LIGAND-BINDING ASSAYS

DEVELOPMENT, VALIDATION, AND IMPLEMENTATION IN THE DRUG DEVELOPMENT ARENA

Edited by

Masood N. Khan
John W.A. Findlay

A JOHN WILEY & SONS, INC., PUBLICATION

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PREFACE

Ligand-binding assays in the form of radioimmunoassay were first developed and applied to protein analysis about 50 years ago. This new technique was sensitive and was also characterized by the unique specificity conferred by the key antibody binding reagent. Since the development of this radioimmunoassay for insulin, ligand-binding assay methodology has undergone many cycles of evolution, primarily along two lines. The first of these is concerned with the technology for detection of the end point of the ligand-binding assay. Progress in this area included detection by enzyme activity assays, such as in enzyme-linked immunosorbent assays (ELISAs), by fluorescence and luminescence end points in many formats (including, most recently, electrochemiluminescence), by surface plasmon resonance, and by immuno-polymerase chain reaction (IPCR). All of these approaches allow the user to be relieved of the hazards of working with radioactivity and the burden of radioactive waste disposal, while maintaining or improving on the sensitivity of radioactivity-based ligand-binding assays. The other line of progress relates to the key binding reagent employed in the assay. Polyclonal antibodies were initially, and are still, widely used in this role. However, the development of monoclonal antibody technology offered reagents of greater specificity (although sometimes of somewhat lower affinity). Receptors and transport proteins have also been used in these assays, but more intriguing has been the work done on molecularly imprinted polymers, also known as "synthetic antibodies" which offers the prospect of unlimited supply of a single batch of binding reagent for use in a large number of assays.

Although ligand-binding assays continue to be applied to the bioanalysis of some classes of low molecular weight molecules, such as some agricultural chemicals, screening of drugs of abuse, and clinical chemistry applications, the continued vitality of ligand-binding assays has been ensured by the burgeoning interest in research and development of biological macromolecules as potential therapeutics. A large number of macromolecules are currently in clinical evaluation in a number of therapeutic areas, with a major emphasis on oncology. Many of these drug candidates are proteins, such as monoclonal antibodies and fusion proteins, but they also include other macromolecules such as antisense and other oligonucleotides. The bioanalysis of such molecules in complex matrices, such as plasma and serum, can not yet be performed by the powerful mass spectrometry based methods that now dominate the bioanalysis of low molecular weight xenobiotics, and ligand-binding assays remain the cornerstone of support for pharmacokinetic and toxicokinetic studies of macromolecules. Until advances in mass spectrometry based methods permit their typically

high specificity and sensitivity to be applied to analyses of macromolecules in complex matrices, ligand-based assays will remain the methods of choice for the bioanalysis of biological macromolecules.

The idea for this book arose from conversations between the editors from time to time after they chaired a roundtable session on challenges in immunoassay validation at the 1998 AAPS Annual Meeting in San Francisco. Validation of bioanalytical methods in general had been a topic of increasing attention over the last decade, with the first Crystal City meeting in 1990 as a milestone. Ten years later, the second Crystal City meeting in 2000 reviewed progress in bioanalytical method validation and a separate meeting was also held on macromolecule bioanalysis. Finally, a third Crystal City meeting in 2006 addressed validation of chromatography-based and ligand-binding assays jointly, as well as in separate sessions. In early 2000 we, along with a number of coauthors, separately published a discussion of validation approaches for ligand-binding assays that provided a framework for the subsequent discussions and workshops.

Reflecting on all of these activities, we felt that there was no text currently available that presented the range of activities related to the development, validation, and implementation of ligand-binding assays in the context of drug development in a truly practical sense. We felt that such a volume would be of great help to practitioners in varied fields, both in the pharmaceutical and allied industries and in related contract research organizations and academic laboratories, and perhaps even in the field of diagnostics and clinical chemistry. To this end, we enrolled a group of authors highly skilled and experienced in the development and implementation of ligand-binding assays to contribute to this book.

The contents of the book cover a wide range of topics related to development, validation, and implementation of ligand-binding assays in the development of drug candidates. These include general discussion of challenges and proven approaches in the development of ligand-binding assays, as well as more detailed examination of characteristics of these assays when applied in support of pharmacokinetic and toxicokinetic studies of compounds at different stages in the discovery or development timeline. A concise, but detailed, discussion of validation of ligand-binding assays for macromolecules is included, as is a practical approach to "fit-for-purpose" validation of assays for biomarkers, those molecules receiving increased attention as potentially demonstrating that the target chosen in Discovery is being modulated by the candidate therapeutic, both in nonclinical and clinical studies. As expected, calibration curves and assay performance are supported by statistical treatments, and this topic is discussed in depth, covering both European and North American perspectives. Deployment of assays in high-throughput format requires automation of ligand-binding assays and, particularly in the current environment, internal capabilities are often reduced, accompanied by increased outsourcing of bioanalytical work. The critical aspects of both of these topics, including successful transfer of assays to a contract research organization, are reviewed in depth. As in most fields of science, technologies related to ligand-binding assays continue to advance. Consequently, newer and emerging ligand-binding assay technologies are reviewed. Given the continuing interest in the question of whether the efficacy or safety of a biological

macromolecule may be compromised by antibodies elicited in response to treatment, assays for the detection and characterization of such antidrug antibodies in animals or humans (immunogenicity assays) are discussed in detail. In light of the critical role of calibration standards in ligand-binding assays, the importance of characterized reference standards in interpreting assay results is discussed. At various stages in the drug discovery and development process, some analyses may be conducted by the use of commercially available assay kits; some of the challenges in qualification or validation and implementation of such assay kits are presented. Finally, the importance of proper documentation of experimental bioanalytical findings in assay validation or study reports from a regulatory viewpoint is emphasized.

The editors are grateful for the energy and expertise of all of our contributors to this book. We trust that basic and applied research scientists will find this book valuable in further understanding and expanding the successful application of ligand-binding assays to bioanalysis of a variety of molecules in complex biological media.

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CHAPTER 1

Ligand-Binding Assays in Drug Development: Introduction and Historical Perspective

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1.1 GENERAL

A ligand-binding assay (LBA) may be defined as an assay in which the key step is an equilibrium reaction between the ligand (analyte) and a binding molecule, most often a protein and, in many cases, a specific antibody or receptor directed against the ligand of interest. This reaction is governed by the law of mass action. The end point of the reaction reflects, either directly or inversely (depending on whether the assay format is competitive or noncompetitive), the concentration of the analyte present in the sample. Although this approach may be applied in a qualitative sense, ligand-binding assays are generally implemented as sensitive, quantitative analytical methods. These assays cover a broad scope. Binding molecules may include antibodies or antibody fragments, receptors, transport proteins, or oligonucleotides such as aptamers or spiegelmers. Detection and quantitation of the reaction end point may involve one of many technologies, including radioactivity or enzyme activity producing UV/visible-absorbing, fluorescent, luminescent, or chemiluminescent products. Ligand-binding assay formats may be competitive or noncompetitive, with solution- or solid-phase configurations.

This chapter will provide a brief history of the development of ligand-binding assays and their increasing and changing applicability to the determination of various types of molecules to provide context for more detailed discussions in subsequent

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chapters. The formation of the Ligand-Binding Assay Bioanalytical Focus Group (LBABFG), sponsored by the American Association of Pharmaceutical Scientists (AAPS), has resulted in active promotion of consistent approaches to the development, validation, and implementation of ligand-binding assays for macromolecule therapeutics. The origins, activities, and numerous contributions of the LBABFG to the field of LBAs are also reviewed in this chapter.

1.2 HISTORICAL REVIEW

Approximately 50 years have passed since Yalow and Berson reported on the binding interaction between insulin and an insulin-binding antibody [1], followed by the first development and application of a ligand-binding assay in the form of a radioimmunoassay (RIA) for insulin [2]. This development marked a major advance in the sensitive and specific measurement of protein hormones in blood-based fluids, taking advantage of the competition between a limited mass of radiolabeled insulin and increasing concentrations of unlabeled insulin for insulin-binding sites on a limited amount of anti-insulin antibody molecules. The greater the amount of unlabeled insulin in the system, the smaller the amount of radiolabeled insulin detected in the antigen–antibody complex; this relationship between antibody-bound radiolabeled and unlabeled insulin concentrations formed the basis of the insulin assay calibration curve, from which insulin concentrations in study samples could be interpolated. The work of Yalow and Berson, who were subsequently awarded the Nobel Prize for Medicine, ushered in an era of widespread development and application of the new immunoassay methodology to many biomedical fields. Despite a subsequent general movement away from the use of radioactivity as an end point detection technology, application of RIA technology in several formats, such as solution-phase, solid-phase, competitive, and immunoradiometric assays, has continued to the present day due to case-specific advantages such as high sensitivity for the analyte compared to alternative immunoassay formats. The work of Landsteiner in 1945 [3] demonstrated that immunization of animals with hapten–protein conjugates, in addition to producing antibodies to the carrier protein, elicited antibodies in the animal against the small molecule hapten. This observation provided the foundation for the subsequent broad application of immunoassay technology to analysis of low molecular weight drugs and other small molecules. Thus, in the same time period as Yalow and Berson reported on assay applications for anti-insulin antibodies, Beiser and coworkers demonstrated that immunization with steroid–protein conjugates resulted in production of antisteroid antibodies [4], which led to widespread applications of radioimmunoassay in the field of steroid biochemistry. Several years passed before these assays were applied to the analysis of therapeutic drugs, initially for the quantitation of digitoxin [5] in biological fluids. Due to the advantages of high sensitivity, relatively good specificity, and high-throughput capabilities, there were extensive and rapid applications of immunoassay to drug analyses, as chronicled in several review articles [6–8]. The impressive potential specificity of antibodies, even in the recognition of very limited, defined molecular structural variations, was illustrated in the stereoselectivity of antibodies to, and immunoassays for, chiral small drug molecules, as reviewed by Got and

Schermann [9]. The other important applications of immunoassay have been in the determination of small molecules in agricultural applications for pesticide analysis [10], in the support of the development of genetically modified crops [11], and in the screening of drugs of abuse [12]. In the latter application, an immunoassay is typically used as a screening approach, often followed by specific identification of the drug involved and quantitation by liquid chromatography/mass spectrometry (LC/MS). Wu [13] and Lepage and Albert [14] have reviewed the major impact of ligand-binding assays, initially in manual and, more recently, automated formats, on rapid progress in the clinical and endocrinology laboratory environments, respectively. In the pharmaceutical industry, the application of immunoassay to determination of small molecules in biological matrices has declined sharply in recent years, being largely surpassed by the advent of highly sensitive and specific LC/MS-based analytical methods [15].

An ongoing migration to the use of nonradioactive immunoassay reaction end point detection started with the introduction of the solid-phase enzyme-linked immunosorbent assay (ELISA) by Engvall and Perlmann [16] and van Weemen and Schuurs [17] and the homogeneous enzyme-multiplied immunoassay technique (EMIT, [18]). Subsequently, nonisotopic detection technology applications such as time-resolved fluorescence [19], electrochemiluminescence [20], and other newer techniques were introduced, as discussed later in this book.

1.3 LBAs FOR MACROMOLECULES

The primary reason for the continued strong interest in ligand-binding assay development and application in the drug development arena is the recent upsurge in interest in the therapeutic potential and promise of various classes of biological macromolecules. Recent years have seen a remarkable increase in research and development of proteins, oligonucleotides (as aptamers and antisense compounds), and other macromolecular therapeutic drug candidates [21]. Macromolecules are in various stages of development in a wide range of therapeutic areas, most notably in oncology, buoyed by recent major clinical and commercial successes of a broad range of biotechnology products [22]. Although applications of various modes of mass spectrometry continue to be evaluated, mass spectrometric assays have not yet evolved to the point required for routine determination of proteins and other macromolecules in complex biological matrices, such as plasma. Thus, immunoassays and other binding assays (e.g., hybridization assays for quantitation of oligonucleotides) remain the mainstay techniques for the determination of these molecules due to their ability to detect macromolecules with high sensitivity and adequate specificity in highly complex biological matrices generally without separation of the analyte of interest prior to assay. Due to the increasing impact of biological macromolecules on therapy of many diseases, development and validation of LBAs for these have received increasing attention. Several workshops [23–26] and review articles [27–29] have discussed validation of these assays in considerable detail. With the increased research and development interest in macromolecules as potential therapeutics has come the need to monitor potential immune response to these large molecules, a situation that is generally rare with low molecular weight drugs. Detection of immunogenicity is

important, since an antidrug antibody response to the potential therapeutic may result in loss of efficacy if the elicited antibodies neutralize the intended beneficial activity of the candidate therapeutic. In this situation, safety is also an important consideration, as exemplified by the case of antibodies elicited to recombinant erythropoietin, which neutralized the effects of the endogenous erythropoietin, resulting in the development of life-threatening pure red cell aplasia [30]. This need has generated a new class of LBAs related to detection of immunogenicity of macromolecular drugs, a topic discussed in detail later in this book.

1.4 ADVANTAGES AND LIMITATIONS OF LBAs

As will be discussed in detail in subsequent chapters, LBAs offer a number of advantages over other bioanalytical methods. Due to the high affinity of the key binding reagent, these assays generally have high sensitivity for the analyte, translating into low limits of quantitation (LLOQ). Thus, some immunoassays have been reported to have zeptomolar sensitivities. Depending on the specificity of the binding protein, LBAs can be highly specific for the analytes of interest. This is particularly true when the binding protein is an antibody carefully crafted against the analyte of interest, as exemplified by the stereospecificity of some small-molecule immunoassays. In such cases, the selectivity of the assay for the analyte of interest can be defined more clearly, due to extensive, accumulated knowledge of analyte metabolism and clearer knowledge of metabolite structures than is typically the case for macromolecule assays, where specific steps in biotransformation are largely unknown. Thus, in the former case, cross-reactivity and potential interference of available synthetic metabolite standards in the assay can be evaluated directly. Although the general pathways of protein anabolism by successive peptide bond hydrolysis leading, eventually, to amino acids are well known, the identity of the intermediary products (catabolites) generally remains unknown. Therefore, in the case of many macromolecule LBAs, the lack of this detailed knowledge means that caution is warranted in interpretation of generated data, since these data will reflect the interaction of all molecular species with affinity for the binding reagent used in the assay.

1.5 LIGAND-BINDING ASSAY BIOANALYTICAL FOCUS GROUP OF AAPS

In view of the increasing use of LBAs to support drug development programs, the inherent complexities of the technology, and the lack of specific regulatory guidance in this area, a group of interested scientists formed the Ligand-Binding Bioanalytical Focus Group of AAPS in 2000. The primary objectives of this group include addressing technical and regulatory issues related to LBAs, making genuine and concerted efforts to reach consensus on issues of general interest, publishing white papers, and providing continuing education and a platform for continuous exchange of ideas related to ligand-binding assays. It is, therefore, of historical interest to

summarize here the sequence of events occurring before 2000 that led to the establishment of the LBABFG.

It is well known that, for years, the proceedings of the 1990 conference on bioanalytical method validation to support bioavailability, bioequivalence, and pharmacokinetic studies served as a *de facto* guidance for bioanalytical method validation in the pharmaceutical industry [23]. Although this report briefly acknowledged the need for some special considerations for nonchromatographic assays, including LBAs and microbiological assays, there was much to be addressed. In 1994, at the Bio-International-2 meeting in Munich, Germany, one of us (J.F.) brought the issues of LBA challenges to the public platform [31]. Around that period, the other of us (M.K.), while establishing an immunoanalytical laboratory at a then emerging contract research organization (CRO) (Phoenix International Life Science (PILS)) in Montreal, had experienced firsthand the difficulties encountered in validating and implementing LBAs in a GLP-compliant environment, and also had the opportunity to address the general deficiencies in approaches to handling and computing LBA-related parameters then practiced across the industry. LBA practitioners in the industry were trying to apply the same acceptance criteria and methods for the computation of validation parameters as were being used for chromatographic assay validation. For instance, computation procedures being used for between-run and within-run variability were particularly inappropriate for application to LBAs.

Typically, within-assay (intra-assay) evaluation was done by analyzing a large number (e.g., 10) of samples at each level of control in a single run. The standard deviation (SD) and percent coefficient of variation (%CV) would be calculated and used as measure of intra-assay (within-run) precision. On the other hand, for between-run variability computation, each control sample in duplicate would be assayed in a minimum of five independent assays and the data would be used to compute the inter-assay precision. However, due to the relatively large imprecision associated with LBAs, one may observe a small intra-assay CV (e.g., 5.2%) one day and a relatively large CV (e.g., 25%) the other day for the same assay, indicating that the estimation of intra-assay precision based on just one run may not depict the true picture. Therefore, it was thought that to obtain better estimates of within-run CV, one could analyze replicate controls (e.g., five aliquots of a control sample in duplicate) in multiple runs (e.g., five runs) preferably on different days. Using the data thus generated, pooled SD and corresponding CV could be calculated. Pooled intra-assay SD is the square root of the pooled intra-assay sample variance, which is just the arithmetic mean of the squared intra-assay standard deviations. Hence, the pooled SD and the corresponding CV should give a more realistic estimate of within-assay precision [32]. In this approach, the same data can also be used for the computation of between-run variability, obviating the need to perform a separate set of experiments. In collaboration with Bob Parks (a statistician colleague at PILS), an Excel worksheet was designed for the computation of pooled statistics for inter- and intra-assay precision and accuracy. This, essentially, marked the beginning of a different and more appropriate computation of the critical validation parameter of assay precision. This Excel worksheet provided a very useful and convenient tool to perform complex statistical calculations even by those lab analysts who were not well versed in statistics. Moreover, this could also be

used to harmonize inter-laboratory practices for computation of accuracy and precision parameters for an easy comparison of interlaboratory performances of the method.

At the same time, accessory calibrators, outside the dynamic range of quantification, that were used to optimize the curve fit were being implemented in routine use at PILS. These accessory calibrators were also referred to by Findlay as "anchor points" [31]. Much of the work done with immunoassays till that time had been routinely conducted with data processing algorithms that attempted to linearize the calibration curve response–analyte concentration relationship. However, it is well recognized that LBA calibration curves are inherently nonlinear and best described by four-parameter or five-parameter logistic algorithms. Inclusion of high- and low-concentration anchor points beyond the declared range of the calibration curve often improves the fit of the calibration curve to these models, resulting in overall improved assay performance and data quality [31].

Arising from the uncertainty about optimal approaches to LBA validation, M.K. had in-depth discussions in 1997 with several leading LBA practitioners in the industry, in particular with Ron Bowsher (then at Eli Lilly), the late John Gilbert (then at Merck), and Nancy Hopkins (then at Pharmacia & Upjohn) related to the urgent need for a public platform to discuss and exchange ideas on the challenges faced in the LBA arena. In 1998, the current authors organized a roundtable on the topic of "Challenges of Immunoassay Validation and Implementation for GLP Studies" at the AAPS Annual Meeting in San Francisco. Perhaps, this was the first time LBA-specific challenges were discussed at length on a public platform [25,27,33]. Following the roundtable, in collaboration with the speakers at the event (Fig. 1.1), we published a

FIGURE 1.1 (From left to right) *First row*: Binodh DeSilva (P&G), Masood Khan (MedImmune), Ira Das (Searle), Jean Lee (MDS), Wendell Smith (Eli Lilly), and Ronald Bowsher (Eli Lilly); *second row*: Gerald Nordblom (Parke-Davis) and John Findlay (Searle).

FIGURE 1.2 (From left to right) *First row*: Jeffrey Sailstad (GSK), Masood Khan (MedImmune), Jean Lee (MDS), Marian Kelley (J&J), Binodh DeSilva (P&G); *second row*: Ronald Bowsher (Eli Lilly), John Ferbas (Amgen), Russell Weiner (BMS), and Richard Tacey (PPD).

white paper on the industry perspective on LBA validation in 2000 [27]. This white paper directly influenced the discussions on LBA issues at the AAPS- and FDA-sponsored workshop on Bioanalytical Method Validation for Macromolecules, held in Crystal City, VA, in 2000. Later in 2000, a day before the 2000 AAPS Annual Meeting in Indianapolis, Ron Bowsher and his wife, Penny, graciously hosted a notable gathering of some very enthusiastic and concerned LBA scientists from the industry (Fig. 1.2). At this meeting, we discussed and formulated the basic framework and future directions for the focus group that was to be formally inaugurated at the AAPS Annual Meeting. Later, in a very lively e-mail contest, the name Ligand-Binding Assay Bioanalytical Focus Group was chosen for the newly formed focus group. The primary mandate of this focus group is to provide a forum to address LBA-specific issues and promote education on the bioanalysis of a broad range of analytes using this technology.

The LBABFG is an independent entity operating within the BIOTEC section of AAPS. It has an online newsletter and has also created an online forum for public discussion of LBA-related issues at the AAPS Web site. Over the past 8 years, the focus group has played a key role in organizing short courses, roundtables, symposia, and hot topic discussions and in increasing the LBA-related content in overall programming at the AAPS national meetings and workshops. In 2003, one of us (M.K.) organized the first short course on "GLP-Compliant Validation of Ligand-Binding Assays: A Practical and Rational Approach," at the AAPS Annual Meeting in Salt Lake City, Utah. Faculty members of this short course included Howard Hill, Jean Lee, Jeffrey Sailstad, Tony Mire-Sluis, and Wendell Smith. This short course was audio recorded for the AAPS Continuing Education Series. Three years later, in 2006, Ron Bowsher and colleagues redesigned the course as a 2-day course that has been offered every year

since then. Faculty members of this course included Binodh DeSilva, Bonita Rup, Jeffrey Sailstad, Marie Rock, Ronald Bowsher, Viswanath Devanarayan, and Wendell Smith. LBABFG continues to provide training and developmental tools to the practitioners of ligand-binding assay technology.

Since the inception of the focus group, several subcommittees were formed that addressed unique issues related to the application of LBAs in various disciplines. These committees have published white papers related to the validation of LBAs for quantitative drug analysis [28], "fit-for-purpose" method validation of biomarkers [29], antidrug antibody assay method validation [34], and validation of assays for neutralizing antidrug antibodies [35].

1.6 SCOPE OF THE PRESENT VOLUME

Despite the current extensive work on the development of biologic macromolecules as potential therapeutics, there does not appear to be a broad-based text collating experience with LBAs, which are still the bioanalytical methods of choice for supporting the nonclinical and clinical development of these compounds. The editors hope that this book will prove a useful reference text for approaches to the development, validation, implementation, and documentation of LBAs in the context of drug development to personnel from laboratory analysts to managers in the pharmaceutical and biotechnology, contract research, diagnostic and related industries, as well as in academic and hospital laboratories. The scope of the book is broad, and practical situations and examples of challenges likely to be encountered are presented.

In Chapter 2, the authors review the characteristics of ligand-binding assays intended for supporting the pharmacokinetic or toxicokinetic studies of biological macromolecules. These are put in the context of a discussion of the differences in assay methods for small-molecule xenobiotics and macromolecules, as well as the marked differences in the pharmacokinetic disposition of these two general categories of drugs. Pharmacokinetics of low molecular weight xenobiotic drugs is generally more clearly defined than in the case of macromolecules. In the former case, metabolite pathways are relatively easily defined and specificity of LBAs for these compounds is more readily identifiable. Protein macromolecules are catabolized by peptide hydrolytic pathways, with the possibility of unidentified intermediate products that may interfere with the LBA by cross-reactivity with key binding reagents. The role of the Brambell receptor in the clearance of IgG-based antibody drugs is also discussed. This chapter describes the requirements for a successful LBA for supporting pharmacokinetic studies in the context of these complexities. The chapter also addresses critical ligand-binding assay considerations at different stages of the research and development process.

To have an assay to validate, a robust assay must be developed. Chapter 3 discusses, in a systematic and pragmatic way, the approaches to the development of LBAs. This discussion focuses on the practical aspects of LBA development for use in the GLP-compliant environment. A structured strategy for the development of a validatable LBA that would withstand the rigor of prestudy and in-study method validation

processes is presented. The application of the analyst's tools and the assay components are reviewed in depth, and the optimization of the assay is also discussed. In the latter section of the chapter, all major steps for successful optimization of the assay are discussed, including evaluation of such characteristics as specificity and selectivity, plate coating for multiwell assays, and evaluation of any prozone effects. LBAs "act up" in practice as often, or even more frequently than, do chromatographic assays. Chapter 3 also provides an in-depth discussion on troubleshooting LBAs.

Validation of bioanalytical assays in general and LBAs in particular has been the subject of intensive debate for the past 18 years or more. Chapter 4 focuses on the key agreements on a phased approach to the validation of LBAs, including evaluation of all critical validation parameters prior to implementation of the method to the analysis of any study samples (prestudy validation) as well as in-study validation to assure high performance of the assay during analysis of actual study samples. Also covered in this chapter are the topics of when and how to conduct full validations, partial validations, and cross-validation.

Chapter 5 discusses in depth the statistical considerations related to LBA development and validation. In addition to the most appropriate algorithms for describing the nonlinear calibration curves typically found in LBAs, the authors also provide further insight into the performance characteristics to be evaluated during assay validation, including the concepts of total error in prestudy validation and the use of the "4-6-X rule." The decision rules at the prestudy validation and routine assay implementation stages are also discussed in some detail in Chapter 5.

Identification of biomarkers for animal models of diseases has become crucial in demonstrating the proposed mechanisms of pharmacological action preclinically, in making decisions on whether to allow the progress of a compound from discovery to development, and in providing mechanistic data in support of the desired beneficial effect of the agent in clinical trials in the disease population. Demonstration of the desired effect on a biomarker of disease in early clinical trials is important to the development of the molecule in clinical testing or to the decision to terminate the development prior to large investments in pivotal trials that may fail to demonstrate the desired efficacy in disease patient populations. Chapter 6 addresses the development and validation of assays for these biomarkers, including the considerations in the development and validation of LBAs for biomarkers. The subcategories of biomarker assays (definitive quantitative, relative quantitative, quasi quantitative, and qualitative) are discussed in terms of the degree to which they may be characterized with calibration standards of defined content and the degree to which they may be validated as quantitative assays. The concept of an LBA being "fit for purpose" is also discussed.

A significant amount of LBA work, particularly in diagnostics and the burgeoning field of biomarker assays, employs LBAs in the form of manufacturer's kits. Chapter 7 focuses on considerations for the correct application of these kit assays, including the proper level of validation needed to support specific applications.

Treatment of animals or humans with macromolecular drug candidates may result in an immune response to the macromolecule, most frequently happening upon repeated dosing. This is most often seen with protein molecules, but experience with other macromolecules, such as oligonucleotides, may not yet be sufficient to be

conclusive about the potential immunogenicity of these molecules. It is important to reliably demonstrate the presence or absence of these antidrug antibodies and characterize those detected, because of their potential to antagonize the beneficial therapeutic effects of the macromolecule or, in some cases, to cross-react with endogenous analogues of the new therapeutic. Since the latter situation has been shown to result in serious toxicity in some cases, it is critical to develop assays to reliably detect these antibodies. Chapter 8 deals in detail with the assays for the detection and characterization of antibodies elicited in response to treatment with biological macromolecules as therapeutics or therapeutic candidates. Both LBA and supporting competitive neutralizing and cell-based neutralizing assays are discussed in detail.

Reference standards for macromolecule LBAs are inherently heterogeneous and complex, in contrast to the homogeneity of low molecular weight drugs. This subject is elaborated in Chapter 9, accompanied by a number of illustrative examples. Characterization of USP and non-USP standards is discussed in this chapter in terms of such parameters as purity, potency, and stability. Case studies illustrate the assay effects of variability in reference standard quality.

Outsourcing of bioanalytical work to CROs is widely practiced in the pharmaceutical industry and, indeed, has grown markedly in recent years. The work at the CRO may involve *de novo* assay development, validation, and implementation on behalf of the client company or, more frequently, may involve transfer of an existing assay from a client, followed by its validation and implementation. Transfer of assays between clients and CROs is a challenging process but, because of the current extensive outsourcing of bioanalytical work by pharmaceutical and biotechnology companies, is vital for the continuity of many preclinical and clinical development programs. The challenges involved in the interaction between sponsors and CROs and recommendations for success in this exercise are presented in Chapter 10.

One of the significant advantages of LBAs is their potential for high-throughput sample analysis. To take full advantage of this potential, LBAs may be coupled with automated sample processing and assay systems. Automation of LBAs brings a fresh set of challenges that are discussed in Chapter 11. Logical phased implementation of automation projects is discussed, as are specific automated instrumentation units, including full robotic systems.

In a regulated environment, proper documentation to support both data and reports is of paramount importance; responsibilities for documentation and appropriate documentation practices to support the validation and implementation of LBAs in this regulatory environment are discussed in Chapter 12.

Assay methods, formats, and technologies continue to change, with some newer LBA technologies possessing remarkable sensitivities. Another feature of newer assays is the attempt to miniaturize and automate assays, leading toward the goal of an assay on a chip or compact disk. Chapter 13 discusses some of these newer assay methods (including DELFIA, ELISPOT, immuno-PCR, ECLIA, hybridization-ELISA, SPR, applications of molecularly imprinted polymers, and coupled chromatography–LBA methods). These authors also offer a glimpse of possible future directions for LBA development.

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