Towards Sensitive and Accurate Analysis of Antibody Biotherapeutics by LC/MS

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List of nonstandard abbreviations used in this paper:
ADC, antibody-drug conjugates; DAR, drug-to-antibody ratio; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FASP, filter aided sample preparation
HIC, hydrophobic interaction chromatography; I.S., internal standards; LBA, ligand-binding assays; LC, liquid chromatograph; LOQ, limit of quantification; mAb, monoclonal antibodies; MS, mass spectrometry; OAO, orthogonal-array-optimization; PD, pharmacodynamic; PK, pharmacokinetics; PTM, post-translational modifications; SCX, strong cation exchange; SIL, stable isotope labeled; SISCAPA, stable isotope standards and capture by anti-peptide antibodies; S/N, signal-to-noise ratios; SP, signature peptide; SRM, selected-reaction monitoring; TK, toxicokinetics; TOF, time-of-flight.
Abstract

Remarkable methodological advances in the past decade have expanded the application of LC/MS analysis of biotherapeutics. Currently, LC/MS represents a promising alternative or supplement to the traditional ligand binding assay (LBA) in the pharmacokinetic, pharmacodynamic and toxicokinetic studies of protein drugs, owing to the rapid and cost-effective method development, high specificity and reproducibility, low sample consumption, the capacity of analyzing multiple targets in one analysis and the fact that a validated method can be readily adapted across various matrices and species. While promising, technical challenges associated with sensitivity, sample preparation, method development and quantitative accuracy need to be addressed to enable full utilization of LC/MS. This article introduces the rationale and technical challenges of LC/MS techniques in biotherapeutics analysis, and summarizes recently-developed strategies to alleviate these challenges. Applications of LC/MS techniques on quantification and characterization of antibody biotherapeutics are also discussed. We speculate that despite the highly attractive features of LC/MS, it will not fully replace traditional assays, such as LBA, in the foreseeable future; instead, the forthcoming trend is likely the conjunction of biochemical techniques with versatile LC/MS approaches to achieve accurate, sensitive, and unbiased characterization of biotherapeutics in highly complex pharmaceutical/biological matrices. Such combinations will constitute powerful tools to tackle the challenges posed by the rapidly growing needs for biotherapeutics development.
1. Introduction

Biotherapeutics, especially therapeutic monoclonal antibodies (mAb), have become one of the primary focuses for pharmaceutical industry worldwide (van den Broek et al., 2013). Sensitive, accurate, and high-throughput analytical methods that deliver high-quality quantitative data for pharmacokinetic (PK), pharmacodynamic (PD), and toxicokinetic (TK) studies, are critically important to the development of these agents (Nowatzke et al., 2011; Geist et al., 2013b). Traditionally, ligand-binding assays (LBA), such as enzyme-linked immunosorbent assay (ELISA), are the primary means for quantification of therapeutic proteins, which are often considered to afford sufficient sensitivity and throughput for PK, PD, and TK studies (Urva et al., 2010; Shah and Balthasar, 2014). Nevertheless, LBA methods may fall short in that they are often matrix- and species-dependent (e.g. methods developed in one matrix/species can’t be readily transferred to another), and the quantitative accuracy and specificity may be compromised by interferences from bio-matrices, mAb modification/degradation and anti-mAb antibody, especially when highly specific critical reagents are not available (Damen et al., 2009; Hoofnagle and Wener, 2009). Moreover, the method development is often time-consuming and costly, which is particularly problematic in the phases of discovery and early development (Savoie et al., 2010).

Liquid chromatography coupled to mass spectrometry (LC/MS) has emerged as a promising alternative to LBA for quantitative characterization of biotherapeutics (Heudi et al., 2008). Since the late 1990’s, LC/MS has been a powerful tool for sensitive, accurate and rapid analysis of small-molecule drugs, metabolites and biomarkers (Trufelli et al., 2011). More recently, various LC/MS techniques have been developed for the quantification of proteins of interest in complex biological matrices (Qu et al., 2006; Pan et al., 2009). Although it is possible to quantify proteins by LC/MS on both intact-protein and proteolytic-peptide levels (Kippen et al., 1997; Pan et al., 2009; Duan et al., 2012a; Rauh, 2012; van den Broek et al., 2013), the vast majority of LC/MS-based protein quantifications are performed at peptide levels for several important reasons. First, the sensitivity of MS is far superior at peptide level than at protein level (Blackburn, 2013); second, in a biological system, intact proteins often carry a cohort of post-translational modifications (PTM), which shift the masses of the proteins and introduce considerable analytical variability; per contra, when quantifying protein at peptide level, the quantification is usually based on the selected peptide domains where modifications are not likely to occur, and thus ensuring high reliability and reproducibility (Hopfgartner et al., 2013); third, the upper m/z limits of most MS analyzers are often
too low to analyze the multiply-charged precursor ions of a relatively large protein such as a therapeutic mAb, whereas the m/z of most peptide precursors can be readily detected by almost all MS analyzers (Blackburn, 2013).

For protein quantification at peptide level, Selected Reaction Monitoring (SRM) operated on a triple-quadrupole MS is by far the most commonly utilized technique. Briefly, the first quadrupole analyzer selects a specific peptide precursor ion from the complex matrix, which is then fragmented in a downstream fragmentation chamber filled with collision gas; then the second quadrupole analyzer monitors a specific fragment from the target peptide (Qu and Straubinger, 2005). Compared with other tandem MS techniques, SRM-MS exhibits higher sensitivity, better quantitative accuracy and wider dynamic range for targeted protein quantification, and can be easily multiplexed (i.e. quantification of multiple analytes in one LC/MS analysis) by quickly switching among different precursor/product transitions (Qu and Straubinger, 2005). When the excellent specificity of SRM is combined with sufficient LC separation, the LC/SRM-MS constitutes a versatile and powerful tool for the quantification of proteins in complex matrices. A typical procedure for LC/SRM-MS-based quantification includes sample treatment/cleanup, digestion using enzyme, and quantification of the target proteins based on selected signature peptides (SP) derived from the target. Stable isotope labeled (SIL) SP surrogate or SIL-full-length-protein is used as the internal standard (I.S.). Extensive reviews on this technique can be found in these references (Lange et al., 2008; Liebler and Zimmerman, 2013).

LC/SRM-MS has several attractive features over LBA for analysis of biotherapeutics, including fast method development and validation, high specificity and small sample consumption per analysis (Jemal and Xia, 2006; Fernandez Ocana et al., 2012; Liu et al., 2013; van den Broek et al., 2013). Furthermore, many proteins can be simultaneously quantified in one LC/SRM-MS analysis (Xiao et al., 2014) and a method developed in one matrix or species can often be readily transferred to another (Savoie et al., 2010; Pendley and Shankar, 2011; Bronsema et al., 2012; Duan et al., 2012a; Li et al., 2012; Geist et al., 2013b; Jiang et al., 2013). Finally, LC/MS techniques can be employed to obtain critical information on the molecules of biotherapeutics that poses a daunting challenge for LBA, such as the chemical degradation (e.g. oxidation or deamidation of residues) (Huang et al., 2005).

With the emphasis on the LC/SRM-MS technique, this article will discuss the comparison of LC/MS techniques vs. LBA for quantification of antibody biotherapeutics, and then the technical challenges of LC/SRM-MS and emerging approaches to alleviate these challenges. Finally, recent applications of LC/MS techniques to the analysis of biotherapeutics are summarized.
2. **Comparison of LC/SRM-MS vs. LBA**

For quantification of biotherapeutics, the sensitivity, specificity, and robustness achievable by LBA are heavily dependent on the quality of critical reagents (often antibodies) raised against specific epitopes of the target proteins (Lee and Kelley, 2011; O’Hara et al., 2012). By comparison, usually LC/SRM-MS method does not require target-specific critical reagents, and thus may provide the following prominent advantages over LBA:

First, the process to develop a LBA method for quantification of therapeutic proteins in complex pharmaceutical matrixes (e.g. plasma and tissues) with sufficient sensitivity, selectivity, and accuracy, is often both time-consuming (e.g. 6-12 months (Savoie et al., 2010)) and costly. These typically include the development of optimal critical reagents, the extensive examination of endogenous interferences and rigorous method validation in samples from various sources (Nowatzke et al., 2011; Pendley and Shankar, 2011). On the contrary, as LC/SRM-MS-based approaches do not require critical reagents, these methods can be developed more rapidly (e.g. in 2-3 weeks) at substantially lower cost (Pan et al., 2009).

Second, the critical reagents used for LBA are generally produced via biological processes, which are inherently prone to variability arising from such factors as PTM, variations in different batches of reagents, and biological interferences (Lee and Kelley, 2011; O’Hara et al., 2012); as LBA does not employ an internal standard to correct quantitative bias and variation, stringently controlled operations are required to prevent deterioration of assay performance (Pandya et al., 2010; Lee and Kelley, 2011). Consequently, it is often challenging to maintain high inter-batch/inter-lab consistency, which is necessary to correlate results among batches and to transfer validated methods between labs (Ezan and Bitsch, 2009). On the contrary, analytical variation is frequently much less a concern for LC/SRM-MS approaches since critical reagents are not required, and that isotope-coded peptides or proteins are prevalently used as I.S. which effectively correct analytical variations introduced by LC/MS analysis and matrix effects (Pan et al., 2009; Bronsema et al., 2012; van den Broek et al., 2013; Nouri-Nigjeh et al., 2014). In practice, the performance of a developed and validated LC/SRM-MS method is quite robust as long as the instrument maintenance and quality control are carried out properly.

Third, measurement of the distribution of biotherapeutics in different matrices (e.g. plasma, tissues, etc.) and across different species is important for the development and evaluation of these agents (Lin et al., 1999; Garg and Balthasar, 2009; Deng et al., 2011; Shah and Betts, 2013), but is difficult to achieve with a single LBA method.
The specificity of LBA is profoundly affected by matrix: in different matrices or species, the extents of interferences and cross-reactions by matrix components vary considerably (Pendley and Shankar, 2011), rendering it difficult to transfer a LBA method among matrices (e.g. from plasma to a tissue or among different tissues) (Damen et al., 2009; Ezan and Bitsch, 2009; Hoofnagle and Wener, 2009). Conversely, as LC/SRM-MS minimizes matrix effect by employing isotope-coded internal standards and sufficient chromatographic separation (Qu and Straubinger, 2005), the methods are often readily transferrable among different matrices. For example, recently we applied the same LC/SRM-MS method for the quantification of therapeutic mAbs in mouse plasma and tissues, such as brain, heart, liver, spleen, kidney, and lung, with rapid and simple verification and re-validation in different matrices (Duan et al., 2012a).

Forth, while it is very challenging to develop a LBA method capable of quantifying multiple proteins in one analysis, LC/SRM-MS approaches can be multiplexed for hundreds of targets in one run (Li et al., 2012; Shi et al., 2012; Xiao et al., 2014). Such multiplexing capacity has been widely utilized in biomarker and proteome-wide mechanism studies (Sakamoto et al., 2011; Percy et al., 2013; Xiao et al., 2014). The multiplexing method is also highly valuable for the research and development of biotherapeutics, such as simultaneous quantification of an mAb and its circulating or tissue-specific targets (Li et al., 2009; Kawakami et al., 2011; Ohtsuki et al., 2012; Chambers et al., 2014), or multiple drug candidates in the same sample obtained from administration strategies such as cassette-dosing (Jiang et al., 2013; Li et al., 2013). The cassette-dosing strategy, which doses multiple drug candidates together to one subject to enable rapid screening, has long been employed in PK profiling and metabolites screening for small-molecule drugs (Bayliss and Frick, 1999; Korfmacher et al., 2001). This approach also substantially reduces the time and resource required for initial screening and development of biotherapeutics (Liu et al., 2008). A multiplexed LC/SRM-MS approach enables the simultaneous albeit specific analysis of different mAb candidates even the sequences are only slightly different (Geist et al., 2013a; Li et al., 2013), and thus is the method-of-choice for cassette-dosing study.

Other merits of LC/SRM-MS over LBA include low sample consumption and high operational robustness (Ouyang et al., 2012; Liu et al., 2013). For example, in our recent studies, each LC/SRM-MS analysis only used peptide digests from ~0.1 µL plasma or ~0.1 mg tissue (Duan et al., 2012a; Duan et al., 2012b; Nouri-Nigjeh et al., 2014), which is much lower than the sample amount required by a typical LBA method.
The main disadvantage of LC/SRM-MS compared with LBA is that an expensive MS instrument is required. Moreover, for these well-established biotherapeutics with industry-grade LBA methods already been developed and validated, sensitive and high-throughput quantification can be achieved following a relatively straightforward LBA procedure (Ezan et al., 2009). Consequently, LBA remains the preferred choice for these targets (Damen et al., 2009).

3. Challenges of LC/SRM-MS and strategies

Despite of the merits of LC/SRM-MS and its rapidly-growing utility in the quantification of biotherapeutics, a number of significant technical challenges still exist. This section discusses the challenges and recent efforts to address them.

3.1 Method development

Till now, the optimal strategy for the development of an LC/SRM-MS method remains elusive (Pan et al., 2009; Duan et al., 2012a). The key aspect for method development is the discovery of optimal SP derived from the target biotherapeutics that ensures sensitive, specific, and robust quantification. Currently, in silico prediction approaches are popularly used which employ tools such as PeptideAtlas, Skyline, and MRMaid, to identify and validate SRM transition with minimum wet lab labor (Cham Mead et al., 2010; Halquist and Thomas Karnes, 2011; Stergachis et al., 2011; Rauh, 2012). Nevertheless, this approach may not accurately predict the most sensitive, stable peptides and matrix-dependent parameters, such as chemical interferences (Cao et al., 2010; Duan et al., 2012a).

Discovery of optimal SP by experimentally evaluating many proteolytic candidates in the target matrix (e.g. plasma or tissue digest) is the most reliable approach (Cao et al., 2010; Duan et al., 2012a); however, to experimentally evaluate these candidates, it is necessary to obtain optimal SRM conditions (e.g., the optimal parent/product transitions and the declustering/collision energy) for each of the many candidates in a digest mixture, which is challenging. Moreover, it is important to choose stable peptides as the SP (Cao et al., 2010; Duan et al., 2012a) to prevent quantitative variation and bias arising from poor peptide stability, which has been often overlooked. Finally, most methods use a lone SP for the quantification of a mAb, which may carry a significant risk of error where the mAb could be truncated biologically outside the SP domain or certain residues within the SP domain could be biologically modified (Hoofnagle and Wener, 2009).

To address these issues, we devised a new pipeline to facilitate a high-throughput and accurate method development (shown in Figure 1). Instead of using an in silico method to predict the best SP and optimal SRM
conditions, we employed an experimental strategy to discover and optimize many SP candidates, and then evaluate these candidates in target matrices prior to SP selection (Cao et al., 2010; Duan et al., 2012a; Duan et al., 2012b; Nouri-Nigjeh et al., 2014). Briefly, the pool of SP candidates was generated by a data-dependent LC/MS experiment following a stringent filtering step to remove peptides that are not unique to the target, containing labile amino acid (cysteine residues were not excluded as a number of studies showed cysteine-containing peptides may be used in a reliable quantification) (Keshishian et al., 2007; Picotti et al., 2009), known modification or miss cleavage. To evaluate these candidates, the target protein was spiked into the blank matrices (e.g. plasma or tissue extract) and then prepared and digested. The optimal LC/MS conditions of all SP candidates were accurately obtained by a high-throughput and on-the-fly orthogonal-array-optimization (OAO) procedure (Cao et al., 2010; Duan et al., 2012a; Duan et al., 2012b), which has the capacity to develop the SRM conditions for >100 candidates within one single LC/MS run, with high accuracy and reproducibility. Using the developed LC/MS conditions, all candidates were thoroughly assessed for stability and signal-to-noise ratios (S/N) in the matrix digest. Among the stable peptides, two peptides with the highest S/N were selected as the SPs. The use of two SP from different domains of the same protein provides a versatile gauge for the reliability of quantitative methods and results. Details can be found in the following references (Cao et al., 2010; Duan et al., 2012a; Duan et al., 2012b).

Recently Furlong et al. described the use of a ‘universal surrogate peptide’ derived from the constant Fc region of human antibody for quantification of human antibodies in non-human animal models (Furlong et al., 2012). This method may greatly simplify and expedite the method development for the study of human antibodies in pre-clinical animal models.

3.2 Sample preparation

In order to achieve a sensitive and accurate quantification with LC/SRM-MS, it is critically important to achieve efficient and reproducible sample preparation, including effective sample clean-up, high and quantitative recovery of protein and efficient, reproducible peptide recovery (Qu et al., 2006; Pan et al., 2009). So far a universal and optimal preparation procedure for the quantification of biotherapeutics in pharmaceutical matrices has yet been established, largely due to the fact that tissue and plasma samples are highly complex, which contain numerous proteins and small-molecule compounds, and that the structure of a typical mAb renders it resistant to enzymatic digestion (Ouyang et al., 2012; Yuan et al., 2012). Moreover, it is challenging to prepare tissue samples for quantification of biotherapeutics, owing to the generally low drug concentrations in tissues and the lack of a
quantitative and high-throughput protein extraction procedure that is compatible with LC/SRM-MS analysis (Duan et al., 2012a).

Recently, we developed a gel- and filter- free procedure that achieved effective protein denaturation and sample clean-up, and high, quantitative peptide yields from plasma and tissue samples (Duan et al., 2009; Duan et al., 2012a). Briefly, plasma or tissue samples were treated or extracted with high concentrations of detergent cocktail, which not only effectively solubilized proteins in the samples to ensure a high recovery, but also completely denatured the proteins to achieve an efficient reduction, alkylation, and digestion (Duan et al., 2009; Tu et al., 2013); the mixture was then cleaned up by precipitation with cold organic solvents, which effectively removed the detergents and significantly reduced non-protein matrix components such as lipids and fragmented or small-molecule nucleic acids that may negatively affect the robustness and consistency of LC/SRM-MS analysis (Qu et al., 2014). After precipitation, an on-pellet-digestion approach was employed without dissolving the protein pellet. This approach consists of two phases: under active agitation, the short phase-I digestion brings the pellets into solution by cleaving the pelleted proteins into soluble albeit large tryptic peptides; these incompletely-cleaved peptides were then subjected to an overnight phase-II digestion for complete cleavage. Compared with other preparation methods used for LC/SRM-MS-based protein quantification, the detergent-aid precipitation/on-pellet-digestion provided higher digestion efficiency and much cleaner sample than in-solution digestion (Duan et al., 2009; Cao et al., 2010; Yuan et al., 2012), while affording higher and more reproducible peptide yields than in-gel digestion (Olsen et al., 2006) and filter aided sample preparation (FASP) methods (Manza et al., 2005; Wisniewski et al., 2009). Therefore, this procedure can be used for high-throughput quantification of mAb in plasma and tissues with excellent analytical sensitivity and robustness.

Recently, a number of techniques have been developed to enable rapid digestion of proteins, such as digestion assisted by microwave (Lesur et al., 2010), ultrasound (Priego-Capote and de Castro, 2007) and infrared radiation (Wang et al., 2008a), and accelerated digestion with immobilized trypsin (Krenkova et al., 2009; Yamaguchi et al., 2009; Yuan et al., 2009). Some products utilizing immobilized trypsin have been commercially available, e.g. Perfinuity Flash Digest™ Kit for rapid and efficient digestion (Rivera-Burgos and Regnier, 2012). The performance of these newly emerged techniques for quantification of biotherapeutics remains to be extensively evaluated.

3.3 Sensitivity
Although LC/SRM-MS is considered a highly sensitive technique, insufficient sensitivity is often a prominent concern for quantification of therapeutic mAb, largely due to two reasons: i) the signal response of LC/MS is dependent on the molar rather than mass amounts of the analyte; consequently, the large molecular weights of mAb pose a considerable disadvantage; ii) owing to the very high protein contents in plasma or tissue samples (Tu et al., 2011), it is often necessary to dilute the samples to a large extent before analysis (Dams et al., 2003; Chambers et al., 2007; Cao et al., 2010; Duan et al., 2012b; Yuan et al., 2012).

In order to improve sensitivity for targeted protein analysis, we’ve developed a robust nano-flow LC/SRM-MS strategy (Cao et al., 2010; Duan et al., 2012a; Duan et al., 2012b), which typically lowers the limit of quantification (LOQ) by ~30-50 fold compared to a conventional-flow LC/SRM-MS. Another approach to improve sensitivity is to enrich target proteins or peptides prior to LC/SRM-MS analysis. For instance, Dubois et al. achieved a LOQ at 0.02 µg/ml for quantification of a chimeric mAb in human serum samples with an enrichment procedure (Dubois et al., 2008); Lin et al. utilized immunoprecipitation enrichment prior to LC/SRM-MS analysis, which achieved a LOQ of 10 ng/ml for mAb analysis (Lin et al., 2013).

A variety of other techniques were developed to increase the sensitivity for LC/SRM-MS-based protein quantification, although these have yet been applied in quantification of biotherapeutics. To give several examples: Stable Isotope Standards and Capture by Anti-peptide Antibodies (SISCAPA) technique was developed to enrich signature peptides using polyclonal antibodies (Anderson et al., 2004). More recently, Neubert et al. developed a series of affinity-based methods for quantitative enrichment of target proteins and/or SPs in plasma, achieving ultra-sensitive quantification of circulating biomarkers in plasma (Ocaña and Neubert, 2010; Neubert et al., 2013; Palandra et al., 2013). Furthermore, except increase sensitivity, affinity capture based method can quantify specific targets, such as free or total mAb (Fernandez Ocana et al., 2012), which will be reviewed in application section. Other approaches to improve the sensitivity of targeted quantification include SCX fractionation (Keshishian et al., 2009), high-pH fractionation before LC/MS analysis (Shi et al., 2012), and the use of long columns to obtain high S/N of target peptides (Shi et al., 2013).

3.4 Quantitative accuracy

The correct measurement of drug concentrations in plasma and tissues is essential for the research and development of biotherapeutics; consequently highly accurate quantification methods are important (Wang et al., 2008b). SIL-I.S. is prevalently used for LC/SRM-MS quantification of therapeutic proteins, which greatly enhance
the analytical reproducibility (Pan et al., 2009; Li et al., 2012; Nouri-Nigjeh et al., 2014). However, insufficient quantitative accuracy frequently represents a daunting problem. Most current works on LC/MS-based protein quantification employ synthesized peptides as the calibrator and SIL-peptides as I.S. (spiked after digestion) (Bronsema et al., 2012; van den Broek et al., 2013). Such peptide-level calibration approaches enable straightforward development of quantitative methods, and both the calibrators and SIL-I.S. are readily available from commercial sources. However, the use of an SIL-peptide I.S. only addresses variations/biases caused by LC/MS analysis, but not the upstream steps, such as sample preparation and digestion (van den Broek et al., 2013); furthermore, these approaches assume nearly 100% efficiency of the preparation and digestion procedures which may not be true (Cao et al., 2010), e.g., tryptic digestion is rarely complete and can be partially non-specific (Picotti et al., 2007). Our recent investigations showed severe negative biases by the peptide calibration approaches (Duan et al., 2012b; Nouri-Nigjeh et al., 2014), and that the quantification using two different SP from distinct domains of the same mAb resulted in profoundly discordant quantitative results.

To address problems related to digestion efficiency, the extended-peptide calibration approaches, which use synthesized extended-peptide containing the SP sequence and (typically) 3-6 flanking residues extended from both the N- and C- termini, were introduced. A SIL-extended-peptide is used as the I.S., which is spiked prior to digestion (Ocaña and Neubert, 2010; Rauh, 2012; Neubert et al., 2013). This approach may help to compensate for the bias and variation introduced in the digestion step (e.g. missed cleavage or peptide degradation (Ocaña and Neubert, 2010)). Neubert et al. recently demonstrated that the extended-peptide calibration approach enabled accurate and sensitive quantification of small protein biomarkers (e.g. nerve growth factors) in plasma (Neubert et al., 2013; Palandra et al., 2013). Our recent study showed that the extended-peptide calibration method still resulted in considerable negative bias when quantifying a much larger protein (mAb) (Nouri-Nigjeh et al., 2014). Protein-level calibration methods that employ full-length protein calibrator with SIL-protein I.S., can correct errors and variations in all preparation and analytical steps, and thus is considered the Gold Standard for accurate quantification (Heudi et al., 2008; Li et al., 2012). However, SIL-proteins are costly to produce and may be impractical for many classes of proteins. Our lab and others demonstrated accurate quantification of regulatory proteins and protein drugs in plasma and tissues using “hybrid” calibration strategies (e.g. protein calibrator with SIL-peptide or SIL-extended-peptide I.S.) (Cao et al., 2010; Duan et al., 2012a; Duan et al., 2012b; Jiang et al., 2013), providing that reproducible sample preparation and digestion are achieved. Our recent study suggested the hybrid strategies may provide a cost-
effective means for accurate quantification without the costly SIL-protein (Nouri-Nigjeh et al., 2014). The quantitative biases by protein-, extended-peptide- and peptide level calibrations and hybrid methods for the quantification of the same mAb in plasma are shown in Figure 2.

4. Applications of LC/MS in the analysis of biotherapeutics

4.1 Quantification of mAb in plasma and tissues

LC/SRM-MS-based strategies have been widely applied to the quantification of mAb in plasma to support PK studies. Some representative works are exemplified here. Heudi et al. developed and validated an accurate quantitative method for a candidate mAb in marmoset serum, using post-digestion SPE cleanup and SIL-full-length-protein as I.S. (Heudi et al., 2008). The method was applied to PK analysis in marmosets at dosing level of 150 mg/kg. It was discovered the concentrations by LC/SRM-MS were higher than these by a parallel ELISA assay, which might reflect the fact that ELISA measures the free form of mAb while LC/SRM-MS measures the total mAb (Heudi et al., 2008). Li et al. developed a universal LC-SRM/MS approach for quantification of a variety of therapeutic mAb based on the utilization of a full-length SIL-mAb as the common I.S.; such a method is valuable for preclinical studies (Li et al., 2012). Hagman and co-workers developed an LC/SRM-MS method to quantify a human mAb in the serum of cynomolgus monkey (Hagman et al., 2008). The study showed that the analytical sensitivity was significantly increased by an albumin depletion procedure prior to LC/MS analysis. Ouyang et al. described the combination of on-pellet digestion with LC/SRM-MS for reproducible analysis of a mAb drug candidate in monkey plasma (Ouyang et al., 2012). Through an extensive comparison with other preparation methods, the authors showed that on-pellet digestion was the optimal technique as it permitted a straightforward and efficient preparation of mAb. Yang et al. established quantification method for somatropin and a therapeutic human mAb in human and rat serum samples, which employed a solid-phase extraction for cleanup and bovine fetuin as I.S. (Yang et al., 2007); the method was applied in a rat PK study at a dosage of 10 mg/kg. Lu and co-workers employed albumin depletion, protein A capture, and antibody capture coupled to LC/SRM-MS for sensitive quantification of a mAb candidate (NT0736); the authors concluded that all LC/SRM-MS-based methods evaluated in the study provided adequate sensitivity for PK study (Lu et al., 2009). Fernandez Ocana et al. established a LC/MS strategy to quantify free and total anti-MadCAM mAb (PF-00547,659) in human serum (Fernandez Ocana et al., 2012), which captured free mAb with a biotinylated anti-idiotypic antibody followed by enrichment with streptavidin magnetic
beads; total target mAb was enriched by protein G magnetic beads. The strategy was successfully applied in a
clinical PK study. Our lab described a sensitive nano-LC/SRM-MS method for quantification of a chimeric mAb
c(T84.66) in mouse serum (Duan et al., 2012b). Owing to the high sensitivity and selectivity achieved, the method
was successfully applied to the preclinical PK study with a subcutaneous dosing at 1 mg/kg. The high sensitivity
achieved in the work made it feasible for PK study at even lower dosages and/or over longer period post
administration.

While the determination of the levels of biotherapeutics in tissues is critical for PK studies, such works
have rarely been reported due to technical challenges such as the low drug concentrations and problems associated
with tissue matrices, as discussed above. Using a sensitive nano-LC/SRM-MS, effective sample preparation and a
high-throughput method optimization strategy, we described sensitive quantification of two mAb (8c2 and cT84.66)
in seven tissues with LLOQ in the range of 0.156 – 0.312 µg/g tissue (Duan et al., 2012a). The method was applied
to the investigation of steady-state tissue distributions of 8c2 in various mouse models. Similar distribution
characteristics was observed among the wild type animals and these deficient in FcγRIIb and FcγRI/RIII; by
comparison the 8c2 tissue levels in the FcRn α-chain deficient group were significantly lower, as expected due to the
absence of FcRn-mediated protection of antibody from catabolism. The work demonstrated that LC/SRM-MS is a
promising alternative to radio-labeling strategies, which may fall short in problems related to assay accuracy and
specificity, degradation of labeled protein, and radiation exposure to investigators and animals (Duan et al., 2012a).

4.2 Application in cassette-dosing

As discussed previously, LC/SRM-MS is capable of simultaneous quantification of multiple targets, and
therefore enabling cassette-dosing study of drug candidates. Though cassette-dosing has been prevalently used to
screen small molecule candidates (White and Manitpisitkul, 2001; Smith et al., 2007), it was found to be even more
suitable for preliminary investigation of mAb candidates (Li et al., 2013), because i) studies of multiple mAb usually
do not carry the risk of drug-drug interaction, and the PK of proteins is not affected by CYP450 and transporters
(Zhou and Mascelli, 2011; Li et al., 2013), and ii) it is fairly straightforward to find an optimal, common formulation
for multiple mAb (Dani et al., 2007; Spencer et al., 2012). Jiang et al. developed and validated an LC/SRM-MS
method for simultaneous quantitation of two co-administrated mAb, which showed good sensitivity, reproducibility
and accuracy for both targets. The method was successfully applied to toxicokinetic study in monkeys (Jiang et al.,
Li. et al. reported an analytical method to quantify four mAb after subcutaneous cassette-administration with LOQ of 0.1–0.5 µg/ml in plasma (Li et al., 2013).

### 4.3 Characterization of intact mAb

The recent technical advances and increasing availability of high-resolution MS instruments have resulted in a rapid growth in using LC/MS to characterize intact mAb. Although most of these studies do not directly quantify antibodies, such works greatly facilitate assay development and/or PK/PD study by affording detailed physicochemical information on these agents. In most cases, a “top-down” strategy, which directly analyzes intact proteins with a high-resolution analyzer, is employed. Despite of the fact that the top-down techniques are generally less sensitive than the bottom-up methods, it is capable of providing overall information and accurate PTM mapping of the target protein (Peng et al., 2013). One important paradigm is the characterization of the critical reagent for LBA (Geist et al., 2013b). Such studies can substantially improve the management of critical reagents and contributes to the development of a robust LBA method by providing essential information such as Fab/Fc sequence, enzymatically produced PTM, and charge-state. Details on the utilization of LC/MS approaches for troubleshooting of LBA methods and critical reagent quality control can be found in a previous publication (Geist et al., 2013b). Another prominent utility of top-down technique is the quality control of mAb products. Thompson et al. performed a top-down profiling of glycosylation on mAb (Thompson et al., 2014). Wang et al. established a series of top-down and bottom-up ESI-TOF-MS methods to comprehensively investigate N-terminal pyroglutamate formation, cleavage of C-terminal lysine, glucosylation, and deamidation of a recombinant mAb (Wang et al., 2005). Dillon and co-workers developed a LC/MS method with a TOF analyzer to obtain accurate mass and unique terminal ladder sequences of a recombinant antibody, which provided glycosylation profile and heterogeneity information of the molecule (Dillon et al., 2006). In another study, a LC-ESI-TOF method was developed to determine glycosylation pattern of a therapeutic mAb (siltuximab) after immunoaffinity purification (Geist et al., 2013a). The work quantified therapeutic mAb produced in two different host cell lines. Xie et al. developed an LC/TOF-MS method for comprehensive comparison between innovator mAb vs. a biosimilar, in terms of intact protein mass, sequence, and peptide mapping, which provided valuable information for assessment of biosimilar (Xie et al., 2010).

### 4.4 Characterization of antibody-drug conjugates (ADC)

Antibody-drug conjugates (ADC), which utilize mAb for targeted delivery of highly potent small molecule drugs, constitute a new class of biotherapeutics for effective targeted therapy (Doronina et al., 2003).
Drug-to-antibody ratios (DAR) and drug load distribution are critical parameters that profoundly determine the \textit{in vivo} efficacy and toxicity of an ADC (Ducry, 2013; Kaur et al., 2013). For example, even a small decrease in DAR (e.g. loss of conjugated drug in circulation) will lead to a significant change of exposure at the targeted site (Xu et al., 2011) and thus determination of DAR is essential for ADC development and their PK/PD studies. LC/MS with high resolution analyzer has become a promising technology for characterization of ADC in the recent years. An affinity capture capillary LC coupled to quadrupole-TOF MS have been employed to analyze anti-MUC16 TDC, which obtained both \textit{in vitro} and \textit{in vivo} DAR information (Xu et al., 2011). Valliere-Douglass et al. developed a native LC/MS method for the determination of DAR via analysis of intact protein (Valliere-Douglass et al., 2012). Recently, a more sensitive method was reported by Chen et al., which employed a native nano-ESI-TOF analysis in conjunction with a limited digestion by cysteine protease to obtain DAR information (Chen et al., 2013); the method was also demonstrated substantially higher sensitivity than the traditional hydrophobic interaction chromatography (HIC).

5. Conclusion and future perspective

LC/MS represents a promising alternative to traditional LBA methods for the analysis of biotherapeutics, because LC/MS \textit{i}) can be readily adapted to quantification in plasma and tissues and across various species; \textit{ii}) provides extraordinary specificity and high reproducibility with low sample consumption, and low inter-lab/batch variance; and \textit{iii}) is capable of simultaneous quantification of multiple proteins (e.g. biotherapeutics and/or their targets) in one analysis. Furthermore, the development and validation of a LC/MS method is rapid at a relatively low cost, which is a highly desirable feature that facilitates the rapidly-growing developments of biotherapeutics. Nonetheless, LC/MS methods still face challenges associated with sensitivity, sample preparation, method development and quantitative accuracy. Recent technical advances helped to overcome these problems; to name a few: the use of target-specific enrichment and low-flow-LC/MS substantially improved analytical sensitivity; robust and efficient preparation methods are emerging; hybrid calibration methods are demonstrated to provide high quantitative accuracy without using expensive SIL-protein I.S.

In spite of the drastically increasing role of LC/MS in biotherapeutics analysis, most likely it will not fully replace traditional methods such as LBA in the foreseeable future. First, in the event that a well-developed LBA method is available for the target molecule, LBA may be advantageous over LC/MS in that it could be more sensitive (de Dios et al., 2013) and does not require expensive LC/MS instruments; moreover, LBA can be
developed to quantify free or total mAb, while LC/MS only detect the total mAb unless specific affinity capture enrichment is employed (Lee et al., 2011). Second, the combination of LC/MS and traditional biochemical strategies provides a powerful tool to acquire critical information on biotherapeutics in highly complex pharmaceutical and biological systems. For instance, immunocapture and immunodepletion have been demonstrated to greatly enhance the quantitative sensitivity of LC/MS; size-exclusion, immunoprecipitation, or native gel separation prior to LC/MS analysis will provide important information on the target protein, such as binding, aggregation, and degradation states. In a broad sense, antibody capture of a target molecule from biological matrix followed by LC/MS analysis may be considered a new form of LBA method with LC/MS as the means of detection. As LC/MS affords much higher selectivity and sensitivity than the spectrophotometers conventionally used for LBA, it will greatly facilitate and expedite method development, e.g. development of critical reagents may be markedly faster, because the requirements on selectivity and sensitivity of the critical reagents is far less stringent when LC/MS is used as the downstream detection approach.

In summary, LC/MS is a versatile and powerful tool for analysis of biotherapeutics, which can be utilized either alone or as the detector downstream of various biochemical procedures. We expect its application in the research and development of biotherapeutics will continue to expand rapidly in the future.
Authorship Contributions:

Wrote or contributed to the writing: An, Zhang and Qu.
Reference:


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Footnote:

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**Figure Legends:**

Figure 1. Flow chart of novel LC-SRM-MS method development process based on OAO optimization, detailed procedure can be found in previous publications.

Figure 2. Two-dimensional representations of the quantitative biases by (A) peptide-, extended-peptide- and protein-level calibration approaches and (B) the two “hybrid” calibration approaches (protein calibrator with SIL-peptide/SIL-extended-peptide I.S.). QC samples were prepared by spiking blank plasma with pure protein at three levels: 1.6, 10 and 80 µg/mL. The purities of all standards were accurately measured by quantitative amino acid analysis method to eliminate bias arising from possible inaccurate purity. Five aliquots of each QC sample were individually prepared and analyzed in replicates by the five calibration approaches. Each sample was analyzed three times in each of two different days (day 1 and day 14, N=6, shown as individual data points). For every calibration method, the quantitative values were obtained independently using the two signature peptides (SP), i.e. the GPS and TVA peptides. The two axes represent the quantitative biases by the two SP. The red box in the center of each panel denotes the zone of <20% bias, while the golden box signifies the zone of <10% bias. Reproduced from (Nouri-Nigjeh et al., 2014)
Figure 2

 QC: 1.6 µg/mL  QC: 10 µg/mL  QC: 80 µg/mL

 Peptide calibration  🔹 Extended-peptide calibration  🔹 Protein calibration

 🔹 Hybrid calibration with SIL-extended-peptide I.S.  🔹 Hybrid calibration with SIL-peptide I.S.