Hybridizing LBA with LC–MS/MS: the new norm for biologics quantification

“By combining traditional LBA immunoaffinity assays with the latest LC–MS/MS technologies, bioanalysts can achieve efficient immunocapture and sample extraction of large molecules, followed by highly sensitive and selective data analysis...”

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Over the past 20 years, protein and peptide drug development has advanced significantly, becoming a rapidly growing segment of the biotherapeutics market. It has generated treatments for a wide variety of diseases ranging from inflammatory disorders to cancer [1,2]. This expansion of biologics-based medicines has pushed drug manufacturers to adopt new bioanalytical and analytical approaches to quantify and characterize multiple modalities of protein-based therapeutics efficiently with particular focus on the high-throughput needs of early-stage PK studies. Ligand-binding assays (LBAs) are widely used for bioanalysis of small and large molecules, but these assays can be lengthy to develop and have limited selectivity and sensitivity. LC–MS/MS-based bioanalysis can offer important advantages over immunoassay-based techniques, but LC–MS/MS with large molecules introduces several challenges affecting throughput, operating expertise and ease of use.

Bioanalytical techniques for large-molecule quantification

Traditional approaches for quantification of biologic drugs typically involve either LBAs, such as ELISA, or UV detection of individual peptides using HPLC separation [3,4]. One of the challenging parts of biologic drug quantification is developing a selective and sensitive assay, which can accurately measure and not over- or underestimate the drug con-

centration levels. This is very important during preclinical or clinical safety/efficacy testing. The gold standard has been LBAs but due to lack of specificity, this technique has been recently complemented with LC–MS. More recently, LC–MS/MS-based methods [5–7] have come to the forefront as a feasible approach for the quantification of various therapeutic peptides, proteins and monoclonal antibodies (mAbs) in biological matrices with many of these methods relying on proteolytic digestion of the target mAb and quantification of multiple unique signature peptides, which are equivalent to levels of the whole protein. The LC–MS/MS method complements the traditional LBA approach and provides the specificity and sensitivity necessary for overcoming LBA limitations, such as matrix interferences and time-consuming reagent development. Further incorporation of immunoaffinity-enrichment steps, which concentrate the target biologic drug prior to proteolysis, not only lowers the background complexity but also captures the active and circulating isoform of the drug, which makes the assay functionalized [8,9].

Pros & cons of ligand-binding & LC–MS/MS techniques for large-molecule bioanalysis

The main driver behind exploring alternate techniques is to increase the selectivity of an assay. Each assay platform, either LBA or LC–MS/MS, has demonstrated its strengths

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and limitations [10]. The ultimate end goal for any technique is to provide most reliable and accurate results. Traditionally, large-molecule PK assays have employed LBAs that rely on immunoaffinity detection of a unique epitope on the protein or peptide of interest. This approach can detect both physiologically active and 'free' forms of circulating large-molecule drugs, which makes the assay functionalized. Even though, the high specificity of the antibody-based interactions can track an analyte with high sensitivity but are typically expensive and time-consuming to develop. Specific antibody reagents to be developed for each mAb variant, a process that is often not compatible with the compressed time frames encountered during the initial stages of drug development. On the other hand, LC-MS/MS is widely used for highly selective and sensitive bioanalysis of small molecules. However, large-molecule bioanalysis using LC-MS/MS also presents challenges such as the need for extensive and tedious sample preparation and the expertise to operate complex LC-MS instrumentation including the software to process the data limits wider adoption of the technique. LBAs have limited dynamics and are subjected to limited selectivity and antibody cross-reactivity. This results in lack of specificity from interference and high-background levels that are not suitable for meeting the biopharmaceutical industry's requirements to detect diverse peptide, and protein therapeutics with ever-increasing sensitivity and reproducibility. LC-MS/MS assays can bring all the benefits of further selectivity with either multiple reaction monitoring (MRM) quantification or high-resolution quantification, in addition to the ability to measure multiple components and even biotransformation products such as catabolites of the drug [11]. Each technique shares opportunities and limitations to keep up with the ever-increasing demand for selective and sensitive biologics bioanalysis [12]. By combining traditional LBA immunoaffinity assays with the latest LC-MS/MS technologies, bioanalysts can achieve efficient immuno-capture and sample extraction of large molecules, followed by highly sensitive and selective data analysis. Hybrid LBA-LC-MS/MS opens up new prospects to accelerate the drug discovery and development [13].

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Hyphenating LBA & LC-MS/MS opportunities

With the increasing complexity of multidomain therapeutic drug candidates (e.g., antibody-drug conjugates, pegylated proteins and fusion proteins), the

specificity of LC-MS/MS detection combined with the selective enrichment advantages of ligand binding will help to fully characterize the PK/PD properties of these new drug entities. LC-MS/MS methods have the advantage of simultaneous detection of multiple peptides or molecules derived from a protein construct. This offers the opportunity to characterize the metabolism, catabolism and protein stability of both bioactive and inactive regions of the construct and can provide complimentary information to LBAs. Additional information complimentary to LBAs can be obtained by the ability of LC-MS/MS to quantitate multiple proteins in the same sample such as target and therapeutic or therapeutic and antidrug antibodies.

Hybrid LBA-LC-MS/MS assays for biologics bioanalysis

A recent example of the utility of combining ligand binding with the specificity of LC-MS detection was provided by Liu *et al.*, who described a hybrid immune-capture LC-MS/MS assay for the quantification of the payload molecules of an ADC [14]. The hybrid antibody-conjugated payload assay has three steps: immunoaffinity-enrichment with an anti-idiotypic capture mAb, payload cleavage using a specific lysosomal enzyme and LC-MS/MS analysis of the cleaved payload. This combined approach provides direct measurement of the bioactive species, payload molecule linked to the mAb and not the other possible analytes, which include unconjugated payload and its metabolites and unconjugated mAb. A unique characteristic of multidomain peptide therapeutics is their heterogeneity, this is reflected in the drug-antibody ratio (DAR) of ADCs. The DAR *in vivo* is dynamic and changes over the time course of PK measurements, so that the calibration curve ADC reference material might not behave the same as the ADC with varying DAR. The authors showed their hybrid assay with direct measurement of the payload molecule could quantify ADCs in QC samples of DAR 2.0 and 4.0 using calibration samples prepared with a DAR of 3.0. An example of the complementary nature of a hybrid LBA-LC-MS/MS assay and LBA was provided by their characterization of the change in DAR over the time course of the PK measurement. The data were generated by using the ratio of the antibody-conjugated payload concentration from the hybrid assay to the total-Ab concentration from an LBA and showed that the average DAR decreased from 3 to 1 postdosing. This data, generated from multiple assays can give unique insights into the metabolism and disposition of the ADC that a singular assay could not. Finally, the multiplexing ability of MS detection was demonstrated by monitoring an inactive form of the ADC

that consisted of a metabolite of the payload still conjugated to the mAb. This study is an excellent example of the power of hybrid LBA–LC–MS/MS techniques to deliver a comprehensive bioanalytical strategy for ADC PK characterization [14].

The complementarity of integrating the data from orthogonal techniques was illustrated in a recent presentation by Krantz at EBF 2015 [15]. In a preclinical PK study of a PEGylated protein–drug candidate known to be proteolytically unstable, both an LBA and a multiplexing LC–MS/MS assay were employed to characterize its PK behavior. The multiplexed LC–MS/MS assay simultaneously measured three peptides from distinct regions of the protein. The PK profile of the drug candidate as measured by LBA only correlated with the PK profile as measured by one of the three signature peptides. It was confirmed that the LBA results and the LC–MS/MS results of the correlating peptide represented only the bioactive portion of the drug candidate. The major species present in serum were truncated PEGylated forms of the protein which were represented by the assay of the other two peptides. The LBA on its own was not able to detect these metabolites. In a follow-up clinical study of the unconjugated therapeutic protein, a second LBA employing a different capture pAb was employed along with the three peptide multiplexed LC–MS/MS assay [15]. When examining the PK data correlation between the assays from the unconjugated protein study, it was discovered that the converse was true. The LBA PK profile correlated with the LC–MS/MS assay of the peptides representing the inactive region of the protein. In this example, LC–MS/MS was used to characterize the LBA and helped to determine what the LBA was measuring.

Conclusion

The shift from small molecule to large molecule is happening exponentially, so are the techniques to characterize and quantify these molecules in the biological matrices. There are many challenges in dealing with large-molecule drugs and one of the most important and a common challenge is a selective and specific bioanalytical assay. A Gold standard assay such as LBA still has its place given throughput and ease of use. But the expectation around patient safety and efficacy demands more selective assay platforms such as LC–MS/MS

system, perhaps hybrid LBA–LC–MS/MS assay is the strategy to go. Comparable cost analysis [16] between different LBAs and LC–MS/MS assays makes it easier for scientists to put more emphasis in choosing scientifically sound and selective techniques for the study. Hybrid LBA–LC–MS/MS assay can bring all the benefits of further selectivity with either MRM quantification or high-resolution quantification in addition to the ability to measure multiple components and even metabolite's/catabolite's down the road.

Future perspective

Although LC–MS/MS technologies have certainly advanced to be more suitable for biologics bioanalysis, the array of MS technologies and techniques, tedious sample preparation methods and reagents could be daunting for nonexperts needing to develop a new biologics bioanalysis workflow. An integrated package for biologics analysis that includes sample preparation kits, automated immunocapture-enrichment process, MS technologies and standardized software with ready-to-use protocols makes the process easier to implement without compromising data quality. Significantly improved selectivity can also be achieved in biological samples that have numerous highly abundant protein interferences by using MRM [3] and differential ion mobility separation technology. Further advances in software and automation are being made to increase throughput, and enhance method development and optimization. The latest instrumentation and software developments will deliver significant improvements in the quality and reliability of bioanalysis studies, delivering more robust and compliant data that have important implications for patient safety. These developments are making LC–MS/MS more accessible for biologics bioanalysis, helping scientists to accelerate their large-molecule drug development.

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