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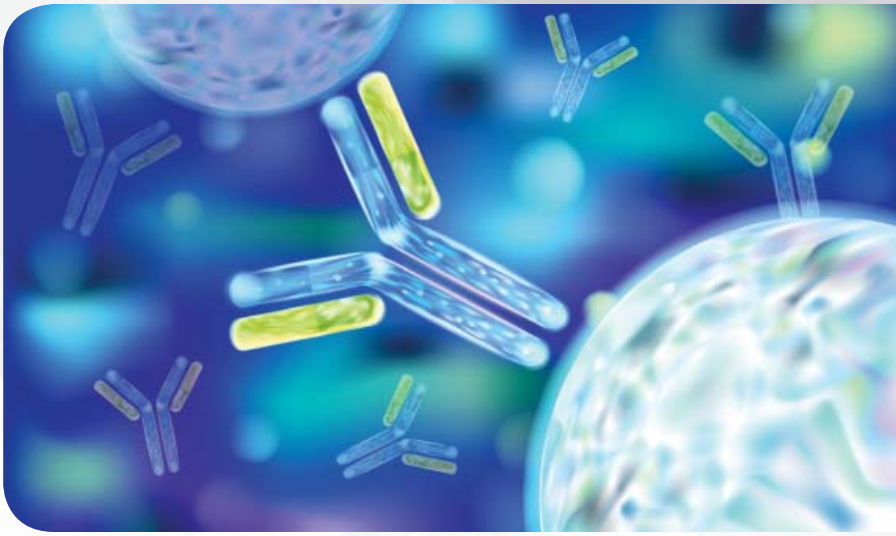
BIOANALYSIS OF LARGE MOLECULE THERAPEUTICS

INTRODUCTION

Bioanalysis is defined as the quantitative measurement of a therapeutic modality or its metabolites in biological samples that include blood, plasma, serum, urine or tissue extracts[1]. Bioanalytical studies are an integral part of pharmacokinetics (PK) and pharmacodynamics (PD) studies, as well as the evaluation of drug toxicity. Typically, an array of methodologies is used to obtain a comprehensive profile of the behavior of the therapy in a systemic biology model.

Historically, most drug assets were small molecules that had defined chemical structures and could be analyzed using mass spectrometry-based methods such as LC-MS (liquid chromatography-mass spectrometry) and GC-MS (gas chromatography-mass spectrometry). In contrast, large molecule drugs are, by definition, larger and more complex than small molecules. Several types of drugs fall under the large molecule umbrella including monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), gene therapies, cell-based therapies, oligos, RNAs, proteins and peptides. The methods used in large molecule bioanalysis vary depending on the type of entity that is being tested, and it is important to note that ADME processes and methodologies to test these modalities are still evolving.



While small molecule drugs still comprise the majority of approved therapies, the approval rate of biologics has been relatively steady and is forecasted to have a robust growth rate. In 2019, 48 NMEs or new molecular entities were approved out of which 10 were biologics[2]. Over the past decade, approximately a quarter of all approved NMEs were biologics[3], but it is forecasted that biologics will comprise half of the top 100 drugs in 2021[4], driven by the accelerated rate of biologics development preclinically. While this forecast is ambitious, it is important to note that about 50% of the global biopharma pipeline is estimated to be large molecules, so it is possible that the large molecule approval rates will increase at a rapid rate.

The global large molecule bioanalysis testing service market is valued at USD 1.3B in 2019 with a CAGR (compound annual growth rate) of 9.1% through 2027[5]. It is estimated that about 35% of the large molecule bioanalysis market is in the preclinical space where a majority of the studies are outsourced to CROs (contract research organizations) who have the processes, personnel and operational infrastructure to perform bioanalytical testing for various therapeutic modalities.

There are several key differences in the development of small molecules and large molecules including dosing and ADME (absorption, distribution, metabolism and excretion) characteristics. Small molecules are typically dosed orally when possible, and have a broad distribution to various organs. They are typically metabolized enzymatically into active and inactive metabolites that are then excreted via the kidneys (renal excretion) or via the intestine (biliary excretion). In contrast, large molecules typically require more invasive dosing routes including intravenously and subcutaneously, and have limited distribution via the vascular and lymphatic systems. Due to the difference in biodistribution, biologics typically require more time to achieve peak concentrations compared to small molecules and also have slower clearance times. Additionally, biologics are typically recycled by the body instead of being excreted[6], [7]. The primary objective of bioanalysis studies is the assessment of ADME characteristics, so the methods used in large molecule bioanalysis are different from small molecules.

LARGE MOLECULE BIOANALYSIS

Bioanalytical studies of large molecule drugs can be segmented as follows: pharmacokinetics or PK studies, toxicokinetics or TK studies, pharmacodynamics or PD studies, immunogenicity assays and biomarker studies. Pharmacokinetic studies give information on the duration and intensity of a therapeutic response, where the large molecule drug is introduced into an *in vivo* model and the amount of drug in the serum or plasma is assessed using either ligand binding assays (LBAs) or mass spectrometry methods to identify the bioavailability and clearance of the drug. Toxicokinetic studies are similar to PK studies except the drug dosage is significantly higher and the study objective is to determine acceptable drug exposure levels. Pharmacodynamic studies ascertain the effect of the drug on the *in vivo* model and in the case of large molecule drugs, target binding and impact on downstream signaling or gene expression are assessed. Several large molecule drugs, especially those that target cancer, trigger an immune response, so immunogenicity assays that measure changes in the immune system or assess the neutralizing antibody responses are critical readouts.

Biomarkers are critical endpoints to measure drug efficacy in preclinical and clinical models, so it is essential to validate identified biomarkers in preclinical models. Biomarker validation is considered to be a part of bioanalysis, and the same standards including specificity, sensitivity, robustness and reproducibility are applied. The FDA's bioanalytical method validation guidelines also highlights the requirement for validation biomarkers using bioanalysis standards[8].

METHODS USED IN LARGE MOLECULE BIOANALYSIS

In vitro assays

In vitro binding and potency assays are commonly used to assess drugs prior to more detailed ADME studies in *in vivo* models. For large molecules, cell-based assays are used to assess biological activity and the assay of choice depends on the drug modality. The potency of therapeutic monoclonal antibodies is typically assessed using binding assays. If a therapeutic protein has enzymatic function, then enzyme activity assays can be used to evaluate potency. Enzyme activity assay readouts can be chemiluminescent, fluorometric, optical or spectrophotometric. Cell-based assays are widely used to assess large molecule activity in a physiological context where other proteins, co-factors etc. are present. Cell-based assay readouts include cell proliferation or death assays, reporter gene readouts or changes in downstream signaling molecules such as an increase in cyclic AMP downstream of GPCR (G-protein coupled receptor) activation.

Ligand Binding Assays

Ligand binding assays (LBAs) cover a broad range of assays from radioimmunoassay (RIA) to ELISA to high throughput assays such as MSD (Meso Scale Diagnostics). These assays use the sandwich format where an immobilized capture antibody binds the analyte of interest that is then detected using another antibody, which is typically conjugated to a readout for detection (colorimetric, chemiluminescent, or optical). LBAs for large molecules typically use sample dilution to reduce background signal, and the assay design is dependent on the study objective – for PK/PD studies, it is important to measure the

amount of active drug that binds to the target and triggers downstream responses whereas for a toxicology study, the total amount of circulating drug needs to be measured to assess the amount and duration of the drug in the body[9]. Several types of LBA platforms are available to test large molecules and some platforms include washing steps to remove unbound drug and other interfering biomolecules while others are "mix and measure" where washing away unbound drug and background molecules is not required. Increasingly, LBAs are being performed on platforms with automated liquid handling to increase accuracy and reproducibility by reducing manual errors[10] and reduce time to results.

Some of the popular LBA methods include the Gyrolab platform, MSD platform and Luminex platform. The Gyrolab technology has been adopted in several biopharma companies and CROs. The immunoassay technology uses affinity columns in a CD format that requires small sample volumes and has shorter time to results. The MSD assay platform is used widely in bioanalytical studies and the platform uses electrochemiluminescence as a readout of the sandwich assay. The Luminex assay platform has been available for some time and is a bead-based multiplexed immunoassay system. The beads are color coded and coated with capture antibodies, so once the analyte is bound it can be detected using a conjugated detection antibody that can be read using a laser. Another trend is the development of hybrid assays where LBAs are combined with mass spectrometry assays to get a more complete view of the activity and downstream effects of the large molecule drug.

Measuring neutralizing antibody responses is one of the key readouts for mAbs and protein therapeutics that trigger an immune response. It is estimated that about 45% of the clinical and nonclinical large molecule bioanalysis market consists of ADA (anti-drug antibodies) assays[5]. Neutralizing antibody assays include competitive LBAs and cell-based assays[11]. LBAs are used to assess large molecule drug binding to the target in the presence of competing antibodies, in an *in vitro* setting while cell-based assays are used to evaluate the drug's immune activation in cell and tissue systems. Cell-based assays can have many endpoints including luciferase mediated luminescence, ELISA assays for competitive binding and fluorescence or electrochemical based readouts of cell proliferation, death or signaling changes. They are more complex than LBA assays but also provide more information on the drug activity in a more physiologically relevant system.

Mass Spectrometry

Mass spectrometry (MS) methods are widely used in small and large molecule bioanalysis and has distinct advantages over LBAs. MS methods are more selective, amenable to multiplexing and can simultaneously identify and quantify both total drug and metabolites. Mass spectrometry is combined with either liquid or gas chromatography (LC-MS or GC-MS). LC-MS is widely used to analyze large molecule therapeutics including mAbs and antibody-drug conjugates (ADCs)[12]. Typically, mAbs are digested into smaller fragments using proteases prior to LC-MS analysis. Bioanalysis of ADCs is more complex as the modality consists of a small molecule drug conjugated to a mAb. LC-MS is uniquely suited to analyze both the mAb carrier and the chemotherapeutic payload, and can also be used to analyze the drug-to-antibody (DAR) ratio and identify the physicochemical properties of a given ADC[13]. LC-MS does have a limitation in the resolution of similar masses of intact large molecules, so in some bioanalytical studies, High Resolution MS (HRMS) may need to be used. HRMS calculates the analyte mass to several decimal places to better resolve and identify analytes that have similar masses[14]. LC-MS can be combined with LBAs either as complementary assays or orthogonal assays where the LBA data is confirmed independently by LC-MS analysis[15].

Single Molecule Analysis

Single molecule analysis assays are an emerging tool in the bioanalysis toolbox. These assays offer extremely high levels of sensitivity molecules and are also amenable to automation to improve throughput. Some of the better-known platforms are the Simoa from Quanterix and SMC Erenna from EMD Millipore. Simoa (single molecule array) used bead conjugated antibodies to capture low abundance protein. Each bead containing protein-antibody complexes is then loaded into wells for fluorescent imaging.

The Simoa technology is ideally suited for large scale bioanalysis as it is amenable to automation, and offers a short time to results. Additionally, the technology is estimated to be up to 1,000 times more sensitive than ELISA, requires low sample inputs that can be multiplexed[16]. The Erenna immunoassay system can detect low abundance proteins in PK studies and evaluate the immune response to large molecule drugs. The platform captures the analytes via capture and fluorescently conjugated detection antibodies. The protein-antibody complex is then dissociated and the labeled detection antibody is quantified using a laser.

Flow Cytometry

Flow cytometry has been the gold standard in many areas including immune cell profiling and cell signaling studies. The technology measures the properties of single cells and provides multiplexed data on different cell populations. With the increased interest in developing cell therapies and therapies that induce an immune response, flow cytometry is increasingly being used in the bioanalysis of large molecule drugs. Whole cell therapies such as CAR-T cells can be comprehensively assessed using relevant flow cytometry panels[17]. Additionally, flow cytometry is also used to detect the presence of anti-drug antibodies that can inhibit the function of the drug. A recent study reported the development of a flow cytometry-based immunogenicity assay for the CAR-T therapy Kymriah®[18].

Given the active development of large molecule therapeutics for various diseases, it is clear that bioanalysis methods for these modalities will continue to develop at a very rapid pace and will include a mix of established methods as well as new technology platforms.

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