Bioanalytical Method Validation

Guidance for Industry

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Veterinary Medicine (CVM)

May 2018
Biopharmaceutics

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TABLE OF CONTENTS

Contains Nonbinding Recommendations

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I. INTRODUCTION

This guidance helps sponsors of investigational new drug applications (INDs) or applicants of new drug applications (NDAs), abbreviated new drug applications (ANDAs), biologic license applications (BLAs), and supplements validate bioanalytical methods used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies that require pharmacokinetic, toxicokinetic, or biomarker concentration evaluation.[2] This guidance can also inform the development of bioanalytical methods used for nonclinical studies that require toxicokinetic or biomarker concentration data. For studies related to the veterinary drug approval process such as investigational new animal drug applications (INADs), new animal drug applications (NADAs), and abbreviated new animal drug applications (ANADAs), this guidance may apply to blood and urine BA, BE, and pharmacokinetic studies.

The information in this guidance applies to bioanalytical procedures such as chromatographic assays (CCs) and ligand binding assays (LBAs) that quantitatively determine the levels of drugs, their metabolites, therapeutic proteins, and biomarkers in biological matrices such as blood, serum, plasma, urine, and tissue such as skin.

This final guidance incorporates public comments to the revised draft published in 2013 and provides recommendations for the development, validation, and in-study use of bioanalytical methods. The recommendations can be modified with justification, depending on the specific type of bioanalytical method. This guidance reflects advances in science and technology related to validating bioanalytical methods.

In general, FDA's guidance documents do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only

[1] This guidance has been prepared by the Office of Clinical Pharmacology in the Center for Drug Evaluation and Research and the Center for Veterinary Medicine at the Food and Drug Administration.

[2] This guidance applies to both sponsors and applicants. The use of the word *sponsor* applies to both sponsors and applicants and hence, INDs, NDAs, BLAs, and ANDAs.

as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

The 2001 guidance for industry on *Bioanalytical Method Validation* was originally based on the deliberations of two workshops described in publications entitled:

- *Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies*[3]
- *Bioanalytical Methods Validation: A Revisit With a Decade of Progress*[4]

Additional workshops, summarized in the following publications, have informed subsequent revisions (e.g., the 2013 draft guidance for industry entitled *Bioanalytical Method Validation*[5]):

- *Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays*[6]
- *The AAPS/FDA Workshop on Incurred Sample Reanalysis*[7]
- *The AAPS Workshop on Crystal City V — Quantitative Bioanalytical Method Validation and Implementation: 2013 Revised FDA Guidance*[8]

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[3] Shah, VP, KK Midha, S Dighe, IJ McGilveray, JP Skelly, A Yacobi, T Layloff, CT Viswanathan, CE Cook, RD McDowell, KA Pittman, S Spector, 1992, Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies, Pharm Res, 9:588-592.

[4] Shah, VP, KK Midha, JW Findlay, HM Hill, JD Hulse, IJ McGilveray, G McKay, KJ Miller, RN Patnaik, ML Powell, A Tonelli, CT Viswanathan, A Yacobi, 2000, Bioanalytical Methods Validation: A Revisit With a Decade of Progress, Pharm Res, 17:1551-1557.

[5] When final, this guidance will represent the FDA's current thinking on this topic. For the most recent version of a guidance, check the FDA Drugs guidance Web page at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

[6] Viswanathan, CT, B Surendra, B Booth, AJ DeStefano, MJ Rose, J Sailstad, VP Shah, JP Skelly, PG Swann, R Weiner, 2007, Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays, Pharm Res, 24:1962-1973.

[7] Fast, DM, M Kelley, CT Viswanathan, J O'Shaughnessy, SP King, A Chaudhary, R Weiner, AJ DeStefano, D Tang, 2009, Workshop Report and Follow-Up — AAPS Workshop on Current Topics in GLP Bioanalysis: Assay Reproducibility for Incurred Samples — Implications of Crystal City Recommendations, AAPS J, 11:238-241.

[8] Booth, B, ME Arnold, B DeSilva, L Amaravadi, S Dudal, E Fluhler, B Gorovits, SH Haidar, J Kadavil, S Lowes, R Nicholson, M Rock, M Skelly, L Stevenson, S Subramaniam, R Weiner, E Woolf, 2015, Workshop Report:

Validated analytical methods for the quantitative evaluation of analytes (i.e., drugs, including biologic products, and their metabolites**)** and biomarkers in a given biological matrix (e.g. blood, plasma, serum, or urine) are critical for the successful conduct of nonclinical, biopharmaceutics, and clinical pharmacology studies. These validated methods provide critical data to support the safety and effectiveness of drugs and biologic products. Validating the analytical method ensures that the data are reliable by addressing certain key questions, including:

- Does the method measure the intended analyte? For example, does anything interfere with the measurement, and is the method specific or selective for the analyte?

- What is the variability associated with these measurements? For example, what are the accuracy and precision of the method?

- What is the range in measurements that provide reliable data? For example, what is the sensitivity of the method (e.g., what is the lower limit of quantitation (LLOQ) of the method, and what is the upper limit of quantitation the method (ULOQ)?)

- How do sample collection, handling, and storage affect the reliability of the data from the bioanalytical method? For example, what steps need to be followed while collecting samples? Do the samples need to be frozen during shipping? What temperatures are required to store the samples, and how long can the samples be stored?

When changes are made to a validated method, the sponsor should conduct additional validation (i.e., partial or cross validation).

The fit-for-purpose (FFP) concept states that the level of validation should be appropriate for the intended purpose of the study. The key questions listed above should be evaluated relative to the stage of drug development. Pivotal studies submitted in an NDA, BLA, or ANDA that require regulatory decision making for approval, safety or labeling, such as BE or pharmacokinetic studies, should include bioanalytical methods that are fully validated. Exploratory methods that would not be used to support regulatory decision making (e.g., candidate selection) may not require such stringent validation. This FFP concept applies to drugs, their metabolites, and biomarkers.

The analytical laboratory conducting toxicology studies for regulatory submissions should adhere to 21 CFR 58, Good Laboratory Practices (GLPs).[9] The bioanalytical method for human BA, BE, and pharmacokinetic studies must meet the criteria specified in 21 CFR 320 Bioequivalence and Bioavailability Requirements (i.e., 21 CFR 320.29).

Crystal City V — Quantitative Bioanalytical Method Validation and Implementation: The 2013 Revised FDA Guidance, AAPS J, 17:277-288.

[9] For the Center for Veterinary Medicine, all BE studies are subject to Good Laboratory Practices.

The following sections discuss the development, validation, and in-study use of bioanalytical methods and how best to document validation methods and results. Refer to the Glossary for the definitions of assay parameters and analytical terms used in this guidance.

III. BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION

A. Guiding Principles

The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

Before the development of a bioanalytical method, the sponsor should understand the analyte of interest (e.g., determine the physicochemical properties of the drug, in vitro and in vivo metabolism, and protein binding) and consider aspects of any prior analytical methods that may be applicable.

The elements and acceptance criteria of method development and validation are summarized in Table 1. Table 2 describes how the sponsor should document the development and validation of the bioanalytical assay and where it should be stored or submitted.

Method development involves optimizing the procedures and conditions involved with extracting and detecting the analyte. Method development includes the optimization of the following bioanalytical parameters (which are discussed in greater detail in section III.B) to ensure that the method is suitable 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

Bioanalytical method development does not require extensive record keeping or notation. However, the sponsor should record the changes to procedures as well as any issues and their resolutions during development of the bioanalytical method to provide a rationale for any changes during the development of the method.

Bioanalytical method validation proves that the optimized method is suited to the analysis of the study samples. The sponsor should:

- Conduct a full validation of any new bioanalytical method for the analysis of a new drug entity, its metabolite(s), or biomarkers.

- Conduct a full validation for any revisions to an existing validated method that adds a metabolite or an additional analyte.

- Establish a detailed, written description (e.g., protocol, study plan, and/or standard operating procedure (SOP)) for the bioanalytical method before initiating validation. The description should identify procedures that control critical parameters in the method (e.g., environmental, matrix, procedural variables) from the time of collection of the samples to the time of analysis to minimize their effects on the measurement of the analyte in the matrix.

- Document and report (in the method validation report) all experiments used to make claims or draw conclusions about the validity of the method.

- Validate the measurement of each analyte in the biological matrix. The specific recommendations and acceptance criteria for each bioanalytical parameter are listed in Table 1.

B. Bioanalytical Parameters of CCs and LBAs

The bioanalytical parameters applicable to CCs and LBAs are discussed below. Issues unique to either CCs or LBAs are specifically identified.

1. Reference Standards and Critical Reagents

The sponsor should appropriately characterize and document (e.g. determine the identity, purity, and stability) all reference standards and critical reagents, such as antibodies, labeled analytes, and matrices and store them under defined conditions.

a. Reference standards

The purity of reference standards used to prepare calibrators and QCs can affect the study data. Therefore, the sponsor should use authenticated analytical reference standards with known identities and purities to prepare solutions of known concentrations. The reference standard should be identical to the analyte; however, when this scenario is not possible, the sponsor can use an established chemical form (e.g., free base, free acid, or salt) of known purity.

The sponsor should provide the certificates of analyses (CoA), including the source, lot number, and expiration date (with the exception of United States Pharmacopeia (USP) standards) for commercially available reference standards. For internally or externally generated reference standards that do not have a CoA, the sponsor should provide evidence of the standard's identity and purity in addition to the source and the lot number. When using expired reference standards, the sponsor should provide an updated CoA or re-establish the identity and purity of the standard. If the reference standard expires, the sponsor should not make stock solutions with this

lot of standard unless the standard's purity is re-established. For internal standards (ISs), the sponsor does not have to provide a CoA or evidence of purity if it demonstrates that the IS is suitable for the specific use (e.g., lack of interference with an analyte).

b. Critical reagents

The sponsor should appropriately characterize and document (i.e., determine the identity, purity and stability) the critical reagents, including – but not limited to – any reference standards, antibodies, labeled analytes, and matrices.

Assay validation is important when there are changes to the critical reagents, such as lot-to-lot changes or switches to another reagent. For example, if there are changes to the labeled analytes, detector reagents, or antibodies, the sponsor should:

- Evaluate binding and re-optimize assays
- Verify performance with a standard curve and QCs
- Evaluate cross-reactivities

2. Calibration Curve

During method development, the sponsor should choose the quantitation range of the assay and the concentrations of the calibration standards on the basis of the concentration range expected in a particular study. For LBAs, in addition to the calibration standards, anchor points outside the range of quantification can facilitate the fitting of the curve. Anchor points should not be used as part of the acceptance criteria for the run. For most LBAs, calibration (standard) curves are inherently nonlinear, and in general, more calibration standards are needed to define the fit over the calibration curve range for LBAs than for CCs. In addition, the response-error relationship for LBA standard curves is a variable function of the mean response (i.e., heteroscadisticity).

The sponsor should use the simplest model that adequately describes the concentration-response relationship, as well as an appropriate weighting scheme and regression equation. For LBAs, the concentration-response relationship is most often fitted to a four- or five-parameter logistic model, although other models can be assessed.

When the method is validated, the calibration curve should be continuous and reproducible. The sponsor should prepare the calibration standards in the same biological matrix as the samples in the intended study. Study samples may contain more than one analyte. The sponsor should generate a calibration curve for each analyte in the sample. When surrogate matrices are necessary, the sponsor should justify and validate the calibration curves.

The requirements for the calibration curve, including the LLOQ, ULOQ, as well as the acceptance criteria are listed in Table 1.

3. Quality Control Samples

Quality controls are used to assess the precision and accuracy of an assay and the stability of the samples. Sponsors should prepare QCs in the same matrix as the study samples to be assayed with the validated method. Freshly prepared QCs are recommended for precision and accuracy analyses during method development, as stability data are generally not available at this time.

During method validation, QCs evaluate the performance of a method and the stability of an analyte. Performance QCs are included in validation runs to determine the precision and accuracy of the method (see section III.B). Stability QCs evaluate the stability of an analyte under various stress conditions (Refer to section III.B for the selection of QC concentrations).

The sponsor should prepare any calibration standards and QCs from separate stock solutions. However, if the sponsor can demonstrate the precision and accuracy in one validation run using calibrators and QCs prepared from separate stock solutions, then the sponsor can use calibrators and QCs prepared from the same stock solution in subsequent runs. The sponsor should make up calibrators and QCs in lots of blank matrix that is free of interference or matrix effects.

4. Selectivity and Specificity

During method development, the sponsor should verify that the substance being measured is the intended analyte to minimize or avoid interference. Selectivity of the method is routinely demonstrated by analyzing blank samples of the appropriate biological matrix (e.g., plasma) from multiple sources. Depending on the intended use of the assay, the impact of hemolyzed samples, lipemic samples, or samples from special populations can be included in the selectivity assessment. When using liquid chromatography/mass spectrometry (LC/MS) methods, the sponsor or applicant should determine the effects of the matrix on ion suppression, ion enhancement, or extraction efficiency. Internal standards should be assessed to avoid interference with the analyte. Potential interfering substances in a biological matrix include endogenous matrix components such as metabolites, decomposition products – and from the actual study – concomitant medications and other xenobiotics. If a stabilizer or enzyme inhibitor is used during sample collection, the sponsor should evaluate the potential for interference on the quantitation of the analyte. Sponsors should make a scientific judgment about the need to assess these (and any other) potential interferences during method development.

During validation, the sponsor should confirm that the assay is free of potential interfering substances including endogenous matrix components, metabolites, anticipated concomitant medications, etc. If the study sample contains more than one analyte and the analytes are intended to be quantified by different methods, the sponsor should test each method for interference from the other analyte.

The sponsor should analyze blank samples of the appropriate biological matrix (e.g. plasma) from at least six (for CCs) or ten (for LBAs) individual sources. The sponsor should ensure that there are no matrix effects throughout the application of the method. Refer to Table 1 for details of selectivity and specificity requirements and acceptance criteria.

For LBAs, it is important to investigate any interference originating from structurally or physiologically similar analytes (i.e., exogenous interference) or matrix effects (i.e., endogenous interference). Investigating exogenous interference involves determining the cross-reactivity of molecules that could potentially interfere with the binding interaction, including molecules structurally related to the drug, any metabolites, concomitant medications (and their significant metabolites), or endogenous matrix components. The sponsor should evaluate each factor individually and in combination with the analyte of interest to determine its ability to cause interference. Matrix effects evaluation involves comparing calibration curves in multiple sources of the biological matrix against a calibration curve in the matrix for parallelism (serial dilution of incurred samples) and nonspecific binding. The sponsor should eliminate or minimize any significant interference. If such attempts are unsuccessful, the sponsor could consider the development of an orthogonal method to eliminate or minimize the interference.

Carryover between samples can occur in analytical methods. The sponsor should eliminate any carryover during method development. If carryover cannot be eliminated, the sponsor should assess the impact of any carryover during method validation on the accuracy of the study sample concentrations.

5. Sensitivity

The LLOQ defines the method sensitivity and should be determined during method development. The method should be developed and validated such that it will be able to meet the requirements necessary for the intended study samples. The LLOQ evaluation can be done separately or as part of the precision and accuracy assessment for the calibration range. The specific recommendations to validate sensitivity are listed in Table 1.

6. Accuracy, Precision, and Recovery

Evaluating the accuracy and precision across the quantitation range during method development is essential to determine whether the method is ready for validation and involves analyzing replicate QCs at multiple concentrations across the assay range. Specifically, the sponsor should evaluate the performance at the LLOQ, low, mid and high QCs (and the ULOQ for LBAs) to determine if the method is suitable to analyze study samples.

Method validation experiments for estimating accuracy and precision should include a minimum of three (for CCs) and six (for LBAs) independent runs (i.e., accuracy and precision (A & P) runs; see Table 1) conducted over several days. Each A & P run should include a calibration curve and multiple QC concentrations that are analyzed in replicates. The sponsor should determine the accuracy and precision of the method based on the performance of the QC in the A & P runs. The specific validation requirements for accuracy and precision and A & P runs are listed in Table 1. The sponsor should use freshly prepared **c**alibrators and QCs in all A & P runs. Use of freshly prepared QCs in all A & P runs is preferred; however, if this is not possible, the sponsor should use freshly prepared QCs in one or more A & P runs.

The sponsor should optimize the recovery of the analyte to ensure that the extraction is efficient and reproducible. Recovery need not be 100 percent, but the extent of the recovery of an analyte

and of the ISs should be consistent and reproducible. The sponsor should perform recovery experiments by comparing the analytical results of extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction (i.e., to represent 100 percent recovery). Recovery evaluation is not necessary for LBAs unless sample extraction is involved. Recovery experiments should be performed as described in Table 1.

7. Stability

During method development, the sponsor should determine the chemical stability of the analyte in a given matrix, including the effects of sample collection, handling, and storage of the analyte. The sponsor should assess autosampler, benchtop, processed or extracted samples, freeze-thaw, stock solution, and long-term stability of the analyte. The sponsor should assess the stability in the same matrix as that intended for in-study samples; however, when the matrix is rare, the sponsor can explore the use of suitable surrogate matrices.

For drugs administered as fixed combinations, or part of a specific drug regimen, the stability of the analyte should be assessed in the presence of the other drug. The sponsor should also consider the stability of the analyte in the presence of other co-medications that are known to be regularly administered to patients for the indication of the drug under development.

Depending on the analyte as well as the sample collection and assay conditions, evaluating the stability of the analyte in whole blood during method development can be useful. For example, a drug can be unstable in whole blood or adsorb to cellular components during collection.

During validation, stability evaluations should cover the expected sample conditions before receipt at the analytical site (e.g., at the clinical site, during shipment, and at all other secondary sites) as well as during receipt and analysis at the analytical site. Validation of drug stability in a biological fluid is a function of the storage conditions, the physicochemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems.

If the storage conditions changed or the sample analysis occurred outside of the validated storage condition , the stability should be re-established under these new conditions. Stability testing of the analyte in whole blood should be revalidated if necessary (e.g., if the analytes are unstable during blood collection). The specific recommendations and acceptance criteria for stability are listed in Table 1.

Matrix-related stability experiments should compare stability QCs against freshly prepared calibration curves and freshly prepared QCs. Although the use of freshly prepared calibrators and QCs is the preferred approach, in some cases, (e.g., for macromolecules), it may be necessary to freeze them overnight. In such cases, the sponsor should provide valid justification and demonstrate the freeze-thaw stability.

All stability determinations (see list below) should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix.

- **Autosampler stability**: The sponsor should demonstrate the stability of extracts in the autosampler only if the autosampler storage conditions are different or not covered by extract (processed sample) stability.

- **Bench-top stability**: The sponsor should determine the stability of samples under the laboratory handling conditions that are expected for the study samples (e.g., the stability of samples maintained at room temperature or stored in an ice bucket).

- **Extract (or processed sample) stability**: The sponsor should assess the stability of processed samples, including the residence time in the autosampler against freshly prepared calibrators.

- **Freeze-thaw stability**: The sponsor should assess the stability of the sample after a minimum of three freeze-thaw cycles. QC samples should be thawed and analyzed according to the same procedures as the study samples. QC samples should be frozen for at least 12 hours between cycles. Freeze-thaw stability QCs should be compared to freshly prepared calibration curves and QCs.

- **Long-term stability**: The sponsor should determine the long-term stability of the sample over a period of time equal to or exceeding the time between the date of first sample collection and the date of last sample analysis. The storage temperatures studied should be the same as those used to store study samples. Long-term stability QCs should be compared to freshly prepared calibration curves and QCs. Determination of stability at minus 20ºC would cover stability at colder temperatures.

- **Stock solution stability**: Stock solutions should not be made from reference materials that are about to expire unless the purity of the analyte in the stock solutions is re-established. When the stock solution exists in a different state (e.g., solution versus solid) or in a different buffer composition (which is generally the case for macromolecules) from the certified reference standard, the sponsor should generate stability data on stock solutions to justify the duration of stock solution storage stability.

8. Dilution Effects

If the method measures diluted samples, the integrity of the dilution should be monitored during validation by diluting QC samples above the ULOQ with like matrix to bring to within quantitation range, and the accuracy and precision of these diluted QCs should be demonstrated. Dilutions used during the validation should mimic the expected dilutions in the study. The prozone effect should be demonstrated in LBAs. Refer to the specific recommendations and acceptance criteria in Table 1.

9. Partial and Cross Validations

The following section defines other types of methods validation.

a. Partial validation

Partial validations evaluate modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision determination to a nearly full validation. Raw data on partial validations should be retained at the analytical site for inspection when requested. Typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following:

- Bioanalytical method transfers between laboratories
- Changes in analytical methodology (e.g., a change in detection systems)
- Changes in sample processing procedures
- Changes in sample volume (e.g., the smaller volume of pediatric samples)
- Changes in instruments and/or software platforms
- Extensions of the assay range
- Changes in the anticoagulant (but not changes in the counter-ion) in harvesting biological fluids (e.g., heparin to EDTA)
- Changes in the matrix within species (e.g., switching from human plasma to human blood) or changes to the species within the matrix (e.g., switching from rat plasma to mouse plasma)
- Changes to the matrices (e.g., cerebrospinal fluid)
- Demonstrating the selectivity of an analyte in the presence of concomitant medications
- Changes in LBA critical reagents (e.g., lot-to-lot changes, changes in reagents)

b. Cross validation

Cross validation is a comparison of validation parameters of two or more bioanalytical methods or techniques that are used to generate data within the same study or across different studies. Also, cross validation is necessary when sample analyses within a single study are conducted at more than one site or more than one laboratory. In such cases, cross validation with shared matrix QCs and nonpooled subject samples should be conducted at each site or laboratory to establish interlaboratory reliability. Pooled incurred samples can be used when insufficient volume exists. An SOP or validation plan should define the criteria a priori.

C. Validated Methods: Expectations of In-Study Analysis and Reporting

This section describes the expectations for the use of a validated bioanalytical method for routine drug analysis. The specific recommendations and acceptance criteria are listed in Table 1.

- If system suitability is assessed, a specific SOP should be used. System suitability, including apparatus conditioning and instrument performance, should be determined using samples that are independent of the current study calibrators, QCs, and study samples. Records of system suitability should be maintained and available for audits.

- Calibration curves and QCs should be included in all analytical runs (see Table 1 for details). The QCs should cover the expected study sample concentration range.

- Typically, the same curve fitting, weighting, and goodness-of-fit determined during validation should be used for the calibration curve within the study. Changes in the response-function relationship between the validation and study sample analyses indicate potential problems. A SOP should be developed a priori to address such issues.

- Total QCs should number at least five percent of the total samples analyzed, or be at least six in number (low-, mid-, and high-QCs, in duplicate), whichever is greater (see Table 1 for details). Duplicate low-, mid-, and high-QCs should be used on all distinct processing batches within a run.

- If the study sample concentrations are clustered in a narrow range of the standard curve, additional QCs should be added to cover the sample range. If the additional QC concentrations are not bracketed by QCs validated before the study, the accuracy and precision of the additional QCs should be demonstrated before continuing with the analysis. If the partial validation is acceptable, samples that have already been analyzed do not require re-analysis.

- The QCs should be interspersed with study samples during processing and analysis.

- In each analytical run, the lack of analyte interference at the LLOQ should be confirmed (see Table 1 for Selectivity and Sensitivity).

- The analytical run fails if the calibration and/or QC acceptance criteria are not met (see Table 1).

- QC results (including outliers) from analytical runs that meet the acceptance criteria should be included in the estimation of accuracy and precision during the study's sample analysis. The QC results from all analytical runs (passed and failed) should be reported, but QCs results from failed runs need not be included as part of the estimation of accuracy and precision.

- If the bioanalytical method necessitates separation of the overall analytical run into distinct processing batches (e.g., groups of samples processed at distinctly different times or by different analysts), each distinct batch should process duplicate QCs at all levels (e.g., low, middle, high) along with the study samples. Examples might include when the number of samples exceeds the capacity of a 96-well plate or when a solid phase extraction manifold cannot accommodate all samples. See Table 1 for what constitutes an acceptable run based on QC acceptance criteria. A distinct batch or batches in an analytical run may be rejected when it fails to meet QC acceptance criteria, but the remaining batches may pass provided that the analytical run meets the overall QC acceptance criteria.

- Study samples with concentrations listed below the LLOQ should be reported as below the LLOQ (BQL). Study samples with concentrations above the ULOQ should be diluted and re-analyzed, or the standard curve should be extended and revalidated.

- Study sample dilutions should use the same matrix (e.g., human plasma to human plasma).

- Assays of all study samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. If sample handling conditions are changed or exceed validated stability data, then the stability of the sample should be established at the new conditions.

- For CCs, the IS response should be monitored for variability. An SOP should be developed a priori to address issues with IS variability.

- Drift should be monitored and its impact on the accuracy of the estimated unknown sample concentrations, if any, should be addressed (e.g., the impact of drift on the accuracy of interspersed QCs).

- All study samples from a subject should be analyzed in a single run, especially for studies designed with repeated measures from individual subjects (e.g., crossover or sequential design required for BE studies). If other approaches are taken, the sponsor or applicant should justify the approach and take steps to minimize the variability between periods.

- Carryover, if any, should be monitored, and its impact on the quantitation of study samples should be addressed.

- Incurred sample reanalysis (ISR) should be performed (See section IV, Table 1 and Table 2).

- An SOP or guideline describing the reasons for a repeat analysis should be established a priori. Repeat analysis is acceptable only for assignable causes (e.g., the samples are above the ULOQ, there are sample processing errors, there is an equipment failure, the chromatography is poor). The SOP should include the acceptance criteria for re-analysis, and the sponsor or applicant should report final values. The specific recommendations

are described in Table 1 and Table 2. The rationale, approach, and all data for the repeat analysis and reporting should be clearly documented.

- For study samples involving multiple analytes, a valid result for one analyte should not be rejected because of another analyte failing the acceptance criteria.

- If a unique or disproportionately high concentration of a metabolite is discovered in human studies, a fully validated assay may need to be developed for the metabolite, depending upon its activity (refer to the FDA guidance for industry entitled *Safety Testing of Drug Metabolites*[10]).

- An SOP or guideline for sample data reintegration for CCs should be established a priori. This SOP or guideline should define the criteria for re-integration and how the re-integration will be performed. The rationale for the re-integration should be clearly described and documented. Audit trails should be maintained. Original and re-integrated data should be documented and reported.

IV. INCURRED SAMPLE REANALYSIS

ISR is a necessary component of bioanalytical method validation and verifies the reliability of the reported study sample analyte concentrations. ISR is conducted by repeating the analysis of a subset of subject or patient samples from a given study in separate runs, preferably during the study, to critically support the precision and accuracy measurements established with the QCs. The original and repeat analyses should be conducted using the same bioanalytical method procedures. If a bulk frozen calibration curve was used for the original analysis, then it is acceptable to use a frozen curve for the ISR evaluation. The calibration curve, QCs, and study samples for the ISR evaluation should be extracted or processed separately from those used in the original runs. Incurred samples should not be pooled. ISR should be conducted in all studies submitted in an NDA, BLA, or ANDA that provide pivotal data for the approval or labeling of the product, regardless of the matrix. For instance, ISR is expected for all in vivo human BE studies in ANDAs, or all pivotal pharmacokinetic, pharmacodynamic, and biomarker studies in NDAs or BLAs. For nonclinical safety studies, the performing laboratory should conduct ISR at least once for each method and species. Table 1 lists the sample requirements and acceptance criteria for ISR. Written SOPs should be established for the conduct of ISR and to guide an investigation in the event of ISR failure to resolve the lack of reproducibility. All aspects of ISR evaluations should be documented to allow reconstruction of the study, as well as guide any investigations (see Table 2).

The percentage difference of the results between the original study and the repeat study is determined with the following equation:

[10] This guidance is available on the Internet at http://www.fda.gov/Drugs/default.htm under Guidances (Drugs).

$$\frac{(\text{Repeat} - \text{Original}) * 100}{\text{Mean}}$$

V. ADDITIONAL ISSUES

A. Endogenous Compounds

For analytes that are also endogenous compounds, the accuracy of the measurement of the analytes poses a challenge when the assay cannot distinguish between the therapeutic agent and the endogenous counterpart. In such situations, the following approaches are recommended to validate and monitor assay performance. Other approaches, if justified by scientific principles, can also be considered.

- The biological matrix used to prepare calibration standards should be the same as the study samples and free of the endogenous analyte. To address the suitability of using an analyte-free biological matrix, the matrix should be demonstrated to have: (1) no measurable endogenous analyte; and (2) no matrix effect or interference when compared to the biological matrix. The use of alternate matrices (e.g., buffers, dialyzed serum) for the preparation of calibration standards should be justified. The QCs should be prepared by spiking known quantities of the analyte in the same biological matrix as the study samples. The endogenous concentrations of the analyte in the biological matrix should be evaluated before QC preparation (e.g., by replicate analysis). The concentrations for the QCs should account for the endogenous concentrations in the biological matrix (i.e., additive) and be representative of the expected study concentrations.

- Parallelism should be evaluated for assays for endogenous compounds.

B. Biomarkers

The recommendations in this guidance only pertain to the validation of assays to measure in vivo biomarker concentrations in biological matrices such as blood or urine. Considerable effort also goes into defining the biological function of biomarkers, and confusion may arise regarding terminology (e.g. biomarker method validation vs biomarker qualification).

Biomarkers are increasingly used to assess the effects of new drugs and therapeutic biological products in patient populations. Because of the important roles biomarkers can play in evaluating the safety, activity, or effectiveness of a new medical product, it is critical to ensure the integrity of the data generated by assays used to measure them. Biomarkers can be used for a wide variety of purposes during drug development; therefore, a FFP approach should be used when determining the appropriate extent of method validation. When biomarker data will be used to support a regulatory decision making, such as the pivotal determination of safety and/or effectiveness or to support dosing instructions in product labeling, the assay should be fully validated.

For assays intended to support early drug development (e.g., candidate selection, go-no-go decisions, proof-of-concept), the sponsor should incorporate the extent of method validation they deem appropriate.

Method validation for biomarker assays should address the same questions as method validation for drug assays. The accuracy, precision, sensitivity, selectivity, parallelism, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method. The approach used for drug assays should be the starting point for validation of biomarker assays, although the FDA realizes that some characteristics may not apply or that different considerations may need to be addressed.

C. Diagnostic Kits

Diagnostic kits are sometimes co-developed with new drug or therapeutic biological products as analytical methods that are used during the development of new drugs and therapeutic biologics. The recommendations in this section of the guidance do not apply to commercial diagnostic kits intended for point-of-care patient diagnosis (e.g., companion diagnostic kits), which are addressed in the following CDRH guidance documents:

- *Principles for Codevelopment of an In Vitro Companion Diagnostic Device with a Therapeutic Product*[11]

- *In Vitro Companion Diagnostic Devices*

However, when commercial diagnostic kits are repurposed as analytical methods to measure the concentrations of drugs, therapeutic biologics, or biomarkers in development, the FDA has the following recommendations:

- LBA kits with various detection platforms are sometimes used to determine analyte concentrations in pharmacokinetic or pharmacodynamic studies when the reported results must exhibit sufficient precision and accuracy. Because such kits are generally developed for use as clinical diagnostic tools, their suitability for use in such studies should be demonstrated.

- Diagnostic kit validation data provided by the manufacturer may not ensure that the kit method is reliable for drug development purposes. In such situations, the performance of diagnostic kits should be assessed in the facility conducting the sample analysis. Validation considerations for kit assays include, but are not limited to, the following examples:

[11] When final, this guidance will represent the FDA's current thinking on this topic. For the most recent version of a guidance, check the FDA Drugs guidance Web page at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

- Site-specific validation should be performed. The specificity, accuracy, precision, and stability of the assay should be demonstrated under actual conditions of use. Modifications from kit processing instructions should be completely validated.

- Kits that use sparse calibration standards (e.g., one- or two-point calibration curves) should include in-house validation experiments to establish the calibration curve with a sufficient number of standards across the calibration range as specified in Table 1.

- Actual QC concentrations should be known. Concentrations of QCs expressed as ranges are not sufficient for quantitative applications. In such cases, QCs with known concentrations should be prepared and used, independent of the kit-supplied QCs.

- Standards and QCs should be prepared in the same matrix as the subject samples. Kits with standards and QCs prepared in a matrix different from the subject samples should be justified, and appropriate cross-validation experiments should be performed. Refer to section V.A of this guidance for additional discussion.

- If the analyte source (i.e., reference standard) in the kit differs from that of the subject samples (e.g., the sample is a protein isoform of the reference standard), testing should evaluate differences in assay performance of the kit reagents.

- If multiple kit lots are used within a study, lot-to-lot variability and comparability should be addressed for any critical reagents.

- Individual batches using multiple assay plates (e.g., 96-well ELISA plates) should include sufficient replicate QCs on each plate to monitor the accuracy of the assay. Acceptance criteria should be established for the individual plates and the overall analytical run (refer to Table 1 and section III.B).

D. Bridging Data From Multiple Bioanalytical Technologies

The FDA encourages the development and use of new bioanalytical technologies. However, the use of two different bioanalytical technologies for the development of a drug may generate data for the same product that could be difficult to interpret. This outcome can occur when one platform generates drug concentrations that differ from another platform. Therefore, when a new platform is used in the development of a drug, the data it produces should be bridged to that of the other method. This is best accomplished by assessing the output of both methods with a set of incurred samples (a minimum of 20 samples). In cases where one method produces data with a constant bias relative to the other, concentrations can be mathematically transformed by that factor to allow for appropriate study interpretation. Sponsors are encouraged to seek feedback

from the appropriate FDA review division early in drug development. The use of two methods for BE studies in ANDAs is discouraged.

E. Dried Blood Spots

Dried blood spot (DBS) technology has been under development for several years. The benefits of DBS include reduced blood sample volumes collected for drug analysis as well as ease of collection, storage, and transportation. Additional validation of this sampling approach is essential before using DBS in regulatory studies. This validation should address, at a minimum, the effects of the following issues: storage and handling temperatures, homogeneity of sample spotting, hematocrit, stability, carryover, and reproducibility, including ISR. Correlative studies with traditional sampling should be conducted during drug development. Sponsors are encouraged to seek feedback from the appropriate FDA review division early in drug development.

VI. DOCUMENTATION

General and specific SOPs and good record keeping are essential to a properly validated analytical method. The data generated for bioanalytical method development and/or validation should be documented and available for data audit and inspection. Documentation at the analytical site and for submission to the FDA is described in Table 2.

All relevant documentation necessary for reconstructing the study as it was conducted and reported should be maintained in a secure environment. Relevant documentation includes, but is not limited to, source data, protocols and reports, records supporting procedural, operational, and environmental concerns, and correspondence records between all involved parties.

Regardless of the documentation format (i.e., paper or electronic), records should be contemporaneous with the event, and subsequent alterations should not obscure the original data. The basis for changing or reprocessing data should be documented with sufficient detail, and the original record should be maintained.

A. Summary Information

Summary information should include the following items:

- A summary of assay methods used for each study protocol should be included. Each summary should provide the protocol number, the protocol title, the assay type, the assay method identification code, the bioanalytical report code, and the effective date of the method.
- For each analyte, a summary table of all the relevant method validation reports should be provided, including partial validation and cross validation reports. The table should include the assay method identification code, the type of assay, the reason for the new method or additional validation (e.g., to lower the limit of quantification), and the dates of final reports. Changes made to the method should be clearly identified.

- A summary table cross-referencing multiple identification codes should be provided when an assay has different codes for the assay method, the validation reports, and the bioanalytical reports.

B. Documentation for Method Validation and Bioanalytical Reports

Refer to Table 2 for the FDA's recommended documentation for method validation and bioanalytical reports.

VII. APPENDIX

Table 1. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct (refer to sections III.A and III.B for additional information).

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Calibration Curve	**Elements:** • A blank (no analyte, no IS), a zero calibrator (blank plus IS), and at least six, non-zero calibrator levels covering the quantitation range, including LLOQ in every run. • All blanks and calibrators should be in the same matrix as the study samples. • The concentration-response relationship should be fit with the simplest regression model. **Acceptance Criteria:** • Non-zero calibrators should be ± 15% of nominal (theoretical) concentrations, except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run. • 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run.	**Elements:** • A blank and at least six, non-zero calibrator levels covering the quantitation range, including LLOQ per validation run. • Calibration curves are usually run in duplicate. • Additional calibrators may be used as anchor points. • All blanks and calibrators should be in the same matrix as the study samples. • The concentration-response relationship is usually fit with a four- or five-parameter logistic model. Other models may be acceptable with justification. **Acceptance Criteria:** • Non-zero calibrators should be ± 20% of nominal (theoretical) concentrations, except at LLOQ and ULOQ where the calibrator should be ± 25% of the nominal concentrations in each validation run. • 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run. • Anchor points should not be included in the curve fit.	**Elements:** • A blank, a zero, and at least six, (in duplicate for LBAs) non-zero calibrator levels covering the expected range, including LLOQ per analytical run. • All blanks and calibrators should be in the same matrix as the study samples. • The in-study analysis should use the same regression model as used in validation. **Acceptance Criteria:** • **CC**: Non-zero calibrators should be ± 15%, except at LLOQ where the calibrator should be ± 20% of nominal concentrations in each run. • **LBA**: Non-zero calibrators should be ± 20%, except at LLOQ and ULOQ where the calibrator should be ± 25% of nominal concentrations in each run. • **CC and LBA**: 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each run.
	Only data points that fail to meet acceptance criteria may be excluded. Exclusion should not change the model used.		

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Quality Controls (QC)	**Elements:** • **For A & P Runs**: Four QCs, including LLOQ, low (L: defined as three times the LLOQ), mid (M: defined as mid-range), and high (H: defined as high-range) from at least five replicates in at least three runs • **For Other Validation Runs**: L, M, and H QCs in duplicates **Acceptance Criteria**: • Refer to A & P Runs, Other Validation Runs, and Stability Evaluations.	**Elements:** • **For A& P Runs**: Five QCs, including LLOQ, L, M, H, and ULOQ from at least three replicates in at least six runs • **For Other Validation Runs**: L, M, and H QCs in duplicates **Acceptance Criteria**: • Refer to A & P Runs, Other Validation Runs, and Stability Evaluations.	**Elements:** • ≥ three QC levels (L, M & H) and ≥ two replicates per QC level in each analytical run. • Total QCs should be 5% of unknown samples or ≥ six, whichever number is greater. • If the analytical runs consist of distinct processing batches, the QC acceptance criteria should be applied for the whole run and for each distinct batch within the runs. **Acceptance Criteria**: • **CC**: ≥ 67% of QCs should be ± 15% of the nominal, and ≥ 50% of QCs per level should be ± 15% of their nominal. • **LBA**: ≥ 67% of QCs should be ± 20% of the nominal, and ≥ 50% of QCs per level should be ± 20% of their nominal.

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Selectivity	**Elements:** • Analyze blank samples of the appropriate biological matrix from at least six individual sources. **Acceptance Criteria:** • Blank and zero calibrators should be free of interference at the retention times of the analyte(s) and the IS. • Spiked samples should be ± 20% LLOQ. • The IS response in the blank should not exceed 5% of the average IS responses of the calibrators and QCs.	**Elements:** • Investigate parallelism (for endogenous products). • Conduct an analysis of blank samples in the matrix from ≥ 10 individual sources. **Acceptance Criteria:** • For ≥ 80% of sources, unspiked matrix should be BQL, and spiked samples should be ± 25% at LLOQ, and ± 20% at H QC.	**CC Acceptance Criteria:** • In each analytical run, the blank and zero calibrators should be free of interference at the retention times of the analyte and the internal standard • In each analytical run, the internal standard response in the blank should not exceed 5% of average internal standard response of the calibrators and QCs. **LBA Acceptance Criteria:** • The blank should be free of interference for the analyte. • Parallelism should be conducted if not done during validation.
Specificity	**Elements:** • The method specificity should be assessed for interference by cross-reacting molecules, concomitant medications, bio-transformed species, etc. **Acceptance Criteria:** • See Selectivity above.	**Elements:** • The method specificity should be assessed for interference by cross-reacting molecules, concomitant medications, bio-transformed species, etc. • Potential interfering materials should be added to calibration curves in buffer. **Acceptance Criteria:** • QCs should meet ± 20%, or 25% at the LLOQ and ULOQ.	**Elements:** • Check as needed.

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Carryover	**Elements:** • The impact of carryover on the accuracy of the study sample concentrations should be assessed. **Acceptance Criteria:** • Carryover should not exceed 20% of LLOQ.	• Not applicable	**Elements:** • Carryover, if any, should be monitored, and its impact on the quantitation of study samples should be addressed. **Acceptance Criteria:** • Carryover should not exceed 20% of LLOQ.
Sensitivity	**Elements:** • The lowest nonzero standard on the calibration curve defines the sensitivity (LLOQ). **Acceptance Criteria:** • The analyte response at the LLOQ should be ≥ five times the analyte response of the zero calibrator. • The accuracy should be ± 20% of nominal concentration (from ≥ five replicates in at least three runs). • The precision should be ± 20% CV (from ≥ five replicates in at least three runs).	**Elements:** • The lowest nonzero standard on the calibration curve defines the sensitivity (LLOQ). **Acceptance Criteria:** • The accuracy should be ± 25% of the nominal concentration (from ≥ three replicates in at least six runs). • The precision should be ± 25% CV (from ≥ three replicates in at least six runs). • The total error should be ≤ 40%.	**Acceptance Criteria:** In each analytical run: • The analyte response at the LLOQ should be ≥ five times the analyte response of the zero calibrator (CC). • The A & P for CC should be ± 20% of nominal concentration. • The A & P for LBA should be ± 25% of nominal concentration. • If the above criteria are not met, the next higher calibrator can be selected as the new LLOQ or the next lower point if the ULOQ fails (provided the resulting calibration curve meets acceptance criteria) and does not change the calibration model.

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Accuracy and Precision (A & P)	**Elements:** • A & P should be established with at least three independent A & P runs, four QC levels per run (LLOQ, L, M, H QC), and ≥ five replicates per QC level. **A & P Run Acceptance Criteria:** • The run should meet the calibration curve acceptance criteria and include the LLOQ calibrator. • This run has no QC acceptance criteria. **Accuracy: Within-run and between runs:** • ± 15% of nominal concentrations; except ± 20% at LLOQ. **Precision: Within-run and between runs:** • ± 15% CV, except ± 20% CV at LLOQ **Total Error:** • Not applicable	**Elements:** • A & P should be established with at least six independent A & P runs, five QC levels per run (LLOQ, L, M, H, ULOQ QC), and ≥ three replicates per QC level. **A & P Run Acceptance Criteria:** • The run should meet the calibration acceptance criteria and include the LLOQ calibrator. • This run has no QC acceptance criteria. **Accuracy: Within-run and between runs:** • ± 20% of nominal concentrations; except ±25% at LLOQ, ULOQ **Precision: Within-run and between runs:** • ± 20% CV, except ± 25% at LLOQ, ULOQ **Total Error:** • QCs should be ±30%, except at LLOQ, ULOQ ±40%	**Elements:** • Not applicable **Accuracy: Between runs:** • CC: ± 15% of nominal concentrations • LBA: ± 20% of nominal concentrations **Precision: Between runs:** • CC: ± 15% CV • LBA: ±20% CV **Total Error:** • Not applicable

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Other Validation Runs	**Elements:** • ≥ three QC levels (L, M, H) in at least duplicates in each run. **Run Acceptance Criteria:** • Meet the calibration acceptance criteria • ≥ 67% of QCs should be ± 15% of the nominal (theoretical) values, ≥ 50% of QCs per level should be ± 15% of their nominal concentrations	**Elements:** • ≥ three QC levels (L, M, H) in at least duplicates in each run **Run Acceptance Criteria:** • Meet the calibration acceptance criteria • ≥ 67% of QCs should be ± 20% of the nominal (theoretical) values, and ≥ 50% of QCs per level should be ± 20% of their nominal concentrations	• Not applicable
Recovery	**Elements:** • Extracted samples at L, M, and H QC concentrations versus extracts of blanks spiked with the analyte post extraction (at L, M, and H)	**Elements:** • Need to be demonstrated only if extraction is involved	

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Stability	**Elements:** • For auto-sampler, bench-top, extract, freeze-thaw, stock solution and long-term stability, perform at least three replicates at L and H QC concentrations. **Acceptance Criteria:** • The accuracy (% nominal) at each level should be ± 15%.	**Elements:** • For auto-sampler, bench-top, extract, freeze-thaw, stock solution/reagent and long-term stability, perform at least three replicates at L and H QC concentrations. **Acceptance Criteria:** • The accuracy (% nominal) at each level should be ± 20%.	**Elements:** • Update stability parameters (e.g., long-term) as needed.
Dilution	**Elements:** • QCs for planned dilutions, 5 replicates per dilution factor: o Accuracy: ± 15% of nominal concentrations o Precision: ± 15% CV	**Elements:** • QCs for planned dilutions • Demonstrate dilution linearity • Demonstrate lack of prozone effect, i.e., increasing analyte concentration results in no change or decreased signals compared to the preceding concentration • 5 replicates per dilution factor: o Accuracy: ± 20% of nominal concentrations o Precision: ± 20% CV	**Elements:** • Dilution QC (if not a validated pre-study) **Acceptance Criteria**: • Same as described under 'QCs' above

Continued

Table 1 continued. Recommendations and Acceptance Criteria for Bioanalytical Method Validation and In-Study Conduct

Parameters	Validation Recommendations		In-Study Analysis Recommendations
	Chromatographic Assays (CCs)	**Ligand Binding Assays (LBAs)**	
Incurred Sample Reanalysis (ISR)	• Not applicable	• Not applicable	**Elements:** • Sample size: o 10% reanalysis of the first 1000 samples, and o 5% reanalysis of the remaining samples • Sample selection: o Around C_{max} and in the elimination phase **Acceptance Criteria:** • CC: 67% should be ± 20% of the mean • LBA: 67% should be ± 30% of the mean
Repeat Analysis	• No re-analysis of individual calibrators and QCs is permitted.	• No re-analysis of individual calibrators and QCs is permitted.	• Re-analysis should be based on reasons described in a pre-existing SOP • No re-analysis of calibrators and QCs • At least the same number of replicates for repeats as originally tested • No confirmatory repeats for BE studies

Table 2. Documentation and Reporting (refer to sections III.B and VI for additional information)

Items	Documentation at the Analytical Site	Validation Report*	Analytical Study Report*
System Suitability	• Dates, times, QCs or samples used for suitability testing	• Not applicable	• Not applicable
Synopsis	• Not applicable	• Synopsis of method development (e.g., evolution of methods with multiple revisions, unique aspects) • Overall summary information	• Not applicable
Reference Standards and Critical Reagents	• Certificate of analysis (CoA) or purity, stability/expiration data, batch number, and manufacturer • Log records of receipt, use, and storage. • If expired, recertified CoA, or retest of purity & identity with retest dates • Internal standard CoA, purity or demonstration of suitability	• Batch/lot number, purity, and expiration (see appendix VII, Table 4) • If expired, purity and stability at the time of use and retest dates	• Batch/Lot number, purity, and expiration (see appendix VII, Table 4) • If expired, purity and stability at the time of use and retest dates
Stock Solutions	• Log records of preparation, and use • Storage location and condition	• Brief description of preparation • Preparation dates • Stock solution stability • Storage conditions	• Brief description of preparation • Preparation dates • Stock solution stability • Storage conditions
Blank Matrix	• Records of matrix descriptions, receipt dates, and storage • Records of interference checks • Matrix effect results	• Description, lot number, receipt dates • Description of interference check • Matrix effect results	• Description, lot number, receipt dates • Description of interference check

Continued

Table 2 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Analytical Study Report*
Calibrators and QCs	• Records of preparation • Record of storage (e.g., in/out dates, temperatures)	• Brief description of preparation • Preparation dates • Storage conditions	• Brief description of preparation • Preparation dates • Storage conditions
SOPs	SOPs for all aspects of analysis, such as: • Method/procedure (validation/analytical) • Acceptance criteria (e.g., run, calibration curve, QCs) • Instrumentation • Re-analysis • ISR • Record of changes to SOP (change, date, reason, etc.)	• A detailed description of the assay procedure	Not applicable
Sample Tracking	• Study sample receipt, and condition on receipt • Temperature during shipment • Sample inventory and reasons for missing samples • Location of storage • Tracking logs of QC, calibrators, and study samples • Freezer logs for QC, calibrators, and study samples entry and exit	• Storage condition and location of QCs and calibrators	• Dates of receipt of shipments and contents • Sample condition on receipt • Analytical site storage condition and location • Total duration of sample storage • Any deviations from planned storage conditions

Continued

Table 2 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Analytical Study Report*
Analysis	• Documentation and data for system suitability checks • Instrument use log, including dates of analysis for each run • Sample extraction logs, including documentation of processing of calibrators, QCs, and study samples for each run, including dates of extraction • Identity of QC & calibrator lots, and study samples in each run • Documentation of instrument settings and maintenance • 100% of run summary sheets of passed and failed runs, including calibration curve, regression, weighting function, analyte and IS response, response ratio, integration type • 100% e-chromatograms of original and re-integrations from passed and fail runs • Laboratory information management system (LIMS) • Validation information, including documentation and data for: o Selectivity, sensitivity, precision and accuracy, carryover, dilution, recovery, matrix effect o Bench-top, freeze-thaw, long-term, and extract stability o Cross/partial validations, if applicable	• Table of all runs (including failed runs), instrument ID, and analysis dates • Tables of calibrator concentration and response functions results of all runs with accuracy and precision. • Tables of within- and between-run QC results (from accuracy and precision runs) • Interference/matrix effect, sensitivity, carryover, dilution, recovery • Bench-top, freeze-thaw, long-term, extract, and stock solution stability • Stability QC storage and handling conditions (dates, duration, temperature, etc.) • Partial/cross-validation, if applicable • Append separate report for additional validation, if any • Include total error for LBA methods	• Table of all runs, status (pass and fail), reason for failure, instrument ID, and analysis dates (see appendix VII, Table 4). • Table of calibrator concentration & response function results of all runs (pass and fail) with accuracy and precision • Table of QC results of all runs (pass and fail) with accuracy and precision results of the QC samples and between run accuracy and precision results from successful runs • Table of re-injected runs with results from original and re-injected runs and reason(s) for reinjection • QC graphs trend analysis encouraged • Study concentration results table

Continued

Table 2 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Analytical Study Report*
Chromatograms and Reintegration	• Electronic audit trail: original and re-integration • Reason for re-integration • Mode of re-integration	• Representative chromatograms (original and re-integration) • Reason for re-integration	• Chromatograms from 20% of serially selected subjects for BE studies in ANDAs • Randomly selected chromatograms from 5% of studies submitted in NDAs and BLAs • Original and re-integrated chromatograms and initial and repeat integration results for BE studies • Reason for re-integration • SOP for re-integration
Deviations from Procedures	• Contemporaneous documentation of deviations/ unexpected events • Investigation of unexpected events • Impact assessment • ISR failure investigations	• Description of deviations • Impact on study results • Description and supporting data of significant investigations	• Description of deviations • Impact on study results • Description and supporting data of significant investigations
Repeat Analysis	• SOP for re-analysis (Refer to Analysis) • 100% of repeat data • Contemporaneous records of reason for repeats	• Not applicable	• Table of sample IDs, reason for re-assay, original and re-assay values, reason for reported values, and run IDs and percent difference between original and re-assay values • Re-analysis SOP

Continued

Table 2 continued: Documentation and Reporting

Items	Documentation at the Analytical Site	Validation Report*	Analytical Study Report*
ISR	• SOP for ISR • ISR data: Run IDs, run summary sheets, chromatograms or other electronic instrument data files • Document ISR failure investigations, if any	• Not applicable	• SOP for ISR • ISR data table (original, reanalysis, percent difference, percent passed) • ISR failure investigations, if any
Communication	• Between involved parties (Sponsor, contract research organizations (CROs), and consultants) related to study/assay	• Not applicable	• Not applicable

*The FDA expects the sponsor to maintain data at the analytical site to support summary data submitted in Validation and Analytical Study Reports.

Table 3.[&] Example of an Overall Summary Table for a Method Validation Report* or a Clinical Study Report (this table contains fictitious information, which serves illustrative purposes only)

Items	Results	Hyperlink[†]	Comments
Methodology	LC/MS/MS	01-SOP-001	
Method Validation Report (MVR) Number	MVR-001	MVR-001	
Biological matrix	Human plasma	MVR-001	
Anticoagulant (if applicable)	EDTA	MVR-001	
Calibration curve range	XXX-YYY ng/mL	**Summary tables** 001MVR-01/CCTables **Report text** 001MVR-01/CCText	
Analyte of interest	Compound A	NA	
Internal standard	Compound A internal standard	NA	
Inter-run accuracy (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (e.g., BB ng/mL): Y% High QC (e.g. CC ng/mL): Z%	**Summary tables** 001MVR-01/APTables **Report text** 001MVR-01/APText	
Inter-run precision (for each QC concentration)	Low QC (AA ng/mL): X% Medium QC (BB ng/mL): Y% High QC (CC ng/mL): Z%		

Continued

Table 3 continued. Example of an Overall Summary Table for a Method Validation Report* or a Clinical Study Report

Items	Results	Hyperlink†	Comments
Dilution integrity (specify dilution factors, QC concentrations, and matrices that were evaluated)	Dilution QC: CC ng/mL (dilution factor: X) Accuracy: Y% Precision: Z%	**Summary tables** 001MVR-01/DILTables **Report text** 001MVR-01/DILText	
Selectivity	< 20% of the lower limit of quantification (LLOQ) -list drugs tested	**Summary tables** 001MVR-01/SELTables **Report text** 001MVR-01/SELText	
Short-term or bench-top temperature stability	Demonstrated for X hours at Y°C	**Summary tables** 001MVR-01/STSTables **Report text** 001MVR-01/STSText	
Long-term stability	Demonstrated for X days at Y°C	**Summary tables** 001MVR-01/LTSTables **Report text** 001MVR-01/LTSText	
Freeze-thaw stability	Demonstrated for Y cycles at Z°C	**Summary tables** 001MVR-01/FTSTables **Report text** 001MVR-01/FTSText	
Stock solution stability	Demonstrated for X weeks at Y°C	**Summary tables** 001MVR-01/SSSTables **Report text** 001MVR-01/SSSText	

Continued

Table 3 continued. Example of an Overall Summary Table for a Method Validation Report* or a Clinical Study Report

Items	Results	Hyperlink†	Comments
Processed sample stability	Demonstrated for Y hours at Z°C	**Summary tables** 001MVR-01/PSSTables **Report text** 001MVR-01/PSSText	
ISR	> 67% of samples acceptable	**Summary tables** 001MVR-01/ISRTables **Report text** 001MVR-01/ISRText	
Recovery: extraction efficiency	**Summary tables** 001MVR-01/EXTTables **Report text** 001MVR-01/EXTText		
Matrix effects	**Summary tables** 001MVR-01/MATTables **Report text** 001MVR-01/MATText		

[&]Report Format examples are pertinent for applications to either CDER or CVM. Summary tables should be included in Module 2 of the eCTD.
*Failed method validation experiments should be listed, and data may be requested.
†For eCTD submissions, a hyperlink should be provided for the summary tables and report text.

Table 4. Example of Summary Analytical Runs for a Bioanalytical Study Report* (this table contains fictitious information, which serves illustrative purposes only)

Sponsors and applicants should provide a table summarizing both the failed and accepted runs for each study.

Clinical Study XXYY-0032456

Analytical run *	Batch number within analytical run	Dates of analysis	Results (Accepted /Rejected)	Hyperlink†	Comments (e.g. information on runs that failed)
001-100-01	Not applicable	MM/DD/YY	Rejected	**Summary tables for calibration curve standards and QCs** 001BR-01/01CALTables 001BR-01/01QCTables **Report text** 001BR-01/01CALText 001BR-01/01QCText **Raw Data** 001BR-01/01CALData 001BR-01/01QCData	**001BR-01/01Failure** 67% of the QCs passed; however both QCs that exceeded ±15% were at the low QC concentration. The follow-up investigation concluded that the LC/MS/MS instrument required a recalibration.
001-100-02	Not applicable	MM/DD/YY	Accepted	**Summary tables for calibration curve standards and QCs** 001BR-01/02CALTables 001BR-01/02QCTables **Report text** 001BR-01/02CALText 001BR-01/02QCText **Raw Data** 001BR-01/02CALData 001BR-01/02QCData	This is the reanalysis of the samples from run 001-100-01

*If multiple batches are analyzed within an analytical run, each batch should be separately evaluated to determine if the batch meets acceptance criteria.

†For eCTD submissions, a hyperlink should be provided for the summary tables, report text, and raw data.

VIII. GLOSSARY

Accuracy: Accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. Accuracy is also sometimes termed *trueness*.

Analyte: An analyte is the specific chemical moiety being measured; it can be an intact drug, a biomolecule or its derivative, a metabolite, or a degradation product in a biologic matrix.

Analytical run: An analytical run is a complete set of analytical and study samples with an appropriate number of standards and QCs for their validation. Several runs can be completed in one day, or one run may take several days to complete.

Autosampler stability: Autosampler stability is the stability of the analyte in the processed sample under the conditions in the autosampler.

Biological matrix: A biological matrix is discrete material of biological origin that can be sampled and processed in a reproducible manner. Examples are blood, serum, plasma, urine, feces, cerebrospinal fluid, saliva, sputum, and various discrete tissues.

Batch: For purposes of this guidance, a batch is a number of unknown samples from one or more patients in a study and QCs that are processed at one time.

Between run: Between run refers to the distinct period between or among several analytical or validation runs.

Bench-top stability: Bench-top stability is the stability of an analyte in a matrix under conditions of sample handling during sample processing.

Blank: A blank is a sample of a biological matrix to which no analytes have been added that is used to assess the selectivity of the bioanalytical method.

Calibration curve: The calibration curve — also known as the standard curve — is the relationship between the instrument response and the calibration standards within the intended quantitation range.

Calibrators/Calibration standards: Calibrators, or calibration standards, refer to a biological matrix to which a known amount of analyte has been added. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QC samples and in-study samples are determined.

Carryover: Carryover is the appearance of an analyte in a sample from a preceding sample.

Critical reagents: Critical reagents are requisite components of an assay, which include antibodies, labeled analytes, matrices, etc.

Dilutional linearity: Dilutional linearity demonstrates the accurate measurement of concentrations of spiked samples (i.e., QCs) exceeding the quantitation range when serially diluted to within the quantitative assay range.

Extract: An extract is a sample treated to remove impurities or interfering substances (also known as a processed sample).

Extract stability: Extract stability assesses the degradation of the processed sample relative to the starting material.

Freeze-thaw stability: Freeze-thaw stability refers to the stability of the analyte in the matrix upon freezing and thawing.

Freshly prepared: Freshly prepared refers to QC sample preparation (i.e., spiked) on the day of the experiment; not frozen before use.

Full validation: Full validation refers to the establishment of all validation parameters that apply to sample analysis for the bioanalytical method for each analyte.

Heteroscadisticity: Heteroscadisticity occurs when the variance of a response is not constant but changes with the response.

Hook effect: The hook effect occurs when increasing analyte concentrations result in no change or decreased signals when compared to the preceding concentration.

Incurred samples: Incurred samples are study samples or samples from subjects or patients who were dosed.

Incurred Sample Reanalysis (ISR): ISR is the repeated measurement of an analyte's concentration from study samples to demonstrate reproducibility.

Interference: Interference refers to the action of sample components, including structurally similar analytes, metabolites, impurities, degradants, or matrix components that may impact quantitation of the analyte of interest. Refer to **Selectivity** and **Matrix effect** for further information.

Internal standard (IS): ISs are test compounds (e.g., structurally similar analogs, stable isotope labeled compounds) added to both calibration standards and samples at known and constant concentrations to facilitate quantification of the target analyte(s).

Long-term stability: Long-term stability assesses the degradation of an analyte in the matrix relative to the starting material after periods of frozen storage.

Lower limit of quantification (LLOQ): The LLOQ is the lowest amount of an analyte that can be quantitatively determined with acceptable precision and accuracy.

Matrix effect: The matrix effect is a direct or indirect alteration or interference in response because of the presence of unintended analytes (for analysis) or other interfering substances in the sample.

Method: A method is a comprehensive description of all procedures used in the collection, storage, and analysis of samples.

Non-zero calibrator: A non-zero calibrator is a calibrator to which the internal standard is added.

Nominal concentration: The nominal concentration is the actual or intended concentration of the calibrator or quality control samples.

Parallelism: Parallelism demonstrates that the serially diluted incurred sample response curve is parallel to the calibration curve. Parallelism is a performance characteristic that can detect potential matrix effects and interactions between critical reagents in an assay.

Precision: Precision is the closeness of agreement (i.e., degree of scatter) among a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Processed sample: A processed sample is the final extract (before instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Processing batch: A processing batch is a group of unknown samples from one or more study subjects, calibrators, and a set of QCs that are subjected to the analytical methodology together.

Prozone: The prozone is an effect observed when increasing analyte concentrations result in either no change or decreased detector response when compared to the preceding concentration. (Also see the **Hook effect**)

Quality control sample (QC): A QC is a biological matrix with a known quantity of analyte that is used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of study samples analyzed in an individual run.

Quantification range: The quantification range is the range of concentrations, including the ULOQ and the LLOQ that can be reliably and reproducibly quantified with accuracy and precision with a concentration-response relationship.

Recovery: Recovery refers to the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.

Reintegration: Reintegration is a reanalysis of the chromatographic peak.

Reference standard: A reference standard is a chemical substance of known purity and identity which is used to prepare calibration standards and quality controls. Three types of reference standards are usually used: (1) certified (e.g., USP compendial standards), (2) commercially-supplied, and (3) custom-synthesized.

Reproducibility: Reproducibility is the precision between two laboratories. It also represents the precision of the method under the same operating conditions over a short period of time.

Response function: Response function is the mathematical expression that describes the relationship between known sample concentrations and the response of the instrument (Also refer to **Calibration curve**).

Sample: A sample is a generic term encompassing controls, blanks, unknowns, and processed samples.

Selectivity: Selectivity is the extent to which the method can determine a particular compound in the analyzed matrices without interference from matrix components.

Sensitivity: Sensitivity is defined as the lowest analyte concentration in the matrix that can be measured with acceptable accuracy and precision (i.e., LLOQ).

Specificity: Specificity is the ability of the method to assess, unequivocally, the analyte in the presence of other components that are expected to be present (e.g., impurities, degradation products, matrix components, etc.).

Spiked samples: A spiked sample is a general term that refers to calibrators (calibration standards) and quality controls.

Stability: Stability is a measure of the intactness an analyte (lack of degradation) in a given matrix under specific storage and use conditions relative to the starting material for given time intervals.

Standard curve: Refer to **Calibration curve**.

Stock Solution: A stock solution refers to an analyte in a solvent or mixture of solvents at a known concentration, which is used to prepare calibrators or QCs.

Study samples: Study samples refer to samples from subjects or patients enrolled in a study.

System suitability: System suitability is a determination of instrument performance (e.g., sensitivity and chromatographic retention) by analyzing a set of reference standards before the analytical run.

Total error: Total error is the sum of the absolute value of the errors in accuracy (%) and precision (%). Total error is reported as percent (%) error.

Unknown: An unknown is a biological sample that is the subject of the analysis.

Upper limit of quantification (ULOQ): The ULOQ is the highest amount of an analyte in a sample that can be quantitatively determined with precision and accuracy.

Within-run: Within-run refers to the time period during a single analytical or validation run.

Zero calibrator: A zero calibrator is a blank sample to which the internal standard is added.