FUNDAMENTALS, ROLES & REGULATORY REQUIREMENTS OF BIOASSAYS IN CURRENT PHARMACEUTICAL INDUSTRIES

Abstract

The physicochemical and structural characteristics of proteins utilized as active pharmaceutical ingredients in biopharmaceuticals are critical factors in determining their biological activity. Consequently, the tests designed to assess the functionality of biopharmaceuticals serve as substantiating proof that these substances possess the necessary physicochemical attributes and structural conformation. The validation of the methodologies employed to examine the critical quality attributes of biopharmaceuticals stands as a crucial prerequisite for production within Good Manufacturing Practice (GMP) settings.

Keywords: **P**hysicochemical, GMP, Bioassays, CoA

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I. INTRODUCTION

Biopharmaceuticals represent a diverse group of compounds subject to rigorous quality control measures aimed at ensuring consistent quality across different batches. Consequently, regulatory guidelines outline specific attributes that must be assessed to gauge their quality. Critical quality characteristics (CQAs) pertaining to identity, structure, heterogeneity, purity, and functioning are evaluated using a range of analytical techniques. When assessing the quality of biopharmaceuticals, the assessment of functionality using bioassays is very important [1], since it not only validates the sufficiency of other physicochemical and structural CQAs but also sheds light on their mode of action. Nevertheless, the development, standardization, and implementation of bioassays pose significant challenges due to their reliance on responses from living organisms, the use of critical reagents, and other uncontrollable factors that can impact the system's performance [[1], [2], [3], [4]].

Bioassays should have the ability to replicate in vitro the mechanisms through which a biomolecule exerts its biological activity in patients. Furthermore, these assays should integrate a dependable technique for detecting the interaction between the biomolecule and its target, typically involving colorimetric, luminescent, or fluorometric signals [5]. Consequently, the development of bioassays demands a thorough comprehension of the mechanisms of action exhibited by the biomolecules being studied. This understanding is particularly valuable during the assay's design phase, as it aids in determining the critical attributes for evaluation and assists in the selection of the most suitable analytical approaches for their assessment.

After defining the attributes to be assessed and the assessment approach, it is essential to standardize the experimental conditions of the bioassay, followed by a validation process to confirm its suitability for the intended purpose [[7], [8], [9]]. The validation procedure should primarily focus on assessing attributes that ensure the assay's robustness within the specified experimental conditions. Typically, these include specificity, accuracy, precision, sensitivity, and system suitability [1,7,9,10]. Nevertheless, the rigor and specific characteristics evaluated during the validation of bioassays will be contingent on the unique nature of each assay, insights gained during its development and standardization, and its intended application (e.g., Research and Development, Manufacturing, Quality Control, Batch Release, or Biosimilarity analysis).

To determine the suitability of a bioassay, it is imperative for analysts to precisely define the objectives behind conducting the bioassay. The uses of bioassays are numerous and include, but are not limited to: stability evaluations; characterization of process intermediates and formulations; identification of contaminants and degradation products; facilitation of modifications in the product manufacturing process; lot release of the drug substance (active pharmaceutical ingredient) and drug product.

II. FIT FOR USE CONCEPT

In the context of lot release assays, a linear-model bioassay might provide an adequate measure of similarity. Reviewing improvements to production procedures, qualifying reference materials, crucial reagents, or assay methodology adjustments, or analyzing

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similarity utilizing the asymptote of maximum response for bioassays supporting stability or comparability can all be beneficial.

1. Significance of Biological Assays at Different Levels

- Process development
- Process characterization
- Process intermediates effects
- Product release
- Stability of samples
- Qualification of reagents critical to assays
- Product integrity

In vivo potency experiments are bioassays where animals are given varying concentrations of both the Standard and Test materials, and potency is estimated using dose-response relationships. Certain animal tests have straightforward outcomes (e.g., rat ovarian weight assay for follicle stimulating hormone or rat body weight gain assay for human growth hormone); however, other tests necessitate additional processing of samples taken from treated animals (reticulocyte count for erythropoietin, steroidogenesis for gonadotropins, neutrophil count for granulocyte colony-stimulating factor, or antibody titre following vaccination administration). Animals are used far less frequently to evaluate potency now that cell lines relevant to the proposed physiological mechanism of action (MOA) have been developed. (Ref USP chapter 1032).

2. Drawbacks for In-vivo Assay: There are practical reasons such as limited throughput, cost, ethics, and others that make using animal bioassays objectionable and limit the use of animals in industrial settings.

3. Regulatory Requirement: The Interagency Coordinating Committee on the Validation of Alternative Methods, Mission, Vision, and Strategic Priorities, February 2004. Regulatory bodies have advocated the appropriate limiting of animal use whenever possible.

A suitable physicochemical approach (e.g., IEF, glycan analysis) combined with an in vitro cell-based assay may serve as a substitute for in vivo assays where the results of the latter are not substantially correlated with the former (e.g., EPO).

If in vitro assays are unable to identify variations that could impact in vivo activity, there may still be a need for in vivo assays. It is possible to evaluate a test article's activity by cultivating human or animal donor cells or tissues in a lab. When it comes to cytokines, most assays employ hematopoietic cells as well as subsets of these cells from peripheral blood, such as peripheral blood lymphocytes or peripheral blood mononuclear cells. Growth factors and hormones are examples of proteins that act on solid tissues. These proteins can be isolated from animals, separated, and cultivated as adherent or semi-adherent cells for a brief amount of time. While the advantage of using an ex vivo test technique is that it is similar to the natural environment, there are several drawbacks, including significant donor-to-donor variability and difficult cell supply.

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Bioassays employing live tissues or cells derived from animals, such as the rat hepatocyte glucagon method, necessitate procedural control akin to in vivo assays to mitigate potential variations and biases within the assay. The extent of measures taken to mitigate bias, like randomization, should align with the assay's specific objectives. Various factors can impact assay outcomes, including the time of day, animal weight or maturity, type of anaesthetic employed, components and reagents of the buffer, incubation bath temperature and placement, as well as cell viability.

The design of cell-based bioassays should be based on a thorough understanding of the factors influencing cellular response to the test agent. Variability in response is frequently manifested in parameters like the slope, EC50 of the dose-response curve, or the ratio of maximum to minimum response. While the use of relative potency methodology can mitigate the impact of these parameters on potency estimates, such response variability can still have substantial effects on system suitability, precision, and accuracy. Consequently, it is essential to identify the sources of variability specific to a particular bioassay method and implement control strategies to minimize their influence.

The process of developing a cell-based bioassay commences with the choice or creation of a cell substrate, which is typically the most crucial reagent in such an assay. To guarantee a reliable and consistent source of cells for product testing, it is advisable to establish cell banks containing the chosen cell line(s). Several factors must be taken into account during the development of assay cell banks.

4. **Selection and Identification of cell-substrate or Cell Line:** Regardless of the source—a collaborator, academic institution, culture collection, or product manufacturer—the cell line should have thorough documentation detailing its history from the point of origin to banking and substantiating its appropriateness for commercial use. The cell line's origins, development, and expansion up until it is chosen for use should all be carefully recorded and explained in a way that makes it possible to recreate the cell line in the event that it becomes necessary. Before the cell line is banked and during the assay development stage, information about it must be recorded. Identification (e.g., isozymes, phenotypic markers, genetic analysis), morphology (e.g., archived photographic records), purity (e.g., testing for mycoplasma, bacteria, fungi, and viruses), cryopreservation techniques, thawing and culture conditions (e.g., components of the culture media, thawing temperature and method, propagation methods, seeding densities, harvest conditions), thawed viability (both immediately after freezing and after storage), growth traits (e.g., cell doubling times), and functional stability (e.g., ploidy) are some examples of the types of information that may be included.

III. STATISTICAL ASPECTS OF BIOASSAY FUNDAMENTALS

The statistical aspects of bioassay development encompass factors such as data type, the bioassay model, and the establishment of statistical criteria to evaluate and guarantee the quality of bioassay outcomes. These components constitute the foundation of the bioassay system employed for estimating the potency of a test article.

There are fundamentally two bioassay data types: Quantitative and quantal (categorical)

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1. **Quantitative Data** can be discrete (e.g., endpoint dilution titres), counts (e.g., plaque-forming units), or continuous (e.g., acquired from an instrument).

2. **Quantal Data** are usually binary, such as survival in an animal model that measures the protection provided by a test article by challenging it with a disease or positive in a plate-based infectivity assay that causes a cell monolayer to be destroyed once an infectious agent is administered.

   When a threshold is established that statistically distinguishes a positive reaction from a negative response, quantitative data can be transformed into quantal data. This threshold can be determined using data obtained from a negative control, which may involve adding (or subtracting) a measure of uncertainty, like twice the standard deviation of negative control responses, to the average of the negative control. It's important for analysts to exercise caution when converting quantitative data into quantal data, as this conversion can lead to a loss of information that could impact the measurement of the bioassay, as outlined in USP Chapter 1032.

IV. BIOANALYTICAL METHOD VALIDATION GUIDING PRINCIPLE

Developing bioanalytical methods involves defining their design, constraints, operating conditions, and suitability for the intended use. It also involves making sure the method is optimal for validation.

Analyte extraction and detection process conditions are optimized as part of the method development process. The following bioanalytical parameters are optimized as part of method development to make sure the procedure is appropriate for validation:

- Reference standards
- Critical reagents
- Calibration curve
- Quality control samples (QCs)
- Selectivity and specificity
- Sensitivity
- Accuracy
- Precision
- Recovery
- Stability of the analyte in the matrix

Developing bioanalytical methods does not necessitate a lot of documentation or record keeping. The optimized method's suitability for the analysis of the research samples is demonstrated by bioanalytical method validation (Ref may 2018 biopharmaceuticals 05/24/18). The sponsor should:

- Perform a comprehensive validation of any novel bioanalytical method designed for the analysis of a new drug entity, its metabolites, or biomarkers.

- Execute a complete validation process for any modifications made to an already validated method, especially if these changes involve the inclusion of metabolites or additional analytes.
- Create a detailed written description, such as a protocol, study plan, or standard operating procedure (SOP), outlining the bioanalytical method before commencing the validation process. This description should encompass procedures aimed at controlling critical factors within the method, including environmental, matrix-related, and procedural variables, throughout the sample collection and analysis phases to minimize their impact on analyte measurement.
- Make sure that every experiment carried out to support assertions or draw inferences about the validity of the method is well documented and reported in the method validation report.
- Verify each analyte's measurement that was taken from the biological matrix.

V. BIOANALYTICAL PARAMETER THAT PLAYS CRITICAL ROLES IN METHOD VALIDATION OF BIOLOGICAL ASSAYS

1. **Reference Standards:** The study data may be impacted by the purity of the reference standards used to make calibrators and quality control. In order to prepare solutions of known concentrations, the sponsor should employ verified analytical reference standards with known identities and purities. For commercially available reference standards, the sponsor shall furnish the certificates of analyses (CoA), containing the source, lot number, and expiration date (except for United States Pharmacopeia (USP) standards). In addition to the source and lot number, the sponsor must give proof of the standard's identity and purity for any internally or externally developed reference standards without a certificate of authenticity (CoA). The sponsor must either re-establish the identity and purity of the standard or produce an updated certificate of authenticity when utilizing reference standards that have expired (Ref May 2018 biopharmaceuticals 05/24/18).

2. **Critical Reagents:** When there are modifications to the essential reagents, like switching from one lot to another or to a different brand, assay validation becomes crucial. For instance, if the detector reagents, antibodies, or labelled analytes are altered. The sponsor should:

   - Evaluate binding to specific sites and re-optimize assays if required
   - Verify performance with a standard curve and QCs preparations
   - Evaluate cross-reactivity with other reagents in the same assay
   - Performed the bridging study between the reagents.

3. **Calibration Curve:** The process should be confirmed, and then the calibration curve should be continuous and repeatable. The sponsor should use the same biological matrix that holds the study materials to generate the calibration standards. Multiple analytes may be present in study samples. Every analyte in the sample should have a calibration curve produced by the sponsor. The sponsor must validate and justify the calibration curves in cases where surrogate matrices are required.

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4. **Quality Control Samples:** To evaluate the stability of the samples as well as the precision and accuracy of an assay, quality control samples are utilized. In order for the study samples to be evaluated using the approved methodology, sponsors must prepare QCs in the same matrix. For precision and accuracy studies during method development, freshly created QCs are advised because stability data are typically not available at this time.

   QCs assess an analyte's stability and a method's performance during method validation. Validation runs comprise performance QCs (50%,100%,150%, and 200%) to assess the method's accuracy and precision. Stability QCs assess an analyte's capacity to withstand several types of stress (Ref May 2018 biopharmaceuticals 05/24/18).

   All calibration standards and quality control should be prepared by the sponsor using different stock solutions. Nonetheless, the sponsor may utilize calibrators and QCs made from different stock solutions in subsequent runs if the sponsor can show the accuracy and precision of the calibrations in a single validation run. In order to avoid interference or matrix effects, the sponsor should set up calibrators and quality control in large amounts of blank matrix.

5. **Selectivity And Specificity:** The sponsor must verify during validation that the assay is devoid of any chemicals that could interfere, such as metabolites, endogenous matrix components, planned concurrent drugs, etc. If the study sample includes multiple analytes that are intended to be assessed using various techniques, the sponsor should verify that each method is free from interference from the other analyte.

   Blank samples of the relevant biological matrix, such as plasma, from at least six different preparations, should be analyzed by the sponsor. The sponsor is responsible for making sure that the approach is applied without any matrix effects.

   In the context of LBAs (Ligand Binding Assays), it is imperative to thoroughly examine the possibility of interference stemming from analytes that share structural or physiological similarities (referred to as exogenous interference) as well as matrix effects (known as endogenous interference). The assessment of exogenous interference entails the evaluation of molecules that have the potential to disrupt the binding interaction. This includes molecules that exhibit structural similarities to the drug in question, any metabolites, concomitant medications (along with their significant metabolites), or components within the endogenous matrix. The sponsor should meticulously assess each of these factors individually and in conjunction with the analyte of interest to ascertain their capacity to introduce interference.

6. **Sensitivity:** The method should be formulated and validated to ensure its capability to fulfil the necessary prerequisites for the intended study samples. The assessment of the Lower Limit of Quantification (LLOQ) can be conducted independently or as an integral component of the precision and accuracy evaluation within the calibration range.

7. **Accuracy, Precision, And Recovery:** Experiments for method validation to assess accuracy and precision should encompass a minimum of three independent runs for Cell Culture and six for Ligand Binding Assays (referred to as Accuracy and precision or A &

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P runs). These runs should be conducted over multiple days. In each A & P run, a calibration curve and multiple Quality Control (QC) concentrations should be included and analyzed in replicates. The accuracy and precision of the method should be determined by evaluating the performance of the QC samples in the A & P runs. It is essential to use freshly prepared calibrators and QCs in all A & P runs. While the preference is to employ freshly prepared QCs in all A & P runs, if this is not feasible, freshly prepared QCs should be used in one or more A & P runs.

8. **Stability:** During the validation process, it is essential to assess the stability of samples under various conditions, encompassing the anticipated conditions the samples may encounter before reaching the analytical site (such as at clinical sites, during transportation, and at secondary locations). The stability of the samples after they arrive and are analyzed at the analytical location is also included in this evaluation. A medication's capacity to remain stable in a biological fluid is influenced by a number of variables, including the matrix's properties, the drug's physicochemical makeup, and the storage environment. It is important to remember that an analyte's stability in a given matrix and container system only pertains to that unique combination; it should not be generalized to other matrices or container systems. A set of samples should be prepared using a newly made stock solution of the analyte in the appropriate biological matrix that is interference-free and analyte-free for all stability assessments (as detailed below).

9. **Dilution Effects:** During the validation process, when the method involves measuring diluted samples, it's crucial to monitor the integrity of these dilutions. This can be achieved by diluting quality control (QC) samples that exceed the upper limit of quantification (ULOQ) with a similar matrix to bring them within the quantitation range. The accuracy and precision of these diluted QC samples should be established to ensure the reliability of the method when working with dilutions. It's essential that the dilutions applied during validation closely resemble the expected dilutions that will be encountered in the actual study. Additionally, in ligand binding assays (LBAs), it's necessary to demonstrate the presence of the prozone effect.

VI. CONCLUSION

Based on different approaches it can be concluded that biological assays play significant roles in industries, academics, and research institutes at various levels. Development and validation of biological assays are critical to measure the quality parameters of commercial drugs or products. During development, we need to consider the critical parameters of method development to assess the method variability and to overcome the method problems or errors. Method Validation of biological assays is critical to providing a highly competent and robust method for industrial usage purposes. Method validation defines the selectivity, accuracy, precision, stability, and robustness of the method, which enhance the reliability of the method in its regular usage (*Ref May 2018 biopharmaceuticals 05/24/18*).

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