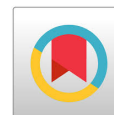


FOREWORD

# Addressing Bioanalytical Needs of Antibody-Based Biotherapeutics by LC-MS



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Antibody-based biotherapeutics are the fastest growing category of therapeutics entering clinical studies [1]. The popularity is attributed to the high selectivity of these drugs which enhances their efficacy and reduces systemic toxicity, in turn, increasing the therapeutic index. In addition, antibody-based biotherapeutics have long circulatory half-lives and are less likely to undergo significant biotransformation in vivo. Since the approval of the first monoclonal antibody (mAb) drug (Orthoclone OKT3®) in the 1980s, the number of antibody-based biotherapeutics on the market and in development has reached several hundred. Recent advancements in antibody engineering and manufacturing techniques have led to generation of new biotherapeutic modalities such as multispecific mAbs, antibody-drug conjugates, Probody™ therapeutics and Probody™ drug conjugates, PEGylated antibody fragments and fragment crystallizable (Fc)-fusion proteins. The increasing complexity of the new biotherapeutic modalities pose newer bioanalytical challenges. In some instances, multiple bioanalytical assays using different analytical platforms are required to address the pharmacokinetic characterization of these new modalities.

## Bioanalysis by LC-MS for Antibody-Based Biotherapeutics

Traditionally, ligand binding assays (LBA), primarily enzyme-linked immunosorbent assay (ELISA), have been the gold standard for protein quantification. ELISA has numerous benefits such as high-sensitivity, high-throughput, low-cost and ease of use. LBAs require high-quality critical reagents, that have high selectivity for the biotherapeutic drug, for capture and detection. The availability of a highly selective critical reagents is a major challenge of LBAs. The time of production of a high-quality critical reagent can prolong the method development time. The narrow dynamic range (typically around 100-fold) and high susceptibility to matrix interferences are other limitations of the LBAs.

Over past decade, liquid chromatography and mass spectrometry (LC-MS) has emerged as an alternate tool for quantification of proteins [2]. Rapid method development, broader dynamic range, higher selectivity and multiplexing capabilities are the main advantages of the LC-MS platform in comparison to the LBAs. The advancements in the mass spectrometric instrumentation and sample preparation methodology has enabled the use LC-MS as a complementary bioanalytical tool and in some applications as a mainstream bioanalytical tool for protein biotherapeutics.

Quantitative bioanalysis of antibody-based biotherapeutics by LC-MS is typically

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conducted using Immunoaffinity (IA) enrichment coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS), also known as (aka) hybrid LC-MS/MS [3]. Hybrid LC-MS/MS combines the selectivity of immunoaffinity enrichment with the selectivity and sensitivity of LC-MS/MS. Hybrid LC-MS/MS involves immunoaffinity isolation of the biotherapeutic molecule followed by proteolytic digestion (typically with trypsin) to yield a characteristic peptide (aka signature peptide) which is analyzed as a surrogate analyte by LC-MS/MS. The immunoaffinity enrichment technique and the surrogate analytes in a hybrid LC-MS/MS are chosen based on the biotherapeutic molecule, concentration range, matrix and analytical purpose. Generic immunocapture reagents such as Protein A or G beads, anti-human Fc or anti-human IgG antibodies can be utilized in a hybrid LC-MS/MS for preclinical evaluations [4]. This mitigates the need for high-quality critical reagents and reduces the method development time. In fact, a generic hybrid LC-MS/MS method utilizing a generic immunocapture capture reagent and a universal peptide from humanized monoclonal antibody, typically from the Fc region, can be utilized for bioanalysis of antibody-based biotherapeutics with a Fc backbone for preclinical applications [4].

### **Applications of LC-MS for Antibody-Based Biotherapeutics**

#### *Total and Active (Free) Drug*

In-vivo, mAb biotherapeutics can exist in multiple forms i.e. free (bivalently unbound), partially free (monovalently bound) and the fully bound drug (bivalently bound). As the free and partially free can engage with the target, these are considered as the pharmacologically active forms of the drug. The capture reagent in a hybrid assay may be chosen to compete or not to compete with the binding site of the interfering component, hence allowing the measurement of total and/or active drug.

#### *Multi-component Biotherapeutics*

Antibody-drug conjugates (ADCs) are multicomponent biotherapeutic drugs designed primarily for cancer therapy. These molecules are comprised of monoclonal antibodies conjugated with a highly cytotoxic agents (aka payload) via synthetic linkers. Bioanalysis of ADCs is challenging due to their heterogeneous structures and their complex catabolism. Typically, three assays are required for PK assessment of ADCs i.e. total antibody, total ADC (ADCs with a Drug-Antibody Ratio (DAR) >1) and unconjugated payload [5]. LBA is the method of choice for measurement of total ADC for ADCs having non-cleavable linkers, while LC-MS/MS is the method of choice for measurement of unconjugated payload. Due to the ease of method development, hybrid LC-MS/MS has emerged as the preferred platform for total antibody assay in animal species. Total ADC assay is evaluated by measuring either the payload-conjugated antibody (LBA) or antibody-conjugated payload (hybrid LC-MS/MS). Hybrid LC-MS/MS is preferred over LBA for measurement of total ADC by measurement of the antibody-conjugated payload, for ADCs with site specific conjugation and cleavable linkers. As a result, hybrid LC-MS/MS Total ADC assays are Drug-Antibody Ratio (DAR) sensitive whereas LBA payload-conjugated antibody assays are not DAR sensitive [5]. Depending on the complexity of the ADC molecule, additional analytes maybe measured. Multiplexing capabilities of the LC-MS/MS platform enables it to reduce the total number of developed methods for ADC bioanalysis [6].

Similarly, LC-MS/MS multiplexing capabilities can address the needs of other multi-component biotherapeutics. Probody™ biotherapeutics and Probody™ drug conjugates are another class of multi-component antibodies that are engineered to have an additional mask peptide blocking the target binding domain of the antibodies. These blocking peptides are cleaved off when the biotherapeutic reaches a tumor micro-environment. Due to multi-component structure of these biotherapeutics, multiple assays are required to ensure the integrity of the molecule in vivo. For example, for Probody™ drug conjugates PK assessment involves the measurement of total antibody, intact Probody™ (contains blocking peptide(s)), total conjugated toxin, as well as unconjugated toxin.

#### *Multi-specific Antibodies*

Multispecific therapeutics are antibodies that contain more than one engineered functional domain that bind two or more independent antigenic targets. Bispecific antibodies (BsAbs) have showed great therapeutic promise for cancer treatment as it can engage the host T-cell immune response towards the tumor cells [7]. Several assays are required to support the pharmacokinetic characterization of bispecific biotherapeutics i.e. intact bispecific assay, functional domain assay and total assay [8]. Using hybrid LC-MS/MS, multiple assays can be combined. For example, intact bispecific assay and total assay can be evaluated in single assay by using an immunocapture reagent directed towards the Fc region or one of the functional domain followed by proteolysis and measurement of signature peptides from one or both binding domains and/or the Fc region. For some BsAbs, hybrid LC-MS/MS platform may be preferred due to steric hindrance encountered in the LBAs due to the proximity of the binding domains.

#### *Combination Therapy*

In many indications, particularly in oncology, a combination therapy approach is employed wherein multiple mAbs or mAb-based biotherapeutics are administered targeting multiple pathways to achieve optimal therapeutic effect. In such instances, it may be optimal to design a single assay to measure multiple mAbs using hybrid LC-MS/MS. Immunocapture isolation can be achieved of multiple mAbs/mAb-based biotherapeutics using a mixture of anti-idiotypic antibodies and detected using signature peptides from the CDR region of the individual mAbs/mAb-based biotherapeutics [9]. Alternatively, a generic capture can be used in animal matrix and possibly in human matrix if the concentration of the co-dosed biotherapeutics are high.

#### *Circulating Target Measurement*

Many mAb-based biotherapeutic have soluble targets such as cytokines. The total target levels often increase in a disease state and following drug treatment due to reduced clearance, as a result of being bound to the therapeutic protein. As the concentrations of both free and total target concentrations provide key evidence of target engagement, the measurements of both free and total target levels for studies in discovery and early development are often recommended. Alternatively, the biotherapeutic may have a membrane-bound target that is known to shed their ectodomain (ECD) physiologically or have a soluble form in systemic circulation. The level of circulating shed/soluble target can impact the effectiveness of the therapy. In some instances, the concentration of the shed or soluble forms of the target, may be used as a disease or predictive biomarker. Hybrid LC-MS/MS assay provide multiple advantages over conventional LBAs for circulating target measurement [10]. Unlike LBAs, only a single immunocapture reagent is required for hybrid LC-MS/MS. This is important for some circulating targets, which due to their small size may have limited availability for capture reagents to bind to separate epitopes as a result of steric hindrance. Different signature peptides can be monitored to determine the presence of different isoforms or post-translational modifications in the circulating targets. Hybrid LC-MS/MS also allows simultaneous measurement of bound therapeutic by measuring signature peptides from both soluble/shed target and biotherapeutic.

#### *Immunogenicity*

Biotherapeutics administration can elicit an anti-drug antibodies (ADA) response. Hybrid LC-MS/MS can be used as a complementary platform for ADA confirmatory test assessment especially when the LBAs show poor drug tolerability [11]. In comparison to the bridging LBA, hybrid LC-MS/MS are more tolerant to drug interferences due to monovalent binding with ADAs and the use of higher concentrations of biotinylated drug for immunocapture. The use of magnetic streptavidin beads provides greater binding capacity in comparison to bridging LBA. As hybrid LC-MS/MS measures signature peptides from

ADAs, detection of false positives is minimized resulting in higher sensitivity. Multiplexing capability of hybrid LC-MS/MS can allow simultaneous ADA isotyping [12]. Hybrid LC-MS/MS for immunogenicity testing is a semiquantitative assay. Over the past few years several reports describing detection and semi-quantification of the ADA responses using hybrid LC-MS have been published. However, some practical considerations, such as assay validation, quality control and regulatory acceptance, need to be addressed before hybrid LC-MS/MS can be routinely employed for ADA testing.

#### *Biotransformation*

Antibody-based biotherapeutics can undergo biotransformation such as oxidation, deamidation, isomerization of amino acids, glycosylation and glycation. Hybrid LC-MS is now the preferred platform for studying the biotransformation of biotherapeutics. Depending upon the type of biotransformation, a top-down high-resolution mass spectrometry (HRMS) approach or bottom-up approach is employed. The need for characterizing and monitoring biotransformations and catabolites increases with the complexity of the biotherapeutic. For example, ADC biotransformation may include payload deconjugation, conjugated payload biotransformation or protein mass adducts/losses. LC-MS is highly beneficial for monitoring biotransformations in ADCs and other multi-component antibody-based biotherapeutics [13].

#### **CONCLUSION**

LC-MS has become a major platform for the bioanalysis of protein biotherapeutics. The ease of method development and the high selectivity has been the key driving factor for the popularity of hybrid LC-MS/MS for bioanalysis of antibody-based biotherapeutics in preclinical species. LBA is still the dominant platform for biotherapeutic bioanalysis for clinical studies. The application of LC-MS to address bioanalytical questions of antibody-based biotherapeutics will rise as we improve the immunocapture and digestion methodology. The scope of intact analysis, particularly for multi-component biotherapeutics, will broaden with development of high quality immunocapture critical reagents and improvements in the HRMS software and instrumentation. Over the last decade, there have been several white papers leading to consensus on the hybrid LC-MS/MS assay validation and acceptance criteria. Some Pharmaceutical companies are reluctant to move forward with using hybrid LC-MS/MS due to a lack of demonstrated regulatory acceptance, i.e. approved drugs. However, with the increase in hybrid LC-MS/MS usage to support biotherapeutic analysis, regulatory review is eminent. As biotherapeutics drugs increase in complexity, a combination of bioanalytical platforms is required for pharmacokinetic characterization. With the development of newer biotherapeutic drugs, the need to address specific bioanalytical question of these multifaceted molecules will necessitate the use of the technology that best addresses the question rather than the one that is conventionally used. In the future, LC-MS application for bioanalysis will continue to grow along with evolution of the biotherapeutic drugs.

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