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Review

Some important considerations for validation of ligand-binding assays☆

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ABSTRACT

Calibration curves for ligand-binding assays (LBAs) are inherently non-linear and standard curve fitting algorithms require careful selection. Reference standards for macromolecule LBAs are more complex than are low-molecular-weight reference standards. Specificity of small molecule LBAs, and accuracy of reported study sample data are easier to assess by cross-validation with a chromatographic method than for macromolecule LBAs. Due to the lack of knowledge of the potential interference of unknown products of catabolism, proteolysis or biotransformation of macromolecules (particularly proteins) in LBAs for the parent molecule, the accuracy of reported concentrations and derived pharmacokinetic data for macromolecules, as determined by LBA, should be viewed with caution. In validation of LBAs, the total error and confidence interval approaches to assessment of the acceptability of an assay for routine implementation for the desired purpose should be given due consideration.

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1. Introduction

The number of biological macromolecules under development by the pharmaceutical industry for a variety of potential indications, particularly in oncology, has increased markedly in recent years [1]. This diverse group of molecules includes recombinant and fusion proteins, monoclonal antibodies and oligonucleotides, such as anti-sense molecules, toll-like receptor-active agents, aptamers and spiegelmers. This interest has been fostered and focused by the major clinical therapeutic and commercial successes of a number of these macromolecules, especially in oncology and anti-arthritic/anti-inflammatory indications [2]. Interest in macromolecular therapeutics has also been fueled by interest in the development and marketing of "generic protein" products, also referred to as "similar biological medicinal products", "biosimilar protein products" or "follow-on protein products" in Europe and the United States. Recent approvals of recombinant proteins such as human growth hormone as a follow-on product in Europe and the United States, following patent expiration for the innovator product, highlight the market forces at work in this area. Interestingly, LBAs are also widely used for determination of genetically modified proteins in agricultural biotechnology [3].

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Given the current limitations of mass spectrometry-based methods for quantitation of biological macromolecules in complex matrices such as serum and plasma, LBAs remain the mainstay quantitative bioanalytical method for support of pharmacokinetic studies of such molecules. Despite the current, almost universal, bioanalytical support of low-molecular-weight xenobiotic drug development by liquid chromatography/mass spectrometric (LC/MS) methods in the pharmaceutical industry, LBAs are still widely used for bioanalysis of low-molecular-weight compounds in clinical chemistry/diagnostics, agricultural chemistry and drugs of abuse screening. Validation of chromatography-based and LBA bioanalytical methods applied to the support of regulatory submissions has been the subject of particular discussion for more than 15 years. General guidance on bioanalytical method validation was provided at a 1990 workshop sponsored by AAPS, FDA and other organizations [4], and several workshops and conferences have followed that initial conference. In 2000, separate workshops addressed the broad topics of validation of bioanalytical methods [5] and specifically validation of bioanalytical methods for macromolecules [6]. In the midst of this activity the United States Food and Drug Administration (FDA) issued a guidance document on bioanalytical method validation that was intended to cover assays for low-molecular-weight molecules and macromolecules [7]. Opinions that current practices for both classes of assays needed to be reviewed in the context of the FDA guidance led to the most recent (3rd) AAPS/FDA workshop on this topic, which was held in 2006, with a stated purpose of "to identify, review and evaluate existing practices, white papers, and articles and clarify the FDA Guidance" [8]. An AAPS-sponsored workshop was also held on the closely related topic of appropriate validation of bioanalytical methods for biomarkers [9], many of which are LBAs. Some publications have also addressed the development and validation of chromatographic bioanalytical methods [10,11], and of LBAs for small molecules, biological macromolecules and biomarkers [12–14]. Several of these publications have advanced the notion of "fit-for-purpose" as a consideration in deciding on the extent of validation needed for support of a particular assay application—exploratory studies requiring less assay validation support and definitive, late-stage studies needing more extensive validation support.

From these discussions several key issues relevant to validation of LBAs emerged, some of which are common to these assays as applied to both small molecules and macromolecules, and some of which highlight differences in validation considerations for LBAs for these two classes of molecules. This review will discuss some of these issues and attempt to provide some focus on their relative importance.

2. Issues common to the validation of LBAs for small molecules and macromolecules

2.1. Non-linear calibration curves

Chromatography-based assays, such as LC/MS methods, are normally characterized by linear calibration curves, often with a weighting function applied, and extend over wide, validated concentration ranges. In contrast, LBAs generally have a much narrower validated concentration range, often only covering two decades of concentration units, and calibration curves are typically non-linear. The signal response in chromatography-based assays is direct and a function of the physicochemical parameters of the molecule being quantitated. For LBAs, the response is not directly related to physicochemical properties of the molecule, but typically involves detection of the endpoint of a reaction between a binding reagent (often an antibody) and the analyte of interest. The response may be directly or indirectly related to the analyte concentration in the sample under analysis. In these assays, the concentration–response relationship is inherently non-linear and heteroscedastic, that is, the variance of the response varies across the concentration range. Several algorithms are available for fitting LBA calibration curve data, as discussed by Rodbard and Frazier [15], Haven et al. [16] and Dudley et al. [17]. The basis of all of these data reduction models is an equation that describes the mean concentration–response relationship, in conjunction with another that describes the relationship between the mean response and the variance of replicate measurements. For LBA assays caution is required in the selection and application of such models, as has been recently emphasized by Findlay and Dillard [18]. Several algorithms which, until relatively recently, were still commonly used to fit LBA concentration–response data (e.g., log-linear and log–log), attempt to convert the inherently sigmoidal response–log concentration relationship of LBA data into a linear relationship while others, such as exponential and quadratic models, attempt, with varying degrees of success, to fit portions of the curvilinear LBA calibration curve. Preferred algorithms for fitting LBA calibration curve data are 4-parameter logistic or, if the curve exhibits asymmetry, 5-parameter logistic models, with appropriate weighting to take into account any observed heteroscedasticity. It is important that LBA calibration curve algorithms are selected based on the goodness of fit of the actual data to the model across the entire validated concentration range of the assay, using the relative error of individual concentrations read back from the calibration curve as a measure of goodness of fit [12,13]. Several of the fitting algorithms available as potential alternatives to 4- or 5-PL algorithms may provide a reasonable fit for different portions of the calibration curve, but generally require marked compromises of fit at the high- or low-concentration (or both) ends of the calibration curve or require a considerable limitation of the range of the curve to that portion which is best described by the algorithm [18]. In contrast, the 4- or 5-PL model, with or without weighting as appropriate, typically provides a good fit of the LBA non-linear calibration curve across a wider concentration range. The use of correlation coefficients or coefficients of determination as a goodness-of-fit guide to selection of a suitable model for fitting LBA data is strongly discouraged. High values of correlation coefficients or coefficients of determination may sometimes not reveal inconsistent fit of data across the entire validated concentration range.

2.2. Incurred sample re-analysis (ISR) and the use of pooled, incurred samples as quality control samples

During the 2006 AAPS/FDA-sponsored workshop on bioanalytical method validation [8], reproducibility of assays for drugs in actual study samples (incurred samples) was discussed, based on some examples of poor such reproducibility observed by FDA inspectors. The primary goal of ISR is to demonstrate that the assay will produce comparable results from study samples, within pre-set limits or criteria, when re-analyzed on a separate occasion. Assessment of ISR involves assessments of components of both accuracy and precision, and criteria are typically set for both of these. For example, many laboratories set as accuracy acceptance limits for ISR that 67% of re-assayed samples must agree with the original values within 30%. Sources of failed ISR results may include errors or differences in sample processing, conversion of unstable biotransformation products to parent during storage or sample preparation, or changes in matrix interferences upon sample storage. ISR has been further debated in depth at numerous informal meetings of specialty groups since that time, as well as being the subject of at least one publication [19]. In this paper Rocci et al. presented the argument that repeat analysis of incurred samples on a separate

occasion is not necessary for study samples analyzed by an LBA, if the samples were analyzed at more than one dilution in the initial assay. However, this approach does not appear to meet the definition of "re-analysis" (analysis again), but would seem more likely to address linearity of dilution. The original FDA concern that stimulated the ongoing debate on ISR arose from the poor agreement of results from repeat analysis of study samples on a separate occasion. This issue is not addressed by analysis of samples at two or more dilutions in the same (initial) assay run. This overall approach also runs the risk of introducing or overlooking a potentially serious error if the two (or more) dilutions in the same run were prepared from the same initial dilution, and the initial dilution had been prepared incorrectly. An independent analysis of the sample on a separate occasion minimizes the likelihood of this occurring. Assay performance for ISR is important for both small molecule and macromolecule LBAs to assure their acceptable reproducibility over time. However, the use of incurred samples raises the broader issue of differences in the types of data that may be derived from incurred samples vs. "spiked" quality control samples, and whether pooled, incurred samples prepared from study samples (when available) should be included as an extension of the currently accepted package of validation experiments. Such "real" samples are closer in overall composition to the study samples to be assayed than are spiked samples. Thus, the use of spiked quality control samples should provide a good estimate of the accuracy and precision of the assay for the analyte of interest in optimal conditions (relatively clean matrix, absence of potentially interfering metabolites, endogenous components or concomitant medications, etc.), while analysis of pooled incurred samples will reflect assay performance more accurately for the analysis of actual study samples. Although such pooled, control samples would not have nominal concentrations for subsequent comparisons (as spiked controls do), initial concentrations could be established by appropriate, analytical runs, designed with input from a biostatistician. Although not directly related to the primary purpose of ISR evaluation, the use of pooled incurred samples may provide additional useful information on stability of the parent analyte or some of its metabolites that is not available from the use of spiked stability samples. Such data may include information on conversion of unstable biotransformation products of the drug to the parent molecule on prolonged storage or on repeated freezing and thawing. Examples, in the case of small xenobiotics, include the potential conversion of some unstable acyl glucuronides or *N*-oxides to parent molecules. The situation for macromolecules is much less clear due to the lack of knowledge of the catabolism, proteolysis and other degradation pathways of such molecules. At a minimum, the inclusion of pooled, incurred samples as routine quality control samples (when available) in addition to the accepted spiked quality controls, should be considered, as these should reflect the performance of the assay in the milieu that more closely reflects that in which the analyte is determined in the actual study samples. The use of pooled incurred samples should also provide an advance indication of the likely assay performance in the actual ISR experiment for a given study. The ongoing evaluation of the performance of pooled incurred quality controls may even obviate the need for conduct of study-specific ISR experiments for individual studies when conducted in the same matrix from a defined patient or healthy volunteer population.

2.3. Establishing acceptance criteria for accuracy and precision

Publication of the proceedings of the first, and subsequent, AAPS/FDA workshops on bioanalytical method validation [4,5,8] and the issuance of an FDA guideline on this topic [7] re-kindled debate on the need for further detailed guidance on validation approaches, in addition to that provided in the workshop reports [4,5,8]. For pre-study validation, the AAPS/FDA workshop reports proposed the use of fixed criteria for accuracy and precision. The suitability of such criteria, in the absence of knowledge about uncertainty of achieving them for a given assay, so that the acceptability of a bioanalytical method for its intended purpose and its likely reliability in routine application can be evaluated, has been challenged. Several authors [11,20–23] have argued that the criteria outlined in the original Crystal City recommendations [4] would not adequately identify bioanalytical methods that would have acceptable performance during practical implementation. They argue that use of a total error and confidence interval approach during pre-study validation increases the probability of accepting an assay that is suitable for implementation and of rejecting prior to implementation an assay that is not suitable for the intended purpose. The use of quality control samples during assay implementation, as recommended by the Crystal City conference [4], has also been criticized as being inadequate [21,22], and arguments have been made for a more statistically based approach to the establishment of acceptance criteria. These views have also been expressed in the report of a commission of the Societe Francaise des Sciences et Techniques Pharmaceutiques (SFSTP) on the validation of bioanalytical methods [10] (with a synopsis discussion of that guide published in 1999 [24]); this discussion of validation of bioanalytical methods has recently been extended in a series of three papers [25–27]. Although the authors of these papers have indicated that their discussion is intended for chromatography-based assays, these general principles may also be considered in the context of LBAs.

It has been pointed out that the trueness and precision of a bioanalytical method is influenced by both a systematic error (bias) and a random error (variability). Total error is defined as the sum of the absolute value of the percent relative error (%RE) and the inter-batch coefficient of variation (%CV), which are indices of the bias and variability, respectively. The applicability of these principles to LBAs has been discussed in recent publications [12,13]. These authors have expressed the view that computation of total error at the pre-study validation stage, coupled with the use of a confidence interval approach, will enhance the likelihood that LBA methods will be accepted into routine use that will not suffer from rejection of a disproportionate number of assays resulting, in turn, in unacceptably high numbers of repeat analyses of study samples. The report from the most recent AAPS/FDA workshop [8] indicated a consensus that, for LBAs, both the inter-batch imprecision (%CV) and the accuracy, expressed as absolute mean bias (%RE) should be ±20% (±25% at lower and upper limits of quantitation), with the additional recommendation that total error be less than ±30% (less than ±40% at the upper and lower quantitation limits). For in-study validation, the recommendation from this conference was that four of six quality control samples should be within 20% of their nominal concentrations (with at least one control at each concentration meeting this criterion. Loosening of the 20% criterion may be considered in cases where the total error in pre-study validation approached or exceeded the limits described above. Clearly, such relaxing of criteria should be considered in the context of the application of the assay, realizing that a major loosening of criteria may significantly decrease the value of study data generated with the assay. Such a decision should be made in the context of subsequent decisions to be made from the data.

The benefit from using the total error/confidence interval approach should be that fewer assays with performance unacceptable for their intended purpose will be implemented into routine use. However, at least in the United States, this more statistically based approach does not appear to be generally favored in the bioanalytical chemistry community within the pharmaceutical industry. In part, this is due to such factors as a lack of familiarity with the concepts and the lack of readily available software

Table 1
Key differences in characteristics of LBAs for small molecules and macromolecules.

Characteristic	Small molecule	Macromolecule
Reference standard quality	Homogeneous/pure	Heterogeneous
Aqueous solubility	Often low	Typically high
Occurrence of endogenous analytes	Unlikely	In some cases
Metabolism	Generally well defined	Typically not defined
Assay specificity	Verifiable by comparator assay (e.g., LC/MS)	Not verifiable

to perform the necessary computations easily and also to concern about potential loss of time that could be productively applied to analytical throughput. Additionally, at least for chromatographic (primarily LC/MS) assays, there does not appear to be a significant issue with the practical use of fixed criteria for trueness and precision, as reflected in the lack of large numbers of unacceptable assays and repeat analyses at the implementation stage. The use of fixed criteria for evaluation of LBAs is also still widely used; however, given the greater imprecision of LBAs, the total error approach may, with more experience, turn out to be a more important consideration for those assays.

3. Small molecule–macromolecule LBA validation differences

Some key differences in LBA-related considerations between small molecules and macromolecules are summarized in Table 1.

3.1. Reference standard differences

Reference standards for small molecule xenobiotics are generally prepared by chemical synthesis and, because of their relatively simple chemical structures, are often available as highly pure, crystalline compounds. Such compounds are typically easy to handle, and can be weighed as solid materials for preparation of stock solutions. Although this favorable scenario is sometimes limited by such properties as hygroscopicity or instability (e.g., if the molecule is an ester sensitive to hydrolysis), small molecule reference standards are generally extensively characterized and are accompanied by a certificate of analysis (COA) documenting their purity and other characteristics. Certified, highly pure reference standards of small molecule drug candidates are generally available relatively early in drug development. Concentrations of stock solutions can be reliably calculated from well-defined parameters, such as molar absorptivities. Stability-indicating assays are generally available for these molecules and degradation products formed in stability studies are relatively readily identifiable by the application of a combination of chromatographic and spectroscopic techniques, such as mass, nuclear magnetic resonance, ultraviolet and infrared spectroscopies. Switching from one batch of reference standard of a small molecule to a subsequent batch typically results in no appreciable change in the LBA calibration curve.

The situation with regard to reference standards for macromolecules is generally quite different from that described above. Many macromolecules, particularly proteins, are produced either by extraction from natural sources or, more commonly in the present day, by recombinant DNA techniques. In some cases, the macromolecule is then modified by incorporation of polyethylene glycol to prolong its in vivo elimination half-life. However, some macromolecules, such as oligonucleotides, may be produced by chemical synthesis and may be somewhat more easily characterized with regard to purity and, thus, suffer fewer of the challenges discussed in this section for proteinaceous macromolecules. In the cases of recombinant synthesis or extraction from natural sources, the final product is typically a heterogeneous mixture of closely related molecules reflecting, in the former case, the product mixture resulting from translational and post-translational (e.g., glycosylation and de-amidation) processes and, in the latter case, the mix of macromolecules with sufficiently similar physicochemical properties to be extracted by the selected extraction and subsequent purification processes. Both the extent and nature of this heterogeneity may vary with the stage of development of the macromolecule, since production processes, including the cell line used in recombinant protein production, may change as development advances. However, throughout this development progression, the protein product will continue to be heterogeneous, albeit with better definition of its components. Lot-to-lot differences for batches produced by the same process in the same cell line may be evident. The components of these mixtures and degradation products formed over time on storage are much more difficult to identify with absolute certainty than are impurities and breakdown products of small xenobiotics due to the complex chemical structures of macromolecules. Degradation products of protein macromolecules include structures formed by de-amidation, methionine oxidation and disulfide bond cleavage. In addition to such chemical changes to the molecule, physical changes, such as aggregation and precipitation, may increase over time. Many of these changes in the physical or chemical nature of the product occurring over time or variations in their content between lots may result in a macromolecule preparation that interacts with the key capture reagents in the LBA in a somewhat different way—resulting in a calibration curve that varies in shape and position on the concentration scale. Similarly, different formulation approaches (e.g., lyophilization) may result in changes in content or nature of aggregates resulting, in turn, in changes in the calibration curve in an immunoassay for the macromolecule. Such variations in LBA sensitivity may result in reporting of erroneous data if the calibration standard and the macromolecule lot used to dose subjects in the study of interest are not well matched in their composition. A true, fully characterized reference standard may not be available until well into the drug development process. In the earlier preclinical and clinical (Phase I and into Phase II) studies, the best “well-characterized” reference material should be used that is representative of the current production process. Regardless of the stage of characterization of the reference material, its key characteristics (purity, source, known stability limitations, etc.) should be documented. It is also important to verify that the reference standard selected for support of a given study reflects as closely as possible the preparation to be dosed in the study to avoid the potential issue noted above of different interactions in the LBA, leading to erroneous results due to use of a “faulty” calibration curve.

The biological activity of the standard material may sometimes be used to characterize a reference material in place of chromatographic or physicochemical characteristics when the latter data are not available. Organizations such as the World Health Organization or the United States Pharmacopeia have standardized the biological activities of a number of protein macromolecules. However, many proteins are not characterized in terms of standardized biological activity [28–30].

3.2. Accuracy and specificity of LBAs for low-molecular-weight molecules and macromolecules

The accuracy and specificity of a bioanalytical method applied to determination of a unique analyte in a complex biological matrix are intimately related since, without specificity for the analyte of interest, accuracy of measurement of the analyte in actual study

samples will be compromised. Assessments of accuracy (trueness) during currently accepted assay validation experiments are clearly defined and largely dependent on analysis of spiked quality control samples consisting of blank matrix to which known quantities of the analyte have been added. Although these procedures define the ability of the method accurately to quantitate the analyte in a relatively "clean" matrix, this approach does little to indicate the effects of any non-specificity on the trueness and precision of the method for analysis of actual study samples. Also, as discussed below, demonstration of the accuracy of an LBA is a considerably greater challenge for macromolecule LBAs than for such assays for low-molecular-weight drugs.

Although favored methodology for bioanalysis of low-molecular-weight drugs applied in support of pharmacokinetic and toxicokinetic studies has generally moved away from LBAs in the relatively recent past, application of LBAs to bioanalysis of selected classes of low-molecular-weight molecules continues, and merits some comment. Thus, LBAs have been applied extensively in the past to the determination of low-molecular-weight xenobiotic drugs [31], in some cases with sufficient specificity even to distinguish between stereoisomers [32]. LBAs are still widely implemented in clinical chemistry and diagnostic applications [33], to drugs-of-abuse screening [34] and determination of some agricultural chemicals [35]. However, liquid chromatography coupled with mass spectrometric detection (LC/MS) has largely replaced LBA as the sensitive method of choice for bioanalysis of these molecules [36]. Such LC/MS methods enjoy enhanced specificity from the combination of chromatographic separation of parent and potentially interfering compounds, such as metabolites, and a detection system that is predicated on the detection of fragment ions characteristic of only the compound of interest. Direct LBAs (i.e., those without a sample processing step before the LBA itself) may match or exceed the sensitivity of LC/MS methods but only occasionally provide an equally high degree of specificity. However, as demonstrated previously [37], judicious selection of the chemical structure of an immunizing drug–protein conjugate, particularly regarding the point of attachment of the small molecule to the protein, can result in an antibody for use in the LBA which is highly specific for the parent drug in the presence of major metabolites. Pathways of metabolism for low-molecular-weight drugs are relatively easy to define by application of modern spectroscopic techniques, such as mass spectrometry and nuclear magnetic resonance spectroscopy [38]. Typically, the major metabolites of such drugs have been isolated and identified by the time the drug has reached advanced stages of development. Metabolites can be synthesized and tested for potential cross-reactivity (and, hence, their effects on the accuracy of the assay) in an LBA used for quantitation of the parent molecule. For a small molecule LBA, specificity can often be assessed by cross-validation with an LC/MS method [39,40] thus increasing confidence that the LBA reports data that are specific and accurate for the analyte of interest.

In contrast to the situation described above for small molecule bioanalysis, direct LBA remains the preferred bioanalytical method for determination of macromolecule therapeutic drugs and drug candidates in biological matrices for support of pharmacokinetic or toxicokinetic studies, typically immunoassay in one of a number of formats. Primarily because of current limitations on application of mass spectrometric detection of macromolecules in serum or plasma, LBAs remain the bioanalytical method of choice for the measurement of biologic macromolecules in such complex matrices, and considerable effort has been expended in defining appropriate experiments for their validation [4–8,12,13]. Unlike the case of small molecules, however, caution needs to be exercised in the final interpretation of the accuracy of concentration data for incurred (study) samples for macromolecules, as determined by LBA [41]. While the accuracy parameter for an assay should address the issue of "closeness of the measured value to the true value" and, in fact, has been referred to as "guaranteeing" this agreement [42], such closeness of measured and true values cannot easily be verified for the typical biological macromolecule in a sample from a dosed animal or human. The main reason for this is that LBAs for macromolecules are typically run without separation of the molecule of interest from any of their biotransformation products or endogenous macromolecules which may also be present in the sample ("direct" LBA), the identities of which, particularly for proteins, are largely unknown. The cross-reactivity of such molecules with the capture reagent (and also their relative biological activity compared with that of the parent macromolecule) is unknown. This is illustrated in recent work on the monoclonal antibody, rituximab [43]. This research demonstrated that the use of different capture reagents in an LBA for rituximab (capture by immobilized target peptide vs. capture by an anti-idiotypic antibody), gave differing plasma concentrations and resulting pharmacokinetic parameters, again showing the inherent difficulty in deriving "true" values from LBA analysis of macromolecules in incurred samples. A variety of other circumstances, including the presence in the sample of antibodies elicited to the macromolecule itself, leading to the possibility of "unbound" and "bound" macromolecule, or the presence in the systemic circulation of soluble receptors for the macromolecule, may also result in interferences in the assay for the macromolecule that are difficult to characterize quantitatively, but which may affect the final reported concentrations of the macromolecule. Another potential effect on the reported LBA concentrations which may vary greatly between subjects is interference from endogenous components in the sample. This may take the form of an endogenous protein that is also given exogenously as a recombinant therapeutic, or an unrelated, but interfering component such as rheumatoid factor, which occurs in some disease populations, or the presence of antibodies cross-reactive with the macromolecule of interest. Advance knowledge of the presence of such factors, their variability between subjects/patients and their effects on apparent concentrations of the macromolecule is generally not available. Appropriate screening of pre-dose samples from each individual may help detect the presence of such factors and provide information on their effects on assay performance, perhaps leading to a more meaningful interpretation of assay results from subjects following administration of the macromolecule. However, in some cases, treatment with the macromolecule may alter the composition of the matrix and, depending on the actual changes elicited in those instances, analysis of the pre-dose sample from the subject may not help with interpretation of data from post-treatment samples.

Use of calibration standards, validation samples and quality control samples, to which the macromolecule of interest has been added in vitro ("spiked" samples), permit evaluation of the trueness and precision of the LBA in relatively optimal, but somewhat unrealistic, conditions. However, given the discussion presented above, we should question whether it is logical for analysts to spend extensive time and resource developing LBAs with better and better accuracy and precision for spiked samples, only to report what may be inherently approximate data for incurred study samples. This potential inaccuracy in LBA data for study samples and the sources of such inaccuracy need to be clearly acknowledged during interpretation of study data and conclusions about pharmacokinetic parameters derived from such data. In some cases, such as support of Discovery or early Development work, this approximation of the study sample concentrations will be acceptable while, in other experiments intended to present pure pharmacokinetic parameters for a macromolecule, additional work should be done to optimize

selection of critical assay binding reagents that are most specific for the macromolecule of interest and, thus, most likely to provide assay results that are closest to true parent macromolecule concentrations. Alternatively, efforts should be made to demonstrate correlation of immunoreactivity data (i.e., data resulting from the binding interactions of all cross-reacting components in the sample with the key binding reagent in the assay), and subsequently calculated pharmacokinetic data, with biological (pharmacodynamic) activity. If a biological activity assay is available, the relationship between LBA concentrations and biological activity should be evaluated. However, it is acknowledged that biological activity assays sometimes have their own challenges in precision and specificity. Nonetheless, in some cases, considerable divergence between immunoreactivity and biological activity over increasing time after in vivo administration of the macromolecule [44] has been noted, indicating that LBA-measured concentrations result from more than just the binding of parent molecule in the assay. The extent of in vivo biotransformation of the macromolecule to mixtures of differentially cross-reacting compounds may also vary with route of administration. In any case, concentration data from in vivo studies of macromolecules should be viewed as “binding equivalents”, “immuno-equivalents” or “LBA equivalents”, until data on the specificity of the LBA are available. It is recommended that a thorough evaluation of methods and results should be conducted on a case-by-case basis, and pharmacokinetic data for biological macromolecules generated by LBAs should generally be viewed with caution.

Given this uncertainty regarding actual concentrations of macromolecules in incurred samples, more emphasis should be placed on the evaluation of incurred samples in LBA applications, as discussed above. While acknowledging the current practical limits on our ability to assess specificity and accuracy of LBAs for macromolecules, additional energy should be devoted to evaluation of new approaches that may elucidate or confirm true macromolecule concentrations in study samples, as determined by LBA analyses. To address this challenge of verification of LBA data for macromolecules, application of advances in automated, high-throughput LBAs coupled with some specificity-enhancing technique, such as prior immunoaffinity or chromatographic separation or subsequent mass spectral characterization, should be pursued more aggressively than they have been to date. Finally, given the fact that LBA specificity for the macromolecule of interest can rarely be defined even for the most highly developed and validated assays, the “fitness” of an LBA for a macromolecule should be honestly assessed in light of the intended purpose of the assay in studies intended to provide definitive pharmacokinetic data for the parent macromolecule. Care should be taken not to over-interpret data from studies supported by an incompletely specific LBA.

4. Conclusions

LBAs are currently the bioanalytical method of choice for support of pharmacokinetic and toxicokinetic studies of biological macromolecules and are still widely employed for analysis of low-molecular-weight molecules in certain fields, such as clinical chemistry/diagnostics, drugs-of-abuse screening and agricultural chemistry. The validation of LBAs raises a number of issues specific to these assays.

For both small molecule and macromolecule LBAs, the inherently non-linear nature of the calibration curve should be recognized and appropriate consideration given to selection of the best algorithm to fit the calibration curve. In most cases, this will be a 4- or 5-PL model, with or without weighting. Another important consideration for both classes of LBAs is conduct of the appropriate type of ISR to demonstrate the occasion-to-occasion reproducibility of the methods in support of individual studies. The evaluation of pooled, incurred quality control samples, when available, should be considered as a potential alternative to study-specific ISR evaluation. Total error and confidence interval approaches should be considered in evaluating pre-study validation data for accuracy and precision, along with fixed criteria, for assessment of the likelihood that an LBA will perform acceptably in routine implementation.

Important differences in LBA validation considerations between small molecules and macromolecules include the characteristics of reference standards for the two classes of assays. Low-molecular-weight reference standards are generally well characterized, highly pure and perform predictably from assay to assay. In contrast, reference standards for macromolecules, often available later in the drug development process than those for small molecules, are generally heterogeneous. Due to variations in composition from batch to batch, different batches may perform differently in the LBA. Finally, the specificity of most LBAs for macromolecules cannot be determined, due to the unknown interferences of biotransformation products, the identities of which, for many macromolecules, are not known. Unlike the case of small molecule LBAs, the accuracy of concentrations of unchanged analytes in study samples determined by LBA cannot be evaluated with a comparator chromatographic assay. Thus, concentration and derived pharmacokinetic data for macromolecules, as determined by LBA, should be viewed with caution.

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