and LLOQ (50 pg/mL) from human plasma for insulin glargine and catabolites M1 and M2.

Immunocapture-LC-MS/MS

When the advanced LC-MS/MS techniques described above fail to eliminate interference from proteolytically digested samples, an alternative pre-digestion workflow can be impleme nted which leverages a drug-specific IC reagent that allows unbound matrix components to be washed away following the drug capture step (17- 18). The advantages of IC prior to digestion are demonstrated in Figure 5 for the 20 kDa pegylated protein Pegvisomant (Somavert®), extracted from plasma by immunoaffinity purification with anti-PEG antibodies followed by on-bead tryptic digestion. When compared to direct digestion, the IC-LC-MS/MS approach furnished a 10-fold response increase whilst eliminating all co-extracted interference for each of the two surrogate peptides used for quantitation.

The hybrid IC-LC-MS/MS approach can also be used to improve quantitation of intact biotherapeutics, as noted for insulin glargine and its two major catabolites (Figure 6). In this instance, the surrogate peptide approach could not be applied to differentiate parent from M1/M2 catabolites, and thus sensitivity was challenged due to highly charged precursor ions exhibiting poor fragmentation characteristics. Further, an initial solid-phase extraction (SPE) approach lacked the selectivity necessary for concentrating extracts and mitigating ion suppression. However, when mouse anti-insulin monoclonal antibody was used to capture all three analytes, significant gains in sensitivity were achieved (Figure 7-8). This application highlights a major advantage in coupling ligand-binding with LC-MS/MS detection, that is, the ability to distinguish catabolites of minor modification from parent drug by measurement of m/z. As LBA alone lacks the ability to discriminate insulin glargine from its major catabolites, measurement would have reported the sum of all species.

CONCLUSIONS

The use of LC-MS/MS for therapeutic protein and peptide quantitation has increased significantly over the past five years, with detection limits approaching or exceeding those of LBA when using highly selective modalities to eliminate interference, such as high resolution/accurate mass, MRM^3 and differential mobility spectrometry. Recent efforts to couple immunocapture purification with LC-MS/MS has resulted in dramatic sensitivity gains, and we anticipate enormous growth in the application of these hybrid techniques to realize continued improvements in method performance.

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JEFFRY B. PLOMLEY
Altasciences Clinical Research
575 Armand-Frappier
Blvd, Laval, QC , H7V 4B3,
Canada

KEVORK MEKHSSIAN
Altasciences Clinical Research
575 Armand-Frappier
Blvd, Laval, QC , H7V 4B3,
Canada