Commentary

Theme: Best Practices for Bioanalytical Methods: Recommendations from the Global Bioanalysis Consortium

Guest Editors: Binodh DeSilva and Philip Timmerman

Recommendations and Best Practices for Reference Standards and Reagents Used in Bioanalytical Method Validation

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Abstract. The continued globalization of pharmaceuticals has increased the demand for companies to know and understand the regulations that exist across the globe. One hurdle facing pharmaceutical and biotechnology companies developing new drug candidates is interpreting the current regulatory guidance documents and industry publications associated with bioanalytical method validation (BMV) from each of the different agencies throughout the world. The objective of this commentary is to provide our opinions on the best practices for reference standards and key reagents, such as metabolites and internal standards used in the support of regulated bioanalysis based on a review of current regulatory guidance documents and industry white papers for BMV.

KEY WORDS: bioanalytical method validation; internal standards; metabolites; reference standards; stock solutions.

INTRODUCTION

The continued globalization of pharmaceuticals has increased the demand for companies to know and understand the regulations that exist across the globe. The goal of the Global Bioanalytical Consortium is to share a current understanding of bioanalysis guidelines and to identify differences in these guidelines or, differences in the interpretation or application thereof, to routine regulated bioanalysis. The objective is to provide globally agreed best practices for bioanalytical method validation (BMV) and application of such methods/technologies to the analysis of drugs of all molecular sizes in support of clinical and nonclinical studies (1). This commentary will focus on reference standards and key reagents, such as metabolites and internal standards used in the support of regulated bioanalysis for new chemical entities (NCEs) and new biological entities (NBEs). At the time of publication, our current understanding is that the assays used to generate biomarker data, which is being

submitted as a primary endpoint, need to be fully validated. Therefore, reference standards used for regulated biomarker assays are only briefly discussed. While we recognize the importance of other assay reagents that are required during BMV, the management and best practices for these will not be addressed in this article, but will be addressed as part of the GBC. In addition, there have been many well-known articles published on this topic previously (2–6).

One hurdle facing pharmaceutical and biotechnology companies developing new drug candidates is interpreting the current regulatory guidance documents and industry publications associated with BMV from each of the different agencies throughout the world (7–17). Current regulatory guidance documents and industry white papers were reviewed in order to provide a consensus interpretation of the content provided. Based on this review, it was clear that gaps still exist between the various publications and that there is a lack of aligned recommendations for reference standards and key reagents, such as metabolites and internal standards. One area demonstrating the biggest gap in recommendations is the preparation and use of these materials, such as preparation of stock solution, preparation of calibrators and quality controls, and preparation of internal standards (7–17).

As new drug candidates evolve in terms of their structural complexity and there is an increase in the number of analytical methods needed to support these programs, it is clear that current guidance documents have not kept pace with this evolution. This increases the pressure on individual companies to ensure they have their own SOPs and best practices in place for support of BMV based on their interpretation of the current guidance documents. Since most

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guidance documents tend to have a bigger focus on NCEs, there has been less guidance and therefore bigger challenges for companies developing NBEs. Fortunately, there are several key industry publications which provide additional guidance and recommendations to companies developing their own internal policies and procedures for support of BMV (14–17). The underlying issue then becomes the wide variability in approach by individual companies as they attempt to control/define these policies and procedures. This publication, while not inclusive, will provide recommendations based on interpretation of current guidance documents, as well as best practices currently in place across the industry.

REVIEW OF CURRENT REGULATORY GUIDANCE AND INDUSTRY PUBLICATIONS

In order to provide a consensus interpretation and to suggest best practices related to BMV, the most current regulatory guidance documents, as well as a combined summary of several key industry publications were reviewed (Table I). During the last few months and years, several key guidance documents have either been revised (10,11) or are in the process of revision (FDA). Despite these recent revisions, we have found that the key industry publications reviewed are more complete and provide more detailed recommendations (8,12). Overall, there is generally good agreement for the recommendations for reference standards in both guidance documents and industry publications as they pertain to well-characterized reference standards, such as certified standards or commercially available standards. However, there is less clarity for reference standards which have not been well characterized in these documents. This presents a challenge in the development of NBEs as many of the reference standards used for ligand binding assays (LBA) tend to be not well characterized. In addition, reference standards to be used in biomarker assays are also not discussed in most guidance documents, but they are discussed in industry publications (14–17). However, in this rapidly evolving industry, the complexity of new analytical methods is beginning to outpace even these key industry publications.

RECOMMENDATION AND BEST PRACTICES

BMV is the process of documenting that the performance characteristics of an analytical method are suitable and reliable for its intended purpose. This is accomplished by spiking blank biological matrix with the analyte of interest using solutions of reference standard. In addition, an internal standard (IS) is normally used in chromatographic methods. Reference standard will be used to spike both calibration standards and quality control (QC) samples for the analysis of drugs and their metabolites in the biological matrix. The purity and identity of these reagents are therefore critical to the success of BMV. The recommendations provided are a consensus formed after careful review and interpretation of the available regulatory guidance documents (7–13) and current industry publications (14–18).

REFERENCE STANDARDS

All reference standards (including certified reference standards or stock solutions, commercially supplied reference

standards, or other material of documented purity) should be provided with a certificate of analysis (CoA) or equivalent documentation that includes, but is not limited to, the following information: lot or batch number, manufacturer, purity, identity, expiration or retest date, appropriate storage conditions, and clearly stated special handling requirements (e.g., light sensitivity).

For LBA, there are often additional design characteristics that need to be considered since LBA reference standards tend to be less well characterized (18). Therefore, the following recommendations should be considered. Reference material should be of the highest purity available and from the same manufacturing process and same batch when possible, as that being dosed for nonclinical and clinical studies. If provided in a buffer or solution, the information on the CoA should be relative to the reference standard in the solution and should include the concentration of the material. Any special requirement (such as freeze/thaw stability, *etc.*) should also be noted. For reference standards which are peptides, peptide content and peptide purity should be provided.

When companies are attempting to validate biomarker assays within the framework of BMV guidance, the following recommendations should be considered. To avoid lot-to-lot variability, it is recommended to use a reference standard from a single lot throughout a study. If it is not possible to use a single lot for the duration of a study, appropriate bridging procedures with defined acceptance should be followed. The same process should be applied if using a commercial kit assay for the purpose of method validation. For a detailed description of a fit-for-purpose approach to the development and validation of biomarker assays, the reader is referred to Lee *et al.* (17).

Where possible, suitable commercial reference standards, including certified standards such as compendial standards (EPCRS, USP, WHO, and NIBSC), or fully characterized standards prepared in-house or by an external noncommercial organization should be used. These are designed to improve lab-to-lab and method-to-method variability and consensus. These standards are considered to be the “gold standard” against which regional, national, and international laboratories and manufacturers calibrate their own working standards (19). However, as is the case with WHO biological reference materials, they are often in limited supply and only distributed to qualified laboratories (20). This means that this type of material may not be suitable for the preparation of all the calibration and QC samples in a study. If this is the case, then careful consideration should be given to how the commercial reference standard is to be used. It could be used to calibrate the concentration of a less well-characterized bulk supplied material, which could then be used in the study.

When using commercial reference standards, careful attention should be paid to how the assigned concentration for the material was generated. For common analytes, such as sex hormones, it is possible that multiple reference standards exist and that they differ in the technology used to generate their assigned concentrations, for example, one might have a concentration based on biological activity assigned by a bioassay, while another has a protein concentration determined by a specific type of immunoassay. In this case, comparison between the two

Table I. Review of Current Regulatory Guidance and Industry Publications

Reference standards and reagents	Reference standard	Metabolite	Internal standard
FDA (7–9)	<ul style="list-style-type: none"> •Authenticated reference standard of known identity and purity •Identical to analyte or established chemical form (free base or acid, salt, or ester) •Source •Lot number •Expiration date •CoA (or evidence of identity and purity provided) <p>Types of reference standards: (1) certified reference standards, (2) commercially supplied reference standards, and (3) other materials of documented purity in report</p> <p>Large molecule: •Reference standards should be characterized appropriately and stored under defined conditions</p>	<ul style="list-style-type: none"> •Cross-reactivity should be evaluated individually and in combination with analyte •Document evidence of purity and identity 	<ul style="list-style-type: none"> •Document evidence of purity and identity
EMA (10)	<ul style="list-style-type: none"> •Authentic and traceable source •Important that quality (purity) is ensured •CoA (to include purity, storage conditions, expiration date, and batch number) <p>Suitable reference standards: (1) certified standards, (2) commercially available standards, and (3) sufficiently characterized standards</p> <p>Document: origin, batch number, CoA, stability, and storage conditions</p> <p>Large molecule: •Reference should be well characterized and documented, <i>e.g.</i>, CoA and origin</p> <p>Purest reference available at the time should be used and should be from the same batch as dosing material for nonclinical and clinical studies. For batch change, analytical characterization and bioanalytical evaluation should be carried out to ensure performance characteristics of the method are not altered</p> <p>Commercial kits: Need to be re-evaluated to ensure LLOQ and QC samples perform accurately and precisely</p>	<ul style="list-style-type: none"> • Investigate interference caused by metabolites • Evaluate possibility of back conversion of metabolite into parent and evaluate impact to study results 	<ul style="list-style-type: none"> •Certified standard not needed as long as suitability of use is shown •CoA is not required. •Demonstrate lack of analytical interference for substance and any impurities •A stable isotope-labeled IS is recommended for MS detection. Labeled standard should be of highest purity and without isotope exchange
ANVISA (11)	Must use certified reference standards. RDC-27/12: use preferably pharmacopeic. For characterized chemical substances, submit the manufacturer documentation with the following data: name, CAS number, formula, molecular weight, shape, properties, impurity profiling care and maintenance and storage, identity, and content validity or retest date	Not addressed specifically. The current understanding based on discussions with ANVISA in 2012 is that they follow the requirements of characterized chemical substances	Not addressed specifically. Current understanding is it is preferable to use the IS labeled with stable isotope. Its use is dispensed if technically justified
MHLW (12)	<ul style="list-style-type: none"> •Chemical structure from an authenticated source •CoA (to include lot number, expiration date, content (purity), storage conditions, and batch number) 	Not addressed specifically	<ul style="list-style-type: none"> •CoA is not required •Demonstrate lack of analytical interference with the analyte
ICH (13)	<ul style="list-style-type: none"> •Well-characterized reference material, with documented purity. Recommend testing for impurities of product vs impurities of assay •CoA (to include purity and stability of analyte) •Record of receipt and storage <p>Document: •Lot numbers (manufacturer), purity, storage, stability, and handling and supporting documents</p> <p>Stability at time of use</p> <p>Large molecule: •Reference standards are often heterogenous and may present unique comparability and stability considerations</p> <p>Biomarker kits: Typically not well characterized and prone to lot-to-lot variability. It is desirable to use reference from a single lot if possible throughout a study; alternatively, appropriate bridging procedures are to be followed</p>	Not addressed specifically	Not addressed specifically
White papers (14–17)	<ul style="list-style-type: none"> •CoA (to include purity and stability of analyte) •Record of receipt and storage <p>Document: •Lot numbers (manufacturer), purity, storage, stability, and handling and supporting documents</p> <p>Stability at time of use</p> <p>Large molecule: •Reference standards are often heterogenous and may present unique comparability and stability considerations</p> <p>Biomarker kits: Typically not well characterized and prone to lot-to-lot variability. It is desirable to use reference from a single lot if possible throughout a study; alternatively, appropriate bridging procedures are to be followed</p>	<ul style="list-style-type: none"> •CoA if available, if not, at least documented purity information 	<ul style="list-style-type: none"> •No CoA is required, but lack of interference between IS and analyte should be established

FDA Food and Drug Administration, *CoA* certificate of analysis, *EMA* European Medicines Agency, *LLOQ* lower limit of quantitation, *QC* quality control, *IS* internal standard, *MS* mass spectrometry, *ANVISA* Agência Nacional de Vigilância Sanitária, *CAS* Chemical Abstracts Service, *MHLW* Ministry of Health, Labour and Welfare, *ICH* International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

materials in any specific method is impossible, as the assigned concentrations will not agree. Ultimately, the use of such a material depends on the intended use of the data being generated. For exploratory biomarkers, then it may not be appropriate, but for a pivotal efficacy biomarker, it could be used to bridge between assay batches and data from different studies.

METABOLITES

Evidence of purity and identity should be documented. Additional cross-reactivity should be evaluated, as well as the possibility of back conversion to parent and its impact on the study. Based on suitability of use (*e.g.*, metabolite is used as a primary endpoint), further

documentation, similar to a reference standard, may be needed to include a certificate of analysis (if available), lot number, manufacturer, expiry (if known), and storage conditions.

INTERNAL STANDARDS

CoA is not required for internal standards, but evidence of purity and identity should be documented. Internal standards should demonstrate a lack of interference and known impurities.

PREPARATION OF STOCK SOLUTIONS

It is recommended that calibration standards and QCs be prepared from separate stock solutions. Typically, for NCE, each stock would be prepared from separate weighings of the reference standards, where for NBE, as these are often already in solution, these would be separate intermediate stocks of the same reference standard solution. Accuracy should be demonstrated by comparing the solutions with defined acceptance between separate preparations, such as within a percent difference as appropriately justified by scientific judgment (*e.g.*, $\pm 10\%$ of nominal). Stock solutions should be used within demonstrated stability. During sample analysis, a single verified reference standard stock solution may be used to prepare both calibrators and quality control provided that the stability and the accuracy of the stock solution have been verified. Stock solution stability should be demonstrated with an appropriate solvent at known concentrations and at appropriate dilution and storage temperature. Short-term stability should be demonstrated based on the intended use of the stock solution at the appropriate storage condition temperature and duration of use. Evaluation of stability at room temperature for at least 6 h is recommended. If stock solutions are to be refrigerated or frozen, appropriate stability for the relevant period should be evaluated. When internal standard material is rare and conduct of formal stability is not possible, stability should be documented through suitability of use (*e.g.*, lack of interference and appropriate assay controls).

PREPARATION OF CALIBRATION STANDARDS AND QUALITY CONTROLS

A calibration curve should be prepared for each analyte in the sample and prepared in a lot of biological matrix deemed to be comparable to the test sample matrix. A single source of matrix may also be used for preparation of calibrators and QCs provided that selectivity has been verified. Calibrators and QCs should be prepared from a stock solution with proven solution stability and where accuracy has been verified. During preparation, stock solutions should not be diluted by more than 5% in any one dilution step, meaning that $>95\%$ matrix should be maintained. However, when working with low concentrations (as in unique matrix) and highly concentrated stock solutions, this approach may not be possible, so best scientific judgment should be used. The method validation must demonstrate that the accuracy of the analytical method has been verified to be specific for the measurement of the therapeutic in the

presence of the endogenous counterparts. Ideally, an analyte-free biological matrix would be used for preparation of the calibrators. If an analyte-free matrix is not available nor can be prepared, a surrogate matrix may be appropriate provided that equivalency has been demonstrated between surrogate matrix and authentic matrix (agreement in the concentration-response relationship between the calibrators and test sample analyte is acceptable), and it has been demonstrated to have no matrix effect when compared to the actual biological matrix of the study samples (8,17). QCs should be prepared in authentic biological matrix of the study samples. Stability should be assessed against freshly spiked calibration curve and compared to nominal concentrations.

BRIDGING REFERENCE STANDARDS FOR LBA

Reference standard lots should be evaluated over time to ensure performance is not trending outside of assay requirements. Bridging between lots of old and new reference standards for LBA should be performed using a scientifically judged appropriate approach with defined acceptance criteria. The run should include calibration curves made from the old lot and QCs prepared from both lots at low, medium, and high concentrations across the standard calibration curve. Evaluation should be per defined acceptance, *e.g.*, two thirds of run acceptance QCs (low, mid, and high) and at least 50% of replicates at each level quantitate within accuracy acceptance limits. These limits may be the assay acceptance or other defined acceptance such as $\pm 20\%$ of theoretical. Intra-assay QCs should also meet these criteria.

CONCLUSIONS

These recommendations and best practices were created after a thorough review of the available regulatory guidance documents and key industry publications. Additionally, a comparison to internal SOPs, policies, and procedures from several companies and knowledge of the industry's best practices were considered. These recommendations are being provided as a consensus interpretation of current regulatory guidance documents and combined summary of several key industry publications to suggest best practices related to BMV.

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