The AAPS Journal 2007; 9 (2) Article 17 (http://www.aapsj.org).

Themed Issue: Bioanalytical Method Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays
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Key Elements of Bioanalytical Method Validation for Macromolecules

Submitted: February 7, 2007; Accepted: April 19, 2007; Published: May 18, 2007

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Abstract

The Third American Association of Pharmaceutical Scientists/US Food and Drug Administration (FDA) Bioanalytical Workshop, which was held May 1 and 2, 2006, in Arlington, VA, addressed bioanalytical assays that are being used for the quantification of therapeutic candidates in support of pharmacokinetic evaluations. One of the main goals of this workshop was to discuss best practices used in bioanalysis regardless of the size of the therapeutic candidates. Since the last bioanalytical workshop, technological advancements in the field and in the statistical understanding of the validation issues have generated a variety of interpretations to clarify and understand the practicality of using the current FDA guidance for assaying macromolecular therapeutics. This article addresses some of the key elements that are essential to the validation of macromolecular therapeutics using ligand binding assays. Because of the nature of ligand binding assays, attempts have been made within the scientific community to use statistical approaches to interpret the acceptance criteria that are aligned with the prestudy validation and in-study validation (sample analysis) processes. We discuss, among other topics, using the total error criterion or confidence interval approaches for acceptance of assays and using anchor calibrators to fit the nonlinear regression models.

Keywords: Bioanalytical validation, ligand binding assays, macromolecules, biological matrices, immunoassay

Introduction

The increased number of biological agents used as therapeutics (in the form of recombinant proteins, monoclonal antibodies, vaccines, etc) has prompted the pharmaceutical industry to review and refine aspects of the development and validation of bioanalytical methods for the quantification of these therapeutics in biological matrices in support of preclinical and clinical studies. Most of these methodologies are used in quantitative assays supporting pharmacokinetic and toxicokinetic parameters of the therapeutic agents.

The methods that are primarily used in these evaluations are ligand binding assays (LBAs [or, for this publication's purposes, immunoassays]), where the specificity and selectivity of the assays depend on the interactions of other biological molecules, such as receptors, antibodies against the therapeutic candidates, and aptamers. The response observed in these methods is indirectly related to the concentration of the therapeutic, that is, the basis of the detection is an enzymatic or radiochemical response tied to a variety of binding interactions. There is no direct physicochemical property of a macromolecule that can be used in this determination (unlike for a small-molecule drug candidate). Because of the nature of these binding interactions, the dynamic range of the standard curves is narrow as well as nonlinear/sigmoidal.

There are several publications that discuss the validation aspects of LBAs in detail.[1,2] The purpose of this article is to highlight the key elements of validation of bioanalytical methods that support the pharmacokinetic and toxicokinetic assessments of macromolecules that were discussed at the Third American Association of Pharmaceutical Scientists (AAPS)/US Food and Drug Administration (FDA) Bioanalytical Workshop in 2006 and that warrant further consideration. These key elements include the selection of reagents for these methods, the format of these assays, the determination of the accuracy and precision of these methods (where there is no extraction procedure that is usually used), the importance of the reference material that is available for use as a standard, and the selectivity of the matrix.

Selection of Reagents and Assay Formats

One of the most important aspects of developing and validating an LBA is the availability of reagents. For most novel and innovative macromolecular therapeutic molecules, there are no commercially available reagents. As a result, unique reagents must be developed within the innovator organizations.

The critical building blocks of LBAs are the ligand reagents, which typically are an antibody or a pair of antibodies for immunoassay-based assays. Other reagents may include

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binding proteins, receptors, oligonucleotides, and peptide fragments. These reagents must be selected in a manner that allows for suitable specificity and selectivity for the intended use and should have binding characteristics that allow for durable and stable antibody/antigen complex formation. Two additional aspects that are overlooked in many situations are the availability of sufficient quantities of these reagents and the stability of these reagents. Some reagents are subject to lot-to-lot variation (eg, conjugated antibodies, radiolabeled ligands). Therefore, it is imperative that sufficient quantities be available to support long-term studies. The reagents that are commonly used in LBAs are macromolecules themselves, and it is essential that the handling and storage be accomplished without destroying the integrity of these reagents. The LBAs are only as good as the reagents that are used; hence, the assay sensitivity and the robustness may be drastically affected if the reagents are unstable.[3] It is important that the quality and characteristics of these critical reagents be fully documented.

In addition to considering the critical reagents used in the LBAs, one must carefully consider the assay diluents, the specific characteristics of the analyte, the intended matrix, and the binding entities (eg, antibodies or receptors). For example, the addition of heavy metals or chelating agents may be required to enable the necessary confirmation for optimal binding. Additionally, the need for detergents (eg, Tween 20 or Triton X-100) or bulking proteins (eg, albumin, casein, or gelatin) must be considered to optimize assay performance.

There are a multitude of assay formats for LBAs, depending on their intended use. Assay formats can include sandwich, competition, direct or indirect binding, inhibition, and solid phase or solution phase assays. In recent years, there have been advances in the detection platforms in addition to the standard colorimetric assays (eg, luminescence, electrochemiluminescence), providing a wide array of assay formats to choose from.

Reference Standard

Macromolecule therapeutic agents are produced in cell culture; hence, they are not characterized as rigorously as small-molecule drug candidates. There is a greater potential for lot-to-lot variability in purity and potency in these preparations. In many instances, a true "reference standard" may not be available; rather, a well-characterized material may be the only choice. It is critical to develop and validate the methods for macromolecules with the appropriate reference material used in the relevant study (ie, the lot of material used in the validation may not be the same as the administered material in the clinical study). The reference material used in the clinical study may have different posttranslational modifications, which could result in the loss of binding activity/epitope for the capture or detection molecule, making the method unsuitable for the intended purpose. At the least, an assessment of the appropriateness of the new lot should be conducted.

When an endogenous counterpart exists in the matrix (eg, erythropoietin), it is critical to have the appropriate reference material for the quantification of therapeutics. The assay specificity and the accuracy of the measurements must be evaluated carefully under these conditions. Depending on the binding properties of the ligands used in the assay, there could be an underestimation or an overestimation of the concentrations measured when an endogenous protein is present in the biological matrix. Table 1 compares the major characteristics of small-molecule and macromolecule compounds.

Specificity and Selectivity

Specificity is the ability to measure the macromolecular therapeutic unequivocally in the presence of other components in the assay matrix. The specificity of the ligands (antibodies, receptors, etc) determines the applicability, sensitivity, and robustness of an LBA. LBAs have to be specific for the macromolecular therapeutic of interest, especially because they measure the therapeutic without extraction procedures. There are 2 types of nonspecificities: specific nonspecificity and nonspecific nonspecificity.[4] Specific nonspecificity results from the interferences of compounds that have physicochemical properties similar to those of the

Table 1. Comparison of the Characteristics of Small-Molecule and Macromolecule Compounds

Characteristic	Small Molecules	Macromolecules
Size	Small (<1000 Da)	Large (>5000 Da)
Structure	Organic molecules	Amino acid biopolymers; could be multimeric
Purity	Homogeneous	Heterogeneous
Solubility	Often hydrophobic	Often hydrophilic
Stability	Chemical	Chemical, physical, and biological
Presence in matrix	Xenobiotic (foreign)	Endogenous
Synthesis	Organic synthesis	Biological production
Metabolism	Defined	Not well defined; could be biotransformed depending on the environment as well as in vivo conditions
Serum binding	Albumin	Specific carrier proteins

analyte (eg, endogenous compounds, isoforms, and variants with different posttranslational modifications that may have similar epitopes). Specific nonspecificity is sometimes referred to as cross-reactivity. Theoretically, an anti-idiotypic antibody reagent is considered to have high specificities toward the analyte and, therefore, low cross-reactivity. For a given critical reagent, data that describe the binding characteristics (eg, cross-reactivity with related compounds) must be taken into consideration before the reagent's use in an LBA. During method development, the specificity of the ligands should be evaluated using compounds that are variant forms of the therapeutic as well as other physicochemically similar compounds, and anticipated concomitant medication.

Nonspecific nonspecificity arises from interferences from unrelated compounds, especially matrix components (eg, heterophilic antibodies, rheumatoid factor, proteases), in the LBA. Nonspecific nonspecificity is sometimes referred to as the matrix effect. This matrix effect is one of the main reasons that LBAs need more method development or validation work to be conducted during the switch from one matrix to another across animal species or even within the same species. It is strongly recommended during clinical study support that the matrix from the relevant disease populations be tested for matrix effects as soon as it becomes available. The matrix effect should be evaluated by comparing the concentration-response relationship of both spiked and unspiked samples of the biological matrix (the recommendation is 10 lots from individual sources) to a comparable buffer solution. It is recommended that the spiked sample concentrations be at the low and high end of the dynamic range.

Nonspecific nonspecificity can usually be reduced or eliminated by dilution of the matrix with a buffer containing chaotropic or chelating agents. This is referred to as the minimal required dilution (MRD). Other sample cleanup procedures such as liquid-liquid, solid phase, or immunoaffinity extractions are also applicable where the nonspecific interferences are stronger. In either situation, the sensitivity (the lower limit of quantification [LLOQ]) should be reported as the concentration of the therapeutic in the 100% matrix.

Specificity and selectivity evaluations verify that the assay is specific for the intended analyte and can select the analyte from a complex matrix without positive or negative interference.

Matrix Selection, Sample Preparation, and MRD

The considerations that pertain to matrix selection are one of the key differences between the assays developed for small-molecule analysis and the LBAs developed for the quantification of macromolecules. Small-molecule assays often include a preassay extraction, which is often helpful to alleviate problems from individual matrix variability. In addition, the use of either analog or stable isotope-labeled internal standards in liquid chromatography/mass spectrometry assays for small molecules normalizes the influence of matrix effects and system fluctuations. The inherent characteristics of macromolecular therapeutics make it difficult and often impossible to extract samples before analysis. LBAs used to quantify macromolecules, therefore, are often developed to measure analyte in complex matrices without extraction. Many macromolecular therapeutics are recombinant or modified variants of endogenous proteins. It is highly unlikely that most of the LBA reagents used will be able to distinguish between the therapeutic and the endogenous counterpart, which could affect the accuracy of measurement of the assay. In these cases, special considerations must be made for matrix selection and for analysis of data.

The matrices collected for bioanalysis include plasma, serum, urine, cerebrospinal fluid, synovial fluid, and homogenized tissue. The characteristics of the macromolecule can be affected by the methods used for sample preparation, the need for additives (anticoagulants, protease inhibitors, etc) at the time of collection, the stability of the macromolecule during collection procedures (whole blood before separation of plasma or serum), and the postcollection processing and storage conditions (temperature, vial type, shipping, freeze-thaw cycles, etc), so these characteristics must be evaluated during the method development phase. Assay format, sample collection conditions, and other factors may influence the choice of matrix in the assay (eg, plasma is the preferred matrix for labile analytes because of the extended time needed for the preparation of serum and because of the presence of proteolytic enzymes).

Spiked samples (ideally at the low and high concentrations) should be prepared in the same matrix as the anticipated matrix of the unknown study samples to evaluate the accuracy (recovery) of the method. In the absence of an endogenous component, simple spiked recovery studies using the nominal concentrations will be sufficient to qualify a matrix.[1,2] The use of a stripped matrix (eg, charcoal, immunoaffinity) or an alternative matrix (eg, protein buffers, dialyzed serum) is not recommended but is necessary when no other strategy for quantification can be designed for measuring endogenous analytes.

Regardless of the source of the matrix interference, validation samples (ie, quality control [QC] samples used during the prestudy validation phase) must be prepared using the same type of neat, unaltered matrix as was used for the study samples for the determination of the assay's precision and accuracy.

The MRD for an assay is the minimum magnitude of dilution to which a sample must be subjected to optimize

accuracy and precision in an assay run. When the standard is prepared in 100% matrix, no MRD exists, and samples can be assayed undiluted or neat. In other cases, where the endogenous material does not generate a linear signal or a background signal is observed due to matrix effects, dilution of the sample may be required to establish acceptable linearity.

If there needs to be a matrix lot change during the course of study sample analysis, appropriate QC samples must be prepared to evaluate the comparability of the data obtained during the prestudy validation.

Nonlinear Standard Curves and Model Selection

LBAs measure the signal of a series of interactions that follow the law of mass action, resulting in a nonlinear and often sigmoidal standard curve. The response error relationship is not constant (heteroscedastic); therefore, the highest precision does not necessarily coincide with the highest sensitivity. In general, it is highly recommended that results from multiple runs be used to estimate the response error relationship. Because of the heteroscedastic nature of the response variance, a weighted, nonlinear, least-squares method is generally recommended for fitting concentration response data from LBAs.[5,6] Four- and 5-parameter logistic calibration models are often used to fit the LBA standard curves. Standard points outside of the range of quantification (anchor calibrators) are often used to assist in fitting these nonlinear regression models.[7] The details of standard curve calibration point selection to be used during method validation are described in DeSilva et al.[2] In summary, it is recommended that at least 3 runs be used to establish the calibration model, with at least 8 non-anchor standard points run in duplicate.[2] The acceptance of the model must be verified by evaluating the relative bias between the back-calculated and nominal concentrations of the calibration standards.[8] The use of the correlation coefficient is not recommended for confirmation of the regression model.[9]

Following prestudy validation, the standard curves for sample analysis (in-study validation) should be monitored using the criteria established during prestudy validation. The general recommendation for acceptance is that at least 75% of the standard points should be within 20% of the nominal concentration (% relative error [RE]), except at the LLOQ and the upper limit of quantification (ULOQ), where the %RE should be 25%. The editing of the standard curve is permitted with only a priori documented criteria and should be independent of and completed before the assessment of the QC acceptance. If edited, the standard curve must be re-regressed and reassessed for acceptance. The final standard curve should have at least 6 nonzero standards besides the anchor points. In situations where the lowest or the highest standard point has been edited, the assay range should be truncated for that particular run and the samples out of the range must be repeated.

Precision and Accuracy

Method precision (random error, variation) and accuracy (systematic error, mean bias) for LBAs should be evaluated by analyzing validation samples (QC samples) that are prepared in the same biological matrix as the anticipated study samples. For analytes with endogenous components, the reader is referred to the section Matrix Selection, Sample Preparation and MRD, for further details about preparing validation samples using an altered matrix. At least 5 concentration levels (anticipated LLOQ, less than 3 times LLOQ, mid, high, and anticipated ULOQ), with at least 2 independent determinations per assay, should be run during the prestudy validation phase. The interbatch variance component is usually higher in LBAs than the intrabatch variance component is. Therefore, it is strongly recommended that at least 6 batches be run during prestudy validation to assess the accuracy, precision, and total error of the method. For each validation sample, the repeated measurements from all runs should be analyzed together using an appropriate statistical method.[2]

Based on our current understanding and the knowledge from the available data discussed in the literature and at recent meetings (3rd AAPS/FDA Bioanalytical Workshop, Round Table discussion at the AAPS Annual Meeting and Exposition, AAPS Bioanalytical Method Validation of Ligand Binding Assays to Support Pharmacokinetic Assessments of Macromolecules: A Post-Crystal City III Perspective 2006), in general an LBA method can be regarded as being acceptable for generating pharmacokinetic and toxicokinetic data if the interbatch precision % coefficient of variation (CV) and the absolute mean bias %RE are both ≤20% (25% at the LLOQ) and the method total error (sum of the %CV and absolute %RE) is ≤30% (40% at the LLOQ). The term "total error" describes the combination of systematic error (the deviation of the calculated value from the nominal value) and random error (deviation of the calculated value from the analytical mean).[1,2] However, it is important to note that in situations where more stringent criteria are needed to support a clinical (ie, bioequivalence) study or a preclinical study, an effort is made to develop and validate LBAs for this purpose.

Once the prestudy validation is completed and the method's accuracy, precision, and total error have been established, this information will be used to set the acceptance criteria for the sample analysis phase. The in-study acceptance criteria must be consistent with the data obtained during the prestudy assessment. If these criteria are inconsistent, there may be higher assay failures than expected. Run acceptance criteria that have been embraced for both chromatographic

assays and LBAs require at least two thirds of all QC results for a run to be within a specific percentage (eg, 15%, 20%, 25%, 30%) of the corresponding nominal reference values, with at least 50% of the results within the specified limit for each QC sample. Assays of conventional small-molecule drugs have adopted a 4-6-15 rule.[10] In contrast, a 4-6-30 rule was proposed for LBAs of macromolecules at the March 2000 AAPS workshop.[7] This was challenged at the 3rd AAPS/FDA Bioanalytical Workshop in 2006, and the survey results indicated that most responders did not use the total error criterion during the assessment of validation data and that the commonly used run acceptance criteria for LBAs was 20% to 25%. Although there was much discussion at this workshop on the use of point estimates for run acceptance criteria, we support the adoption of relevant statistical approaches (eg, total error, confidence intervals, tolerance intervals) that describe the data from the prestudy validation in assigning the run acceptance criteria during in-study validation primarily based on the intended use of these results.[1,8,9,11]

Range of Quantification

The range of quantification for LBAs is based on the lowest (LLOQ) and highest (ULOQ) validation samples that meet the target precision and accuracy criteria. Because of the nonlinear nature of the standard curves in LBAs, it is necessary to define both ends of the standard curve range to obtain the range of quantification. Another difference in the chromatographic assays is that because anchor calibrators are frequently used in LBAs, the LLOQ and the ULOQ concentrations may not be exactly the concentrations of the lowest and the highest calibration standards. LBA standard curves have a narrow dynamic range, so it is necessary to reassay samples that span beyond the ULOQ to be within the range for quantification using appropriate dilutions. Validation samples used to define the range of quantification are prepared in undiluted sample matrix. Therefore, they may be subjected to MRD before analysis. In cases where an MRD is used, it is acceptable to define the range of quantification as the standard concentration values in either neat matrix or as the range of standard concentration values obtained after applying the MRD. As an example, a standard curve of 100 to 1000 ng/mL in neat matrix is equivalent to a standard curve range of 10 to 100 ng/mL with an MRD of 10 (ie, 10% matrix).

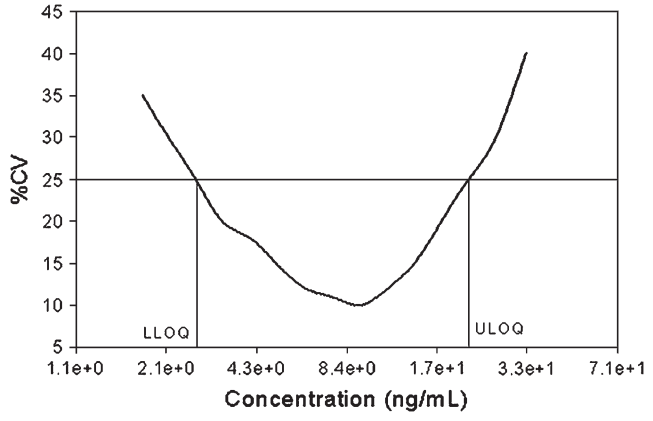

Figure 1. Example of a typical precision profile. %CV indicates percent coefficient of variation; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

A plot of the precision profile can be helpful in assessing the prospective limits of quantification (Figure 1). It is necessary to obtain the data for the precision profile from multiple runs over time rather than using one set of data, because of the high interassay variance over the intra-assay variance. The range of quantification established during prestudy validation is the range into which samples must be diluted if necessary during in-study validation. Samples that fall above the ULOQ must be reassayed at a greater dilution. Samples already at the MRD and below the LLOQ must be reported as <LLOQ (below the limit of quantification). During sample analysis, the LLOQ for a run must be revised upward if editing of the standard curve results in no calibrator at or below the validated LLOQ. In this case the LLOQ is increased to the lowest remaining standard concentration.

Other Validation Parameters

The key elements of method validation of the LBAs were discussed in detail above. There are other validation parameters that should be evaluated and acceptance criteria that should be set up prior to the initiation of study sample analysis (in-study validation). Other publications have offered detailed discussions about evaluating these parameters,[1,2] so only some key points will be reported here.

The stability of the macromolecular therapeutic in the anticipated matrix and in conditions the sample will be subjected to should be demonstrated. In situations where an altered matrix is used for standard curve and QC preparation, stability samples must be prepared in the unaltered matrix. The experiments must mimic the conditions under which the study samples will be collected, stored, and processed. Stability types that need to be assessed include the stability of the analyte in blood when processed into plasma or serum; storage stability such as benchtop, short-term, and long-term storage at –20°C and –70°C; and freeze-thaw stability. It is important to understand the physicochemical properties of the macromolecule during the stability evaluation. For instance, does a protease inhibitor cocktail need to be added during collection? Is the molecule more hydrophobic than others, which may warrant the use of highly proteinaceous buffers for storage? Formal stability experiments must be conducted with an established method during prestudy

validation, but long-term stability experiments may extend into the in-study validation phase. It is important to note that a freshly prepared standard calibrator curve and QC samples or those that are within the acceptable expiration should be used as the reference for comparison of the stability samples. The acceptance criteria applicable to the QC samples may be used for stability evaluations, or other statistically appropriate methods may be used.[12]

Many immunoassays have narrow standard curve ranges (<1 order of magnitude), which necessitates the dilution of samples that are above the ULOQ to within the range of the assay. Dilutional linearity should be evaluated on spiked samples that have been made into the sample matrix (typically 100- to 1000-fold greater than the ULOQ) and then should be diluted into the assay matrix. The back-calculated concentration for each diluted sample should be within 20% of the nominal value, and the cumulative precision should be ≤20%. It is also necessary to conduct dilutional experiments for the identification of a possible prozone or "hook effect" (Figure 2 contains an example of signal suppression caused by high concentrations of analyte).[13]

Dilutional linearity should not be confused with parallelism. Parallelism is a performance characteristic that is typically evaluated during in-study validation using incurred samples (actual study samples). Parallelism can be assessed using C_{max} samples from a given study, pooling the samples to prepare the parallelism validation samples. This approach eliminates the generation of multiple values for individual study samples. The degree of nonparallelism that is acceptable for a method depends on its intended application.[14] It is recommended that the precision (%CV) between samples in a dilution series be ≤30%. The procedure for reporting a result when a sample does not dilute linearly (ie, dilutes in a nonparallel manner) should be defined a priori.

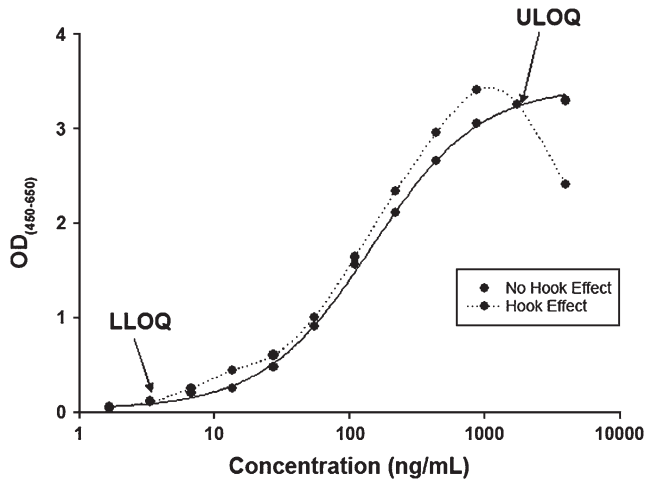

Figure 2. A typical sigmoidal dose-response curve for a 2-site Enzyme Immune Assay (EIA) (solid line), including the high-dose hook effect (dotted line) demonstrating when the higher concentrations of analyte result in a lower than expected response. If there were no hook effect, as shown by the graph with a solid line, higher concentrations of analyte would result in a >ULOQ response. Without a hook effect, the quantification range of the curve is between the LLOQ and the ULOQ. OD indicates optical density; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

Robustness and ruggedness are closely related parameters that should be tested during the prestudy validation phase to ensure the performance of the assay during the in-study phase (during sample analysis for study support). The robustness of the assay is determined by its consistency when changes are implemented that may affect the assay (these should be clearly documented in the method; examples are incubation temperature, light exposure, matrix).[15] The ruggedness of an assay is determined by its consistency when routine changes are implemented, resulting in different operational conditions (eg, analysts, instruments, batch size, environmental factors).[16]

Validations can fall into 3 broad categories: full, partial, and cross. A full validation, which is done for any new method, involves method development, prestudy validation, and in-study validation. Full validations are required for a change in species (eg, rat to mouse) or a change in matrix within a species (eg, rat serum to rat urine). A partial validation is conducted where method changes are considered minor; it can range from a single intra-assay accuracy and precision run to a full validation. Some examples include method transfer, changes to anticoagulant (eg, EDTA, heparin, citrate), changes to critical reagents in a method, sample processing changes (how fast a clot needs to be spun, collection vessels, storage condition), sample volumes, extension of the concentration range, selectivity issues (concomitant medication), conversion of a manual to an automated method, and qualification of an analyst.

Method transfer occurs when a method established in one laboratory (the transfer laboratory) is transferred to another laboratory (the receiving laboratory); it requires at least a partial validation.

Cross-validation is conducted when 2 validated bioanalytical methods are used within the same study or submission (eg, enzyme-linked immunosorbent assay [ELISA] to meso-scale delivery, ELISA to liquid chromatography/mass spectrometry). The general practice is to test samples (spiked and/or pooled incurred samples) with the 2 bioanalytical methods. Data should be evaluated using appropriate predefined acceptance criteria or an appropriate statistical method.[17,18]

DOCUMENTATION

During the discussions on the best practices in validating LBAs, it has been concluded that changes occur in the selection

of critical reagents, the assay format, and the understanding of the drug product during the method development phase. These factors, which would be part of the final selection criteria, require some form of documentation, typically in a lab notebook or binder. As the assay matures during development and the expected format is defined, this too should be documented. With the final format in place, these parameters should be captured in a draft method standard operating procedure (SOP) or an analytical procedure.

The prestudy validation plan guides the validation process and relies on an analytical procedure or method SOP, which may be still in final draft or may be approved. All validation experiments should be documented in a laboratory notebook (or as per the individual company policies). As the validation comes to completion, the details of the experiments and the data that contribute to the validation must be compiled in a validation report. It is important to summarize all the runs to accurately illustrate the assay performance. The prestudy validation data should, to some extent, predict what to expect during sample analysis (in-study validation). Since stability data are frequently obtained over time, the validation report should be updated periodically as new data are generated.

The final validation report can include the following:

1. A description of the assay, including reference standard, critical reagents, and the regression used for analysis
2. The dates that the validation was conducted and last updated
3. The location of stored data
4. The names of analysts who conducted the validation
5. A description of the parameters tested and the methods used to perform the experiments
6. A summary of the standard curve back-calculated values, including overall statistics of accuracy and precision
7. A summary of all the validation samples (QC samples run during validation) used in the conduct of the validation, including overall statistics on accuracy and precision (intra-assay and interassay)
8. Summary tables for each of the parameters included in the validation (eg, dilutional linearity, stability, specificity, selectivity)
9. Deviations from the validation plan
10. A conclusion, including that the validation passed/failed and the final acceptance criteria to be applied during sample analysis

Also, a final summary page of all that constituted the validation is advantageous since this summary page can be associated with sample analysis conducted for studies to document the validity of the sample analysis data.

Conclusions

This article has addressed the key elements in validating an LBA to support pharmacokinetic studies. Most of the parameters that are essential for the successful validation of an LBA must be initiated during the method development phase, confirmed during the prestudy validation phase, and closely monitored during the in-study (sample analysis) phase. The quality of the reagents used in LBAs is pivotal to the development of a robust method; therefore, applying criteria for reagents' lot-to-lot acceptance can be critical. The availability of a well-characterized therapeutic is another important factor to be considered. Most LBAs are quantified in matrices without an extraction procedure, making the assays more susceptible to matrix interferences. For this reason, it is essential that both specificity and selectivity be assessed critically.

Given the complexity and heterogeneity of these macromolecular therapeutics as well as the methods routinely used to quantify these molecules, proper controls must be used and appropriate acceptance criteria established to support these studies. It is essential that appropriate statistical methodologies be used in the assessment of these parameters so that the in-study validation criteria are consistent with the data obtained from the prestudy validation. Furthermore, applying rigorous statistical methods like the concept of total error during the prestudy validation can help ensure that the assay used during sample analysis will not experience significant failures.

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