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A Review on LBA and LC-MS Platforms for Supporting Large Molecule Pharmacokinetics Bioanalysis

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Abstract

Over the past ten years, the global market for large molecule therapeutics has rapidly grown for the treatment of a variety of diseases. LBA and LC-MS are two platforms widely used in pharmacokinetic bioanalysis. In this review, we compare LBA and LC-MS and summarize their strengths and limitations. Strategies for platform selection are provided according to the study purpose, study phase, analyte types, assay requirements and other factors.

Keywords: Ligand binding assay • LC-MS • PK

Introduction

Driven by an increase in the geriatric population and the prevalence of chronic diseases such as cancer, diabetes, inflammatory and autoimmune diseases, large molecule-based therapeutics are the fastest growing class of drugs under development in both academic and industrial sectors in the past ten years. During drug development, the precise quantification of the therapeutic drug concentration is key to delineating the relationship between drug exposure and safety or/and efficacy of the molecule [1-4]. Because of this need, significant efforts have been spent in developing and improving bioanalytical methods to support sample analysis. Among commonly used assay platforms, Ligand Binding Assays (LBA) and Liquid Chromatography and Mass Spectrometry (LC-MS) remain the two most popular options for Pharmacokinetic (PK) assay development. In this review, the key parameters of both bioanalytical platforms are reviewed and at the end, the recommendations are given for selecting a method with regard to different types of therapeutics, including oligonucleotides, proteins, antibody drug conjugates and bispecific antibodies.

Literature Review

Ligand binding assay

A Ligand binding assay is a common analytical procedure that takes advantage of the highly specific interaction of a ligand and a receptor, an antibody, or another macromolecule (collectively called "affinity recognition reagent") (Figure 1) [5]. This principle has been widely used for the accurate, precise and sensitive quantification of large molecule analytes for PK assessment from drug discovery to preclinical and clinical phases [6]. In LBA, the analytes are detected through the formation of the ligand-receptor complexes. The sensitivity and specificity of the assay depend highly on the affinity between the two binding partners and on the specificity of their interaction. The parameters of the assay also need to take into account the inherent properties of these molecules such as their stability, sensitivity to pH or temperature and accessibility of their binding sites.LBA assays are designed to reach and maintain the equilibrium constant of the reactants in order to provide suitable assay performance in terms of assay sensitivity, accuracy, and reproducibility. Based on the method of detection, LBAs can be categorized into optical density-, fluorescence-, and luminescence-based approaches. The Enzyme-Linked Immunosorbent Assay (ELISA), Meso Scale Discovery (MSD), Gyro and Luminex are popular LBA platforms for PK assay development [7-9].

Among these approaches, ELISA is the most commonly applied approach for accurate quantification of biological therapeutics in the context of PK assessment for both preclinical and clinical studies [10-13]. As schematically illustrated in Figure 1, a capture reagent is coated onto a polystyrene plate, the analyte (i.e., drug) is diluted and incubated in order to bind to capture reagents. Unbound analyte is removed from the plate by a washing step and a detection reagent is added, binding to the captured analyte, thereby forming a sandwich complex. The detection reagent is linked to a reporter enzyme (usually Horseradish Peroxidase (HRP)) that is used to generate the assay signal by adding the enzyme's substrate to the plate.

ELISA is a sensitive, specific, reliable and cost effective approach [14-16]. However, the assay sensitivity is limited by two important factors: the affinity of the detection antibody and the detection limit of the absorption spectrophotometer. While ELISA can in theory detect concentrations lower than 1 pg/mL the actual sensitivity of many assays is often only in the ng/mL range. Fluorescence-based detection methods have been implemented into traditional ELISA to improve the assay sensitivity. Fluorescence decay is, however, one challenge that sometimes renders this approach less robust and limits this platform in applications. Electrochemiluminescent detection technology (e.g., MSD) using SULFO-TAG label as detection reagent is another approach to increase ELISA sensitivity [17, 18]. The bottom of the MSD multi-well plates is equipped with carbon electrodes, which conduct electricity from the MSD instrument leading to light emission by the SULFO-TAG labels. The assay signal can be amplified by multiple excitation cycles. At the same time the MSD platform holds promises for low background noise because electric stimulation is decoupled from the light signal, so that only labels near the electrode surface can be detected. Thus, MSD is able to provide great assay sensitivity (pg/mL) and a broad dynamic range (3-4 logs). The main disadvantage of the MSD plate form are the costs of the plates that are much more expensive than ELISA plates.

The Gyrolab is a nanoliter-scale immunoassay that uses flow-through affinity columns which reduce background and matrix effects. The use of micro fluidic technology, automated control of centrifugal and capillary forces and a built-in fluorescence system allows not only to minimize sample volumes but also provides great assay sensitivity and reproducibility without the need of manual pipetting [19-22].

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LC-MS

LC-MS is a platform which combines two analytical technologies. LC provides a simple method for the physical separation of a target substance from the biofluid which contains a complex mixture of components. Solubilized analytes in the mobile phase are passed through a column packed with the stationary phase which separates the compounds based on size, affinity, charge or hydrophobicity. In the interface between LC and MS the separated analytes are fragmented and ionized, after which they can be identified by MS with high specificity. A major limitation of LC/MS-based quantification in comparison to LBAs is the need for sample purification/extraction and enzyme digestion prior to the analysis. Quantification by MS also requires the quantification of selected signature or surrogate peptides derived from the pure target analyte to be used as reference standard (Figure 2) [23-25].

As it is challenging to ensure consistent analyte recovery from each step of sample pre-treatment (e.g., extraction and digestion), a constant amount of a chemical substance referred to as Internal Standard (IS) is spiked into calibration standards, Quality Controls (QCs) and samples. The amount of IS in each sample is quantified and used to normalize the assay compensating for variations encountered during sample preparation, injection and instrumental analysis [26, 27]. A Stable Isotope-Labeled (SIL) form of the analyte is usually the preferred choice for the IS since it mimics the behavior of the intended analyte in the assay. When the ideal IS (e.g., SIL protein) is not available, SIL-peptides or structural analogues of the protein analyte can be used as surrogates [28, 29]. With the incorporation of IS, the assay accuracy has been significantly improved in LC-MS. Yang et al. developed a LC-MS method for quantification of therapeutic proteins, demonstrating the accuracy within 15% [30]. Chiu et al. quantified three IgG-based drugs (Bevacizumab, Nivolumab and Pembrolizumab) using the LC-MS platform following protein G purification. Among all three monoclonal antibodies, the quantification accuracy was within 15% at different concentrations tested [26].

Drug development is a highly competitive business. Naturally there is high pressure to shorten the timelines for drug candidates both in the actual development and in the evaluation phase. Compared to LBA, LC-MS is less limited by reagent availability and quality (e.g., the need for high affinity and specificity capture and detection reagents). Therefore, LC-MS is favored especially in early discovery phases whenever the study purposes can be served. Several highly sensitive LC-MS approaches have been established, but in most cases the sensitivity for bio macromolecules is at ng/mL level. In addition to the intact drug, LC-MS also capable of measuring its metabolites or truncations [31-33] Because of these advantages. LC-MS has been extensively used to quantify a wide variety of therapeutics, including peptides, proteins, monoclonal antibodies, oligonucleotides, and bispecific antibodies [32, 33]. One main limitation of generic LC-MS methods is inability to differentiate the measured drug at different status (e.g., free or bound) [34, 35]. Immunocapture approaches have been developed for extending LC-MS application for these purposes.

Assay Development Considerations

Sensitivity

Assay sensitivity is one of most important considerations in developing a PK method for studies across all phases, though the exact requirements differ with each study phase. Pre-clinical evaluation is focused on establishing the safety and maximal/tolerable dose of the drug. The drug efficacy is investigated in late clinic studies. The quantification range of the

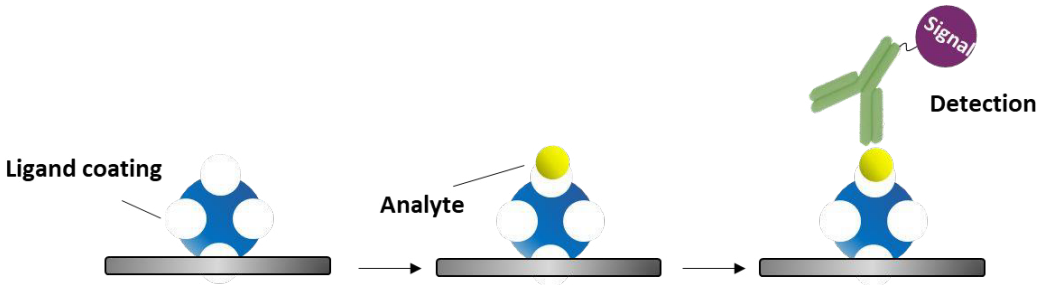

Figure 1. Schematic principles of ligand binding assays.

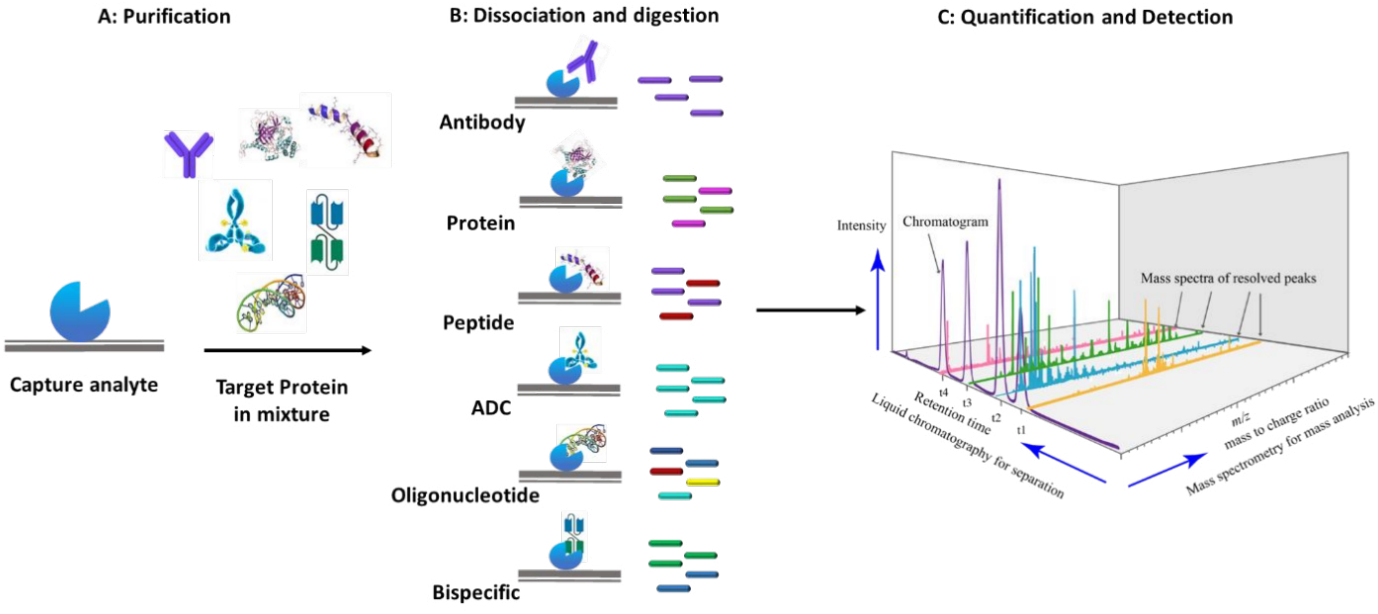

Figure 2. Procedure of LC-MS. (A) Affinity capture of analytes; (B) Digestion of analytes into fragments; (C) LC-MS measurement (from Daniel Norena-Caro).

assay should be designed to cover the concentrations of most samples. It is not recommended to develop an ultra-sensitive method when it is not required by the study since this may compromise the assay robustness. Additionally, the resulting low ULOQ limit requires sample dilutions, which introduces further analytical variability.

Selectivity/specificity

Selectivity/specificity is the ability of a method to measure an analyte in the presence of other potentially interfering or related substances, such as rheumatoid factors, a concomitant compound or structurally related molecules in test samples. A selectivity/specificity test should be designed and performed based on the anticipated levels of an interfering compound and analyte estimated from literature and from the study protocol. In LBA, the analyte is bound between the capture and the detection reagents. The selectivity/specificity of the assay stands and falls with the quality and the specificity of the capture and detection reagents used. Antibodies with insufficient specificity will bind to other compound, leading to variable levels of background signal which poses a major challenge to assay development. In LC-MS, the analyte is extracted and digested to the peptide level. A unique peptide is identified and selected as signature peptide for quantifying the analyte. The non-specific binding related issues does not impair selectivity and specificity in LC-MS.

Accuracy and precision

Another major concern for assay development is the accuracy and reproducibility of the results. A very sensitive method is of little use for bioanalytical purposes, if the measurements are unreliable. Therefore, assay validation has to demonstrate that the accuracy and precision of the method are within acceptable ranges. Accuracy is the closeness of agreement between the reference value and the test results. In bio-analysis, the accuracy assessment takes into account a combination of random and systematic bias components. Precision is defined as the variability among the repeated measurements under stipulated conditions. There are two types of precision, including repeatability and reproducibility. Repeatability compares results from identical testing conditions, i.e. the experimental factors are not changing and thereby do not contribute to the variability. In contrast, under reproducibility testing condition, the experimental factors vary and contribute to the variability, leading to a greater overall variability in the test results.

When evaluating the accuracy and precision of a bioanalytical method, QC samples are prepared as reference by spiking analyte into biofluid at different levels, including lower Limit of Quantification (LLOQ), Low Quality Control (LQC), Medium Quality Control (MQC), high Quality Control (HQC) and Upper Limit of Quantification (ULOQ). According to FDA guidance on bioanalytical method, LBA requires accuracy and precision to be within 20% absolute bias and variation for LQC, MQC and HQC levels, while 25% are acceptable for LLOQ and ULOQ. In LC-MS, the testing result is calculated by referencing the IS that is normally added in sample preparation phase. The incorporation of IS helps to improve the data quality by offsetting lab variations (e.g., extraction and pipetting). FDA recommends LC-MS method to be within 15% absolute bias and variation for LQC, MQC and HQC levels, and within 20% for LLOQ. In both platforms, assay accuracy and precision can be compromised when the assay quantification range is inappropriately set. For example, when the LLOQ level does not generate significant signal to noise ratio or when the ULOQ level is located in the plateau range.

Recovery

Drug escalation dose is often used in the studies to explore the safety and efficacy assessment. This leads to a wide range of the drug concentrations which cannot be covered by the assay quantification range. Samples are diluted in biofluid or assay buffer until they fall into the validated quantification range. Dilution recovery is assessed during validation to ensure that the dilution of samples does not impact the analyte recovery in the measurement. Recovery samples at the estimated Cmax or 2 × Cmax level of the analyte are usually prepared for this assessment. The samples should be measured within the acceptable accuracy and precision range post dilution. Dilution recovery can be a challenge for both LBA and LC-MS methods. The analyte may interact with endogenous matrix molecules leaving most of the analyte after multiple dilutions in a bound state. This results in low analyte recovery in free PK assays. Corrective measures such as interference molecules depletion should be considered. To ensure that the recovery test responds well in study samples, samples with ultra-high drug concentrations should be evaluated in a parallelism test.

Robustness

Robustness is the assay's capacity to remain unchanged despite inevitable variations of method conditions. Robustness tests include critical assay conditions (e.g., incubation time and temperature), reagent lots (e.g., different batches), and instrument variation (same model but a different unit). When establishing a LBA or LC-MS method, the assay condition parameters that potentially could be changed during bioanalytical analysis should be assessed in the robustness test. While high quality reagents are critical for both LBA and LC-MS to produce accurate and reproducible results, performance of LBA is more sensitive to changes in assay reagents, often even between lots of the same reagent. LBA also requires the labeling of capture and detection reagents with functional groups (e.g., Horse Radish Peroxidase, SULFO-TAG), thereby harboring additional variability. The labeling, handling and storage of the assay reagents are critical to control in order to minimize assay variability. The replacement of critical assay reagents with a different lot should be avoided across the study, especially with LBA. The test results from LC-MS are both reagent- and instrument-dependent. Thus, the robustness test of a LC-MS method also requires evaluating different instrument units.

Free, bound and total drug

The accurate quantification of large molecule drugs in preclinical or clinical studies is important because these drug concentration data reveal the relationship between drug exposure and safety or efficacy. In certain studies, the availability of drug concentrations in free versus bound state in circulation is useful in interpreting PK and PD results and their interactions. To better serve the study purposes, bioanalytical assays are designed to measure free drug, bound drug and/or total drug. LC-MS is suited best to measure total drug concentrations because it is detecting the drug through signature peptides whose presence is not affected by binding. Since LBA is based on the availability of binding site on the analyte for both capture and detection reagents it is commonly used to detect the free drug.

Therapeutic Types and Assay Platform

Oligonucleotide

Oligonucleotides (OGNs) have been developed as therapeutics to treat a variety of diseases, including cancer, cystic fibrosis, Alzheimer's, hepatitis B, HPV, Duchenne muscular dystrophy, asthma and inflammatory arthritis. Currently there are numerous types of therapeutic OGNs in development, including Antisense Oligonucleotides (ASOs), Small Interfering RNAs (siRNA) and aptamers [36]. Quantitative Polymerase Chain Reaction (qPCR)-based assay, hybridization ligand binding assay, and LC-MS can be used to measure OGNs in human plasma, urine or various tissues [37].

In a hybridization LBA, the oligonucleotide drug from the sample is hybridized to capture and detection probes. The complimentary design of both probes ensures adequate affinity and specificity for their oligonucleotide drug. The assay signal comes from the labels on the detection probes. Three types of hybridization LBA formats are available, including ligation hybridization ELISA, nuclease-based hybridization ELISA, and dual probe hybridization ELISA. In ligation hybridization assays the oligonucleotide drug is hybridized to a capture probe with an overhang at the 5' end. The overhang binds to the detection probe which is then ligated to the drug using T4 DNA ligase. This approach is not desirable for oligonucleotide drug with long sequences. Nuclease-based hybridization assays utilize a single complementary sequence that serves both as capture and detection

Table 1. the bioanalytical comparison of LBA and LC-MS.

Therapeutic Type	Assay Method	Strengths	Limitations
Oligonucleotides	Hybridization LBA [43-45]	• Excellent sensitivity for large OGNs (> 20 meters) • Little or no sample cleanup required • High throughput	• Needs specific reagents • Relatively narrow assay quantification range (20 fold -50 fold) • Lack of metabolite information
	LC-MS [46-48]	• Excellent specificity, precision and accuracy • Large assay quantification range • No specific reagents required	• Lack of sensitivity • requires intensive sample cleanup • Low throughput
Proteins	LBA [49,50]	• Excellent sensitivity • No purification step	• Potential cross-reactivity between antibodies in a multiplexed immunoassay • Needs specific reagents
	LC-MS [51-53]	• Generally acceptable sensitivity • Excellent specificity, precision and accuracy	• Complex sample preparation • Low throughput
ADCs	LBA [54,55]	• Excellent sensitivity • No purification step • High throughput	• Unable to measure payload, the drug to antibody ratio (DAR) or the overall drug load • Lack of structural/sequence information of the ADCs • Limited multiplexing capability • Needs specific reagents
	LC-MS [56-58]	• Able to provide ADC analyte structure Information • Could be highly multiplexed;	• Relatively low sensitivity for intact ADC analysis • Lower throughput due to additional steps such as proteolytic digestion and chromatographic separation requiring samples to be injected one at a time
Bispecifics	LBA [59-61]	• Excellent sensitivity • Detects the intact molecule	• Need specific reagents • Lack of metabolite information
	LC-MS [51,62,63]	• Generally acceptable sensitivity • Excellent specificity, precision and accuracy	• difficult to simultaneously detect two functional domains

probe with biotin on one end and a detection labeled on the other end. This approach requires great efforts in labeling and purifying the dual labeled molecules. Dual probe hybridization assays detect the drug using two separated sequences both complimentary to the drug with biotin and detection tag labelled, respectively. As each complementary sequence needs sufficient base pairs for effectively and specifically binding, this approach may not be applicable to drugs with a short sequence.

One merit of the hybridization LBA is the high throughput made possible by excluding a purification step, providing the ability to run hundreds of samples per day. LBA equipped with electrochemiluminescence detection offers a high degree of sensitivity with Lower Limits of Quantification (LLOQ) as low as 10 pg/mL. However, hybridization LBA has poor selectivity for the intact drug, especially when dealing with large metabolites of the intended target. Contrary to LBA, LC-MS has been demonstrated to provide excellent selectivity, but LC-MS does not offer the same degree of assay sensitivity. In addition, the LC-MS methods are of low throughput due to the long and sophisticated sample extraction procedures. Therefore LC-MS methods are less suitable to support late phase clinical trials where the sample count is significant.

Protein

Protein-based therapeutics, including monoclonal antibodies (mAB), hormones, enzymes, transporters, immune defenders, and receptors are very common drugs for medical treatment. The increasing demand of protein therapeutics necessitates the development of bioanalytical techniques to support the drug development process. LBA and LC-MS methods have both been used for bioanalysis of protein drugs (10, 25, 27 and 30). Both methods provide decent assay sensitivity, assay selectivity and specificity. The method is often selected based on the study purpose (free or total drug), availability of reagents and timeline.

Antibody-Drug Conjugates

Antibody-Drug Conjugates (ADC) are one of the most recent additions to the therapeutics class and have demonstrated potential in treating cancer [38]. The ideal cancer treatment only targets the cancer cells with toxin, leaving the normal cells unharmed. An ADC generally has these 3 components: a mAb, a cytotoxic payload, and a chemical linker. The mAb is a highly selective agent, which allows the ADC to target only cancerous cells. The cytotoxic payload is a highly toxic drug, which can damage or kill the targeted cells. The combination of these two components, through conjugation via a linker, produces a highly selective therapeutic. Structural complexity and inherent heterogeneity of ADCs create additional challenges for bioanalysis. Both LBA and LC-MS methods have been used for ADC bioanalysis [39]. Investigating the metabolism and disposition of ADC and interpreting the relationship of exposure-efficacy and exposure-safety in the context of their various catabolites is important in designing and subsequently developing clinically successful ADC. For bioanalytical analysis of ADC, the assay should be able to measure intact ADC, total antibody, payload and relevant metabolites.

Both LBA and LC-MS methods have been developed and extensively employed to perform quantitative analysis of ADCs in biological matrices [40]. LBAs, relying on capture and detection reagents to bind to the different components of ADC, have been widely used for measuring intact ADC and total antibody. LBA, however, has a very limited capability for payload quantification as the payload is typically a small molecule compound and LBA reagents for such compounds are lacking. On the other hand, LC-MS is well accepted as method for the accurate and precise quantification of ADCs for bioanalysis. In addition, LC-MS can measure the payload. By sequencing signature peptides, LC-MS is capable of measuring both payload and mAb moieties [41]. The incapability of high assay sensitivity limits its application in certain studies.

Bispecific antibodies

Recently the development of bispecific antibodies as therapeutic agents has showed clinical potential for disease intervention. Unlike regular

antibody therapeutics, bispecifics are recombinant antibodies that can simultaneously recognize two different antigens [42].

Both LBA and LC-MS have been employed to quantitate bispecific antibodies. In LBA, the capture and detection reagents are designed to bind to the different binding arms of a bispecific antibody. This ensures that the assay signal is coming from the intact molecule, rather than from its metabolites. LC-MS is designed to bind one arm of a bispecific Ab and select signature peptides specific for the other arm. The assay development should consider the study need, availability of reagents and the selection of signature peptide (Table 1).

Conclusion

The complexity and diversity of large-molecule therapeutic agents under development require an optimized use of bioanalytical assay platforms to support the pharmacokinetic studies from early discovery to preclinical and clinical phases. In this paper, both LBA and LC-MS, the two major bioanalytical assay platforms, have been reviewed and their strengths and limitations comparatively summarized in the table above. The key assay parameters have been discussed and recommendations have been given on both platforms in the context of different types of therapeutic molecules.

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