Guidance for Industry

Bioanalytical Method Validation

DRAFT GUIDANCE

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Revision 1

Guidance for Industry

Bioanalytical Method Validation

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This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

This guidance provides assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), biologic license applications (BLAs), and supplements in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies that require pharmacokinetic (PK) or biomarker concentration evaluation. This guidance also applies to bioanalytical methods used for nonclinical pharmacology/toxicology studies. For studies related to the veterinary drug approval process (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 PK studies.

The information in this guidance generally applies to bioanalytical procedures, such as gas chromatography (GC); high-pressure liquid chromatography (LC); combined GC and LC mass spectrometric (MS) procedures, such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS; and ligand binding assays (LBAs), and immunological and microbiological procedures that are performed for the quantitative determination of drugs and/or metabolites, and therapeutic proteins in biological matrices, such as blood, serum, plasma, urine, tissue, and skin.

This guidance provides general recommendations for bioanalytical method validation. The recommendations can be modified depending on the specific type of analytical method used.

Originally issued in 2001, this guidance has been revised to reflect advances in science and technology related to validating bioanalytical methods. The guidance is being reissued in draft to enable public review and comment before it is finalized.

[1] This guidance has been prepared by the Bioanalytical Methods Working Group in the Center for Drug Evaluation and Research (CDER) in cooperation with the Center for Veterinary Medicine (CVM) at the Food and Drug Administration.

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

This guidance was originally developed based on the deliberations following two workshops: Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies (December 3-5, 1990[2]) and Bioanalytical Methods Validation: A Revisit With a Decade of Progress (January 12-14, 2000[3]). Since publication of the guidance in May 2001, additional workshops have been held that have helped guide the current revisions to the guidance: the Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays (May 1-3, 2006[4]) and the AAPS/FDA Workshop on Incurred Sample Reanalysis (February 2008[5]).

Selective, sensitive, and validated analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) and biomarkers are critical for the successful conduct of nonclinical and/or biopharmaceutics and clinical pharmacology studies. Validating bioanalytical methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix (e.g., blood, plasma, serum, or urine) is reliable and reproducible for the intended use. Fundamental parameters for this validation include the following:

- Accuracy
- Precision
- Selectivity
- Sensitivity
- Reproducibility
- Stability

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of a method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. For pivotal studies that require regulatory action for approval or labeling, such as BE or PK studies, the bioanalytical methods should be fully validated. For exploratory methods used for the sponsor's internal decision making, less validation may be sufficient.

When changes are made to a previously validated method, additional validation may be needed. For example, published methods of analysis are often modified to suit the requirements of the laboratory performing the assay, and during the course of a typical drug development program, a defined bioanalytical method often undergoes many modifications. These modifications should

[2] Workshop Report: Shah, V.P. et al., Pharmaceutical Research: 1992; 9:588-592.
[3] Workshop Report: Shah, V.P. et al., Pharmaceutical Research: 2000; 17: 1551-1557
[4] Workshop Report: Viswanathan, C.T., Pharmaceutical Research: 2007; 24: 1962-7
[5] Workshop Report: Fast, D., *AAPS Journal*: 2009; 11: 238-241.

be validated to ensure suitable performance of the analytical method. The evolutionary changes needed to support specific studies call for different levels of validation to demonstrate the validity of method performance.

The following define and characterize the different types and levels of methods validation.

Full Validation

Full validation of bioanalytical methods is important:

- During development and implementation of a novel bioanalytical method.
- For analysis of a new drug entity.
- For revisions to an existing method that add metabolite quantification

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. Typical bioanalytical method modifications or changes that fall into this category include but are not limited to:

- Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology (e.g., change in detection systems)
- Change in anticoagulant in harvesting biological fluid (e.g., heparin to EDTA)
- Change in matrix within species (e.g., human plasma to human urine)
- Change in sample processing procedures
- Change in species within matrix (e.g., rat plasma to mouse plasma)
- Change in relevant concentration range
- Changes in instruments and/or software platforms
- Modifications to accommodate limited sample volume (e.g., pediatric study)
- Rare matrices
- Selectivity demonstration of an analyte in the presence of concomitant medications

Cross-Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross-validation would be a situation in which an original validated bioanalytical method serves as the *reference,* and the revised bioanalytical method is the *comparator.* The comparisons should be done both ways.

When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter-laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-

MS/MS vs. ELISA[6]) in different studies are included in a regulatory submission. All modifications to an existing method should be assessed to determine the recommended degree of validation.

The analytical laboratory conducting nonclinical pharmacology/toxicology studies for regulatory submissions should adhere to FDA's Good Laboratory Practices (GLPs) requirements[7] (21 CFR Part 58). The bioanalytical method for human BA, BE, PK, and drug interaction studies must meet the criteria specified in 21 CFR 320.29.

Analytical laboratories should have written standard operating procedures (SOPs) to ensure a complete system of quality control and assurance. SOPs should cover all aspects of analysis from the time the sample is collected and reaches the laboratory until the results of the analysis are reported. The SOPs also should include record keeping, security and chain of sample custody (accountability systems that ensure integrity of test articles), sample preparation, and analytical tools such as methods, reagents, equipment, instrumentation, and procedures for quality control and verification of results.

The following sections discuss in more detail chromatographic methods, ligand binding assays, incurred sample reanalysis, and other issues that should be considered and how best to document validation methods.

III. CHROMATOGRAPHIC METHODS

A. Reference Standards

Analysis of drugs and their metabolites in a biological matrix is performed using calibration standards and quality control samples (QCs) spiked with reference standards. The purity of the reference standard used to prepare spiked samples can affect study data. For this reason, authenticated analytical reference standards of known identity and purity should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used.

Three types of reference standards are usually used: (1) certified reference standards (e.g., USP compendial standards), (2) commercially-supplied reference standards obtained from a reputable commercial source, and/or (3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, expiration date, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference and internal standard (IS) used. If the reference or internal standard expires, stock solutions made with this lot of standard should not be used unless purity is re-established.

B. Bioanalytical Method Development and Validation

[6] Enzyme linked immunosorbent assay.

[7] For the CVM, all bioequivalence studies are subject to Good Laboratory Practices.

A specific, detailed, written description of the bioanalytical method should be established *a priori*. This can be in the form of a protocol, study plan, report, and/or SOP. Each step in the method should be investigated to determine the extent to which environmental, matrix, or procedural variables could affect the estimation of analyte in the matrix from the time of collection of the samples to the time of analysis.

Appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the matrix used for production batches is different from the matrix used during method validation. Matrix effects on ion suppression or enhancement or on extraction efficiency should be addressed. A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report), including a description of validation runs that failed.

Measurements for each analyte in the biological matrix should be validated. Method development and validation for a bioanalytical method should include demonstrations of (1) selectivity; (2) accuracy, precision, and recovery; (3) the calibration curve; (4) sensitivity; (5) reproducibility; and (6) stability of analyte in spiked samples.

1. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Evidence should be provided that the substance quantified is the intended analyte. Analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

Potential interfering substances in a biological matrix include endogenous matrix components; metabolites; decomposition products; and, in the actual study, concomitant medication and other xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

2. Accuracy, Precision, and Recovery

The *accuracy* of an analytical method describes the closeness of mean test results obtained by the method to the actual value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte (i.e., QCs). Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the nominal value serves as the measure of accuracy.

The *precision* of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous

volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run and between-run precision. *Within-run precision* (intra-batch precision or within-run repeatability) is an assessment of precision during a single analytical run. *Between-run precision* (inter-batch precision or between-run repeatability) is an assessment of precision over time and may involve different analysts, equipment, reagents, and laboratories.

Sample concentrations above the upper limit of the standard curve should be diluted. The accuracy and precision of these diluted samples should be demonstrated in the method validation.

The *recovery* of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

3. Calibration Curve

A *calibration (standard) curve* is the relationship between instrument response and known concentrations of the analyte. The relationship between response and concentration should be continuous and reproducible. A calibration curve should be generated for each analyte in the sample. The calibration standards can contain more than one analyte. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. In rare cases, matrices may be difficult to obtain (e.g., cerebrospinal fluid). In such cases, calibration curves constructed in surrogate matrices should be justified. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without analyte or internal standard), a zero sample (matrix sample processed without analyte but with internal standard), and at least six non-zero samples (matrix samples processed with analyte and internal standard) covering the expected range, including LLOQ.

Method validation experiments should include a minimum of six runs conducted over several days, with at least four concentrations (including LLOQ, low, medium, and high) analyzed in duplicate in each run.

a. Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the LLOQ if the following conditions are met:

- The analyte response at the LLOQ should be at least five times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible, and the back-calculated concentration should have precision that does not exceed 20% of the CV and accuracy within 20% of the nominal concentration. The LLOQ should not be confused with the limit of detection (LOD) and/or the low QC sample.
- The LLOQ should be established using at least five samples and determining the CV and/or appropriate confidence interval should be determined.

b. Upper Limit of Quantification (ULOQ)

The highest standard will define the ULOQ of an analytical method.

- Analyte peak (response) should be reproducible and the back-calculated concentration should have precision that does not exceed 15% of the CV and accuracy within 15% of the nominal concentration

c. Calibration Curve/Standard Curve/Concentration-Response

- The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. Standards/calibrators should not deviate by more than 15% of nominal concentrations, except at LLOQ where the standard/calibrator should not deviate by more than 20%.
- The acceptance criterion for the standard curve is that at least 75% of non-zero standards should meet the above criteria, including the LLOQ. Excluding an individual standard should not change the model used. Exclusion of calibrators for reasons other than failing to meet acceptance criteria and assignable causes is discouraged.

d. Quality Control Samples (QCs)

- At least three concentrations of QCs in duplicate should be incorporated into each run as follows: one within three times the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end (high QC) of the range of the expected study concentrations.
- The QCs provide the basis of accepting or rejecting the run. At least 67% (e.g., at least four out of six) of the QCs concentration results should be within 15% of their respective nominal (theoretical) values. At least 50% of QCs at each level should be within 15% of their nominal concentrations. A confidence interval

approach yielding comparable accuracy and precision in the run is an appropriate alternative.

- The minimum number of QCs should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.
- It is recommended that calibration standards and QCs be prepared from separate stock solutions. However, standards and QCs can be prepared from the same spiking stock solution, provided the stability and accuracy of the stock solution have been verified. A single source of blank matrix may also be used, provided absence of matrix effects on extraction recovery and detection has been verified. At least one demonstration of precision and accuracy of calibrators and QCs prepared from separate stock solutions is expected.

Acceptance/rejection criteria for spiked, matrix-based calibration standards and QCs should be based on the nominal (theoretical) concentration of analytes.

4. Sensitivity

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

5. Reproducibility

Reproducibility of the method is assessed by replicate measurements using the assay, including quality controls and possibly incurred samples. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument interruptions.

6. Stability

The chemical stability of an analyte in a given matrix under specific conditions for given time intervals is assessed in several ways. Pre-study stability evaluations should cover the expected sample handling and storage conditions during the conduct of the study, including conditions at the clinical site, during shipment, and at all other secondary sites.

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.

Stability testing should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. If, during sample analysis for a study, storage conditions changed and/or exceeded the sample storage conditions evaluated during method validation, stability should be established under these new conditions.

The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations 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. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations. Stability samples should be compared to freshly made calibrators and/or freshly made QCs. At least three replicates at each of the low and high concentrations should be assessed. Stability sample results should be within 15% of nominal concentrations.

a. Freeze and Thaw Stability

During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles.

b. Bench-Top Stability

Bench top stability experiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples.

c. Long-Term Stability

The storage time in a long-term stability evaluation should equal or exceed the time between the date of first sample collection and the date of last sample analysis.

d. Stock Solution Stability

The stability of stock solutions of drug and internal standard should be evaluated. When the stock solution exists in a different state (solution vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.

e. Processed Sample Stability

The stability of processed samples, including the resident time in the autosampler, should be determined.

C. Validated Method: Use, Data Analysis, and Reporting

This section describes the expectations for the use of a validated bioanalytical method for routine drug analysis.

- System suitability: If system suitability is assessed, a specific SOP should be used. Apparatus conditioning and instrument performance should be determined using spiked

samples independent of the study calibrators, QCs, or study samples. Data should be maintained with the study records.

- Calibration curves and QCs should be included in all analytical runs.

  An analytical run should consist of QCs, calibration standards, and one or more batches of processed samples. A batch may consist of all of the processed unknown samples of one or more subjects in a study and QCs. If the bioanalytical method necessitates separation of the overall analytical run into distinct processing batches (e.g., capacity limit of 96-well plates or solid phase extraction manifold, extraction by multiple analysts), each distinct processing batch should process at least duplicates QCs at all QC levels (e.g., low, middle, high) along with the study samples. In such cases, acceptance criteria should be established for the analytical run as a whole as well as the distinct processing batches.

- The calibration (standard) curve should cover the expected study sample concentration range.

- Accuracy and precision as outlined in section III.B.2. should be provided for both the inter-run and intra-run experiments and tabulated for all runs (passed and failed).

- Concentrations in unknown samples should not be extrapolated below the LLOQ or above the ULOQ of the standard curve. Instead, the standard curve should be extended and revalidated, or samples with higher concentration should be diluted and reanalyzed. Concentrations below the LLOQ should be reported as zeros.

  Any required sample dilutions should use like matrix (e.g. human to human).

- Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available.

- Response Function: Typically, the same curve fitting, weighting, and goodness of fit determined during pre-study validation should be used for the calibration curve within the study. Response function should be determined by appropriate statistical tests based on the actual standard points during each run in the validation. Changes in the response function relationship between pre-study validation and routine run validation indicate potential problems. Internal standard response should be monitored for drift. An SOP should be developed *a priori* to address issues related to variability of the IS response.

- The QCs should be used to accept or reject the run. Runs should be rejected if the calibration standards or QCs fall outside the acceptance criteria stated above (III.B.2).

- QCs should be interspersed with study samples during processing and analysis. The minimum number of QCs to ensure proper control of the assay should be at least 5% of the number of unknown samples or a total of six QCs, whichever is greater.

- 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. Accuracy and precision of the additional QCs should be validated before continuing with the analysis. If the partial validation is acceptable, samples that have already been analyzed do not require re-analysis.

- All study samples from a subject should be analyzed in a single run.

- Carryover should be assessed and monitored during analysis. If carryover occurs, it should be mitigated or reduced.
- Incurred sample reanalysis (ISR) should be performed (See Section V. Incurred Sample Reanalysis).
- Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis. Reasons for repeat analyses could include samples outside of the assay range, sample processing errors, equipment failure, and poor chromatography. Reassays should be done in triplicate if sample volume allows. The rationale, approach, and all data for the repeat analysis and reporting should be clearly documented.
- Samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria.
- The data from rejected runs should be documented but need not be reported; however, the fact that a run was rejected and the reason for failure should be reported.
- 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 *Safety Testing of Drug Metabolites*).
- Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are determined as outliers should also be reported.
- Sample Data Reintegration: An SOP or guideline for sample data reintegration should be established *a priori.* This SOP or guideline should define the criteria for reintegration and how the reintegration is to be performed. The rationale for the reintegration should be clearly described and documented. Audit trails should be maintained. Original and reintegration data should be reported.

IV. LIGAND BINDING ASSAYS

Many of the bioanalytical validation parameters and principles discussed above are also applicable to microbiological and ligand-binding assays (LBA). These types of assays have a variety of design configurations that possess some unique characteristics that should be considered during method validation.

A. Key Reagents

Key reagents, such as reference standards, antibodies, tracers, and matrices should be characterized appropriately and stored under defined conditions.

Assay reoptimization or validation may be important when there are changes in key reagents. For example:

Labeled analytes (tracers)

- Binding should be reoptimized.
- Performance should be verified with standard curve and QCs.

Antibodies

- Key cross-reactivities should be checked.
- Tracer experiments above should be repeated.

Matrices

- Tracer experiments above should be repeated.

B. Bioanalytical Method Development and Validation

A specific, detailed, written description of the bioanalytical method should be established *a priori*. This can be in the form of a protocol, study plan, report, and/or SOP. Each step in the method should be investigated to determine the extent to which environmental, matrix, or procedural variables can affect the estimation of analyte in the matrix from the time of collection of the samples to the time of analysis.

It may be important to consider the variability of the matrix. Appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation. A bioanalytical method should be validated for the intended use or application. All experiments used to make claims or draw conclusions about the validity of the method should be presented in a report (method validation report).

Measurements for each analyte in the biological matrix should be validated. Method development and validation for a bioanalytical method should include demonstrations of (1) selectivity, (2) accuracy, precision, recovery, (3) the calibration curve, (4) sensitivity, (5) reproducibility, and (6) stability of analyte in spiked samples.

1. Selectivity

As with chromatographic methods (described in Section III), LBAs should be shown to be selective for the analyte. The following recommendations for dealing with two selectivity issues should be considered:

a. Interference from Substances Physiochemically Similar to the Analyte

- Cross-reactivity of metabolites, concomitant medications, and their significant metabolites, or endogenous compounds should be evaluated individually and in combination with the analyte of interest.
- When possible, the LBA should be compared with a validated reference method (such as LC-MS) using incurred samples and predetermined criteria to assess the accuracy of the LBA method.

b. Matrix Effects

Matrix effects should be evaluated. For example:

- The calibration curve in biological fluids should be compared with calibrators in buffer to detect matrix effects using at least ten sources of blank matrix.
- Parallelism of diluted study samples should be evaluated with diluted standards to detect matrix effects.
- Nonspecific binding should be determined.

2. Accuracy, Precision and Recovery

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte (QCs). Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The mean value should be within 20% of the actual value except at LLOQ, where it should not deviate by more than 25%.

The *precision* should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected study sample concentrations is recommended. The precision determined at each concentration level should not exceed 20% of the CV except for the LLOQ, where it should not exceed 25% of the CV. Precision is further subdivided into *within-run* and *between-run* precision. *Within-run* (also known as intra-batch precision or repeatability) is an assessment of the precision during a single analytical run. *Between-run* precision (also known as interbatch precision or repeatability), is a measurement of the precision with time, and may involve different analysts, equipment, reagents, and laboratories.

Samples with concentrations over the ULOQ should be diluted with the same matrix as used for the study samples, and accuracy and precision should be demonstrated.

For LBAs that employ sample extraction, the *recovery* of an analyte is the measured concentration relative to the known amount added to the matrix. Recovery experiments should be performed for extracted samples at three concentrations.

3. Calibration Curve

Most LBA calibration (standard) curves are inherently nonlinear and, in general, more concentration points may be recommended to define the fit over the standard curve range than for chromatographic assays. In addition to their nonlinear characteristics, the response-error relationship for immunoassay standard curves is a variable function of the mean response (heteroscedasticity). For these reasons, the standard curve should consist of a minimum of six, duplicate non-zero calibrator concentrations covering the entire range including LLOQ and excluding blanks (either single or replicate). The concentration-response relationship is most often fitted to a 4- or 5-parameter logistic model, although other models may be used with suitable validation. Calibrators should be prepared in the same matrix as the study samples. If an

alternate matrix is used, proper justification should be provided. A calibration curve should be generated for each analyte in the sample.

Method validation experiments should include a minimum of six runs conducted over several days, with at least six concentrations (including LLOQ, low, medium, and high) analyzed in duplicate in each run.

a. Lower Limit of Quantification (LLOQ)

- The lowest concentration on the calibration curve should be the LLOQ if the following conditions are met:
- Analyte peak (response) should be identifiable, discrete, and reproducible and back-calculated concentration should have precision that does not exceed 25% CV and accuracy within 25% of the nominal concentration. The LLOQ should not be confused with the LOD and/or the low QCs.
- The LLOQ should be established using at least five samples and determining coefficient of variation and/or appropriate confidence intervals.

b. Upper Limit of Quantification (ULOQ)

The highest standard will define the ULOQ of an analytical method.

- Analyte response should be reproducible and the back-calculated concentration should have precision that does not exceed 20% CV and accuracy within 20% of the nominal concentration.

c. Calibration Curve/Standard Curve/Concentration-Response

- The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The standard calibrator concentrations should be within 25% of the nominal concentration at LLOQ and within 20% of the nominal concentration at all other concentrations.
- The acceptance criterion for the standard curve is that at least 75% of non-zero standards should meet the above criteria, including the LLOQ. Excluding an individual standard should not change the model used. Exclusion of calibrators for reasons other than failing to meet acceptance criteria and assignable causes is discouraged.
- Total error (accuracy and precision) should not exceed 30%. Values falling outside these limits should be discarded, provided they do not change the established model.

d. Quality Control Samples (QCs)

- At least three concentrations of QCs in duplicate should be incorporated into each run as follows: one within three times the LLOQ (low QC), one in the midrange (middle QC), and one approaching the high end (high QC) of the range of the expected study sample concentrations.
- The results of the QCs provide the basis of accepting or rejecting the run. At least 67% (e.g., at least four out of six) of the QC concentration results should be within 20% of their respective nominal (theoretical) values. At least 50% of QCs at each level should be within 20% of their nominal concentrations. A confidence interval approach yielding comparable accuracy and precision in the run is an appropriate alternative.
- The minimum number of QCs should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.
- It is recommended that calibration standards and QCs be prepared from separate stock solutions. However, standards and QCs can be prepared from the same spiking stock solution, provided the stability and accuracy of the stock solution have been verified. A single source of blank matrix may also be used, provided absence of matrix effects on extraction recovery and detection has been verified. At least one demonstration of precision and accuracy of calibrators and QCs prepared from separate stock solutions is expected.

Acceptance/rejection criteria for spiked, matrix-based calibration standards and QCs should be based on the nominal (theoretical) concentration of analytes.

4. Sensitivity

Sensitivity is defined as the lowest analyte concentration that can be measured with acceptable accuracy and precision.

5. Reproducibility

Reproducibility of the method is assessed by replicate measurements using the assay, including quality controls and possibly incurred samples. Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument interruptions.

6. Stability

The chemical stability of an analyte in a given matrix under specific conditions for given time intervals is assessed in several ways. Pre-study stability evaluations should cover the expected sample handling and storage conditions during the conduct of the study, including conditions at the clinical site, during shipment, and at all other secondary sites.

Stability samples should be compared to freshly made calibrators and/or freshly made QCs. At least three replicates at each of the low and high concentrations should be assessed. Assessments of analyte stability should be conducted in the same matrix as that of the study samples. All

stability determinations should use samples prepared from a freshly made stock solution. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis (e.g., long-term, bench top, and room temperature storage; and freeze-thaw cycles). If, during sample analysis for a study, storage conditions changed and/or exceed the sample storage conditions evaluated during method validation, stability should be established under the new conditions. Stock solution stability also should be assessed. Stability sample results should be within 15% of nominal concentrations.

a. Freeze and Thaw Stability

During freeze/thaw stability evaluations, the freezing and thawing of stability samples should mimic the intended sample handling conditions to be used during sample analysis. Stability should be assessed for a minimum of three freeze-thaw cycles.

b. Bench-Top Stability

Bench top stability experiments should be designed and conducted to cover the laboratory handling conditions that are expected for study samples.

c. Long-Term Stability

The storage time in a long-term stability evaluation should equal or exceed the time between the date of first sample collection and the date of last sample analysis.

d. Stock Solution Stability

The stability of stock solutions of drug should be evaluated. When the stock solution exists in a different state (solutions vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.

e. Processed Sample Stability

The stability of processed samples, including the time until completion of analysis, should be determined.

C. Validated Method: Use, Data Analysis, and Reporting

This section describes the expectations for the use of a validated bioanalytical method for routine drug analysis.

- Standard curves and QCs should be included in all analytical runs.
- The calibration (standard) curve should cover the expected study sample concentration range.

- Accuracy and precision as outlined in Section IV.B.2 should be provided for both the inter-run and intra-run experiments and tabulated for all runs (passed and failed).
- Concentrations in unknown samples should not be extrapolated below the LLOQ or above the ULOQ of the standard curve. Instead, the standard curve should be extended and revalidated, or samples with higher concentrations should be diluted and reanalyzed. Concentrations below the LLOQ should be reported as zeros. Any required sample dilutions should use like matrix (e.g., human to human).
- Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability has been demonstrated.
- Response Function: Typically, the same curve fitting, weighting, and goodness of fit determined during pre-study validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation. Any changes in the response function relationship between pre-study validation and routine run validation indicate potential problems. An SOP should be developed *a priori* to address such issues.
- The QCs should be used to accept or reject the run. Runs should be rejected if the calibration standards or QCs fall outside the acceptance criteria stated above.
- QCs should be interspersed with study samples during processing and analysis. The minimum number of QCs to ensure proper control of the assay should be at least 5% of the number of unknown samples or a total of six QCs, whichever is greater.
- If the study sample concentrations are clustered in a narrow range of the standard curve, additional QCs should be added in the sample range. Accuracy and precision of the additional QCs should be validated before continuing with the analysis. If the partial validation is acceptable, samples that have already been analyzed do not require re-analysis.
- All study samples from a subject should be analyzed in a single run.
- Carryover should be assessed and monitored during analysis. If carryover occurs, it should be mitigated or reduced.
- Incurred sample reanalysis (ISR) should be performed (See Section V. Incurred Sample Reanalysis).
- Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis. Reasons for repeat analyses could include samples outside of the assay range, sample processing errors, and equipment failure. The rationale, approach, and all data for the repeat analysis and reporting should be clearly documented.
- Samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria.
- The data from rejected runs should be documented, but need not be reported; however, the fact that a run was rejected and the reason for failure should be reported.

- 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 on its activity (see guidance for industry *Safety Testing of Drug Metabolites*).
- Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision, excluding values that are determined as outliers, should also be reported.

V. INCURRED SAMPLE REANALYSIS

Incurred sample reanalysis (ISR) is a necessary component of bioanalytical method validation and is intended to verify the reliability of the reported subject sample analyte concentrations. ISR is conducted by repeating the analysis of a subset of subject samples from a given study in separate runs on different days to critically support the precision and accuracy measurements established with spiked QCs; the original and repeat analysis is conducted using the same bioanalytical method procedures. ISR samples should be compared to freshly prepared calibrators. ISR is expected for all in vivo human BE studies and all pivotal PK or pharmacodynamic (PD) studies. For nonclinical safety studies, the performing laboratory should conduct ISR at least once for each method and species.

For regulatory submissions containing only a few studies, it may be advantageous to incorporate ISR into the method development and validation stage by conducting a pilot study prior to the pivotal study. This approach allows for the remediation of methodological issues prior to conduct of the pivotal study. For applications with a greater number of pivotal PK or PD studies, ISR should be monitored in a larger number and variety of studies.

Standard operating procedures should be established and followed to address the following points:

- The total number of ISR samples should be 7% of the study sample size.
- In selecting samples for reanalysis, adequate coverage of the PK profile in its entirety should be provided and should include assessments around Cmax and in the elimination phase for all study subjects.
- Two-thirds (67%) of the repeated sample results should be within 20% for small molecules and 30% for large molecules. The percentage difference of the results is determined with the following equation:

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

Written procedures should be in place to guide an investigation in the event of ISR failure for the purpose of resolving the lack of reproducibility. All aspects of ISR evaluations should be documented to reconstruct the study conduct as well as any investigations thereof. ISR results should be included in the final report of the respective study.

VI. 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 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, may 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 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 is generally not recommended unless an analyte-free biological matrix is not readily available or cannot be prepared. In such cases, use of an alternate analyte-free matrix should be justified, and the calibration standard in the alternate matrix should be demonstrated to have no matrix effect when compared to the actual biological matrix of the study samples.
- The QCs should be prepared by spiking known quantities of analyte(s) in the same biological matrix as the study samples. The endogenous concentrations of the analyte in the biological matrix should be evaluated prior to 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.

B. Biomarkers

The recommendations in this guidance pertain only 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. Information about defining the biological role of a biomarker is available on the FDA Drug Development Tools website.

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 and/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 fit-for-purpose approach should be used when evaluating the extent of method validation that is appropriate. When biomarker data will be used to support a regulatory action, such as the pivotal determination of safety and/or effectiveness or to support labeled dosing instructions, 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 PK assays. The accuracy, precision, selectivity, range, reproducibility, and stability of a biomarker assay are important characteristics that define the method. The approach used for PK assays should be the starting point for validation of biomarker assays, although 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 biologic products. The recommendations in this section of the guidance do not apply to commercial diagnostic kits that are intended for point-of-care patient diagnosis, but rather to analytical methods that are used during the development of new drugs and therapeutic biologics. The reader should refer to the appropriate CDRH guidance documents regarding FDA expectations for commercial diagnostic kits. Furthermore, these recommendations do not apply to Clinical Laboratory Improvements Amendments (CLIA)-regulated entities or to assays designed to quantify or identify genes or genetic polymorphisms.

If a sponsor uses a commercially available diagnostic kit to measure a biomarker, drug, or therapeutic biologic concentration during the development of a novel drug or therapeutic biologic product, FDA makes the following recommendations.

Ligand binding assay (LBA) kits with various detection platforms are sometimes used to determine analyte concentrations in PK or PD 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 PK or PD studies should be demonstrated.

Diagnostic kit validation data provided by the manufacturer may not ensure reliability of the kit method for drug development purposes. 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:

- Site-specific validation should be performed. Specificity, accuracy, precision, and stability should be demonstrated under actual conditions of use. Modifications from kit processing instructions should be validated completely.
- 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.
- 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 the endogenous compounds section of this guidance for additional discussion (see VI.A).
- If the analyte source (reference standard) in the kit differs from that of the subject samples (e.g., protein isoform variation), testing should evaluate differences in immunological activity with the kit reagents.
- If multiple kit lots are used within a study, lot-to-lot variability and comparability should be addressed for critical reagents.
- Individual batches using multiple assay plates (e.g., 96-well ELISA plates) should include sufficient replicate QCs on each plate to monitor accuracy. Acceptance criteria should be established for the individual plates and overall analytical run.

D. New Technologies

FDA encourages the development and use of new bioanalytical technologies. Generally, the use and submission of data based on new technologies should be supported with data generated by established technology, until the new approaches become accepted practice.

Although the Dried Blood Spot (DBS) methodology has been successful in individual cases, the method has not yet been widely accepted. Benefits of DBS include reduced blood sample volumes collected for drug analysis and ease of collection, storage, and transportation. A comprehensive validation will be essential prior to using DBS in regulated studies. This validation should address, at a minimum, the effects of the following issues: storage and handling temperature, 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.

VII. DOCUMENTATION

General and specific SOPs and good record keeping are essential to a properly validated analytical method. The validity of an analytical method should be established and verified by laboratory studies, and the documentation of successful completion of such studies should be provided in the assay validation report. The data generated for bioanalytical method establishment and the QCs should be documented and available for data audit and inspection. Documentation for submission to FDA should include the following:

- Method development and validation data and reports.
- Bioanalytical reports of the application of any methods to study sample analysis.
- Overall summary information including limitations to use.

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 the 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. Electronic audit trails should be available for all chromatography acquisition and data processing software and other means of electronic data capture. Information related to each bioanalytical run should be maintained at the laboratory and should include the analysts performing the run, start and stop times (duration), raw data, integration codes, and/or other reporting codes.

A. System Suitability/Equilibration

System suitability is routinely assessed before an analytical run. Data generated from system suitability checks should be maintained in a specific file on-site and should be available for inspection. System suitability samples should be different from the study samples, standards, and QCs to be analyzed in the run. Therefore, study samples, standards, or QCs should not be used as their own system suitability samples within the analytical run.

B. Summary Information

Summary information should include:

- A summary of assay methods used for each study protocol. Each summary should provide the protocol number, protocol title, assay type, assay method identification code, bioanalytical report code, and 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 assay method identification code, 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, validation reports, and bioanalytical reports.

C. Documentation for Method Validation

Documentation for method validation should include:

- An operational description of the analytical method used in the study.
- A detailed description of the assay procedure (analyte, IS, sample pre-treatment, method of extraction, and analysis).

- A description of the preparation of the calibration standards and QCs including blank matrix, anticoagulant if applicable, dates of preparation, and storage conditions.
- Evidence of purity and identity of drug, metabolites, and IS used at the time of the validation experiments. The chromatography of the analyte should be interference-free. The batch/lot numbers and storage conditions of the reference standards used to prepare the calibration standards and QCs of each assay should be provided.
- A description of potential interferences for the drug or metabolites in LBAs.
- A description of experiments conducted to determine accuracy, precision, recovery, selectivity, stability, limits of quantification, calibration curve (equations and weighting functions used), and a summary of the results including intra- and inter-assay precision and accuracy. QCs results that fail to meet the acceptance criteria should not be excluded from calculations of accuracy and precision unless there is an assignable cause.
- A description of cross-validation or partial validation experiments and supporting study data, if applicable.
- Legible annotated chromatograms or mass spectrograms, if applicable.
- Description and supporting data of significant investigations of unexpected results if applicable.
- Tabulated data including, but not limited to, the following:
  - All validation experiments with analysis dates, whether the experiments passed or failed and the reason for the failure.
  - Results of calibration standards from all validation experiments, including calibration range, response function, back-calculated concentrations, accuracy and precision.
  - QC results from all validation experiments (within- and between-run precision and accuracy).
  - Data from all stability experiments, i.e., storage temperatures, duration of storage, dates of analysis, and dates of preparation of QCs and calibration standards used in the stability experiments.
  - Data on selectivity, LLOQ, carry-over, extraction recovery, matrix effect if applicable, dilution integrity, anticoagulant effect if applicable.

All measurements with the individual calculated concentrations should be presented in the validation report.

D. Documentation for Bioanalytical Report

Documentation of the application of validated bioanalytical methods to routine drug analysis should include:

- Evidence of purity at the time of use and identity of drug standards, metabolite standards, and internal standards used during routine analyses, and expiration or retest dates.
- Step-by-step description of procedures for preparation of QCs and calibrators.

- Sample identification, collection dates, storage prior to shipment, information on shipment batch, and storage prior to analysis. Information should include dates, times, and sample condition.
- Any deviations from the validated method, significant equipment and material changes, SOPs, protocols, and justifications for deviations.
- Equations and regression methods for calculation of concentration results.
- Complete serial chromatograms from 5-20% of subjects, with standards and QCs from those analytical runs. For pivotal bioequivalence studies used to support approval, chromatograms from 20% of serially selected subjects should be included. In other studies, chromatograms from 5% of randomly selected subjects in each study should be included. Subjects whose chromatograms are to be submitted should be defined prior to the analysis of any clinical samples.
- Reasons for missing samples.
- Repeat analyses should be documented with the reason(s) for the repeat analysis, the initial and repeat analysis results, the reported result, assay run identification, and the manager authorizing reanalysis. Repeat analysis of a clinical or nonclinical sample should be performed only under a predefined SOP.
- Data from reintegrated chromatograms should be documented with the reason for reintegration, initial and repeat integration results, the method used for reintegration, the reported result, assay run identification, and the manager authorizing reintegration. Reintegration of a clinical or nonclinical sample should be performed only under a predefined SOP.

The following tables should be included:

- Summary of intra- and inter-assay values of QCs and calibration curve standards used for accepting the analytical run. QC graphs and trend analyses are encouraged.
- A table listing all of the accepted and rejected analytical runs of clinical or nonclinical samples. The table should include assay run identification, assay method, and the subjects that were analyzed in each run. Tables with the individual back-calculated results for all study samples should be submitted.
- Examples of tabular listings of analytical data for reports can be found in the Appendix (IX. Appendix)

VIII. GLOSSARY

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

Analyte: A specific chemical moiety being measured; it can be an intact drug, a biomolecule or its derivative, a metabolite, and/or a degradation product in a biologic matrix.

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

Biological matrix: A 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: A batch is a number of unknown samples from one or more patients in a study and QCs that are processed at one time.

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

Calibration standard: 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 quality control samples and in unknown study samples are determined.

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

Incurred Sample Reanalysis (ISR): A repeated measurement of analyte concentration from study samples to demonstrate reproducibility.

Internal standard (IS): Test compound(s) (e.g., structurally similar analog, stable labeled compound) added to both calibration standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).

Limit of detection (LOD): The lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise.

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

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

Method: A comprehensive description of all procedures used in sample analysis.

Precision: 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: The final extract (prior to instrumental analysis) of a sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Quality Control Sample (QCs): A sample 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 the unknown samples analyzed in an individual run.

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

Recovery: 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.

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

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

Selectivity/Specificity: The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants, or matrix components.

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

Stability: The chemical stability of an analyte in a given matrix under specific conditions for given time intervals.

Standard curve: The relationship between the experimental response values and the analytical concentrations (also called a *calibration curve*).

System suitability: Determination of instrument performance (e.g., sensitivity and chromatographic retention) by analysis of a set of reference standards conducted prior to the analytical run.

Unknown: A biological sample that is the subject of the analysis.

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

IX. APPENDIX

Report Format examples for applications to CDER or CVM. Summary tables should be included in Module 2 of the eCTD.

TABLE 1-EXAMPLE OF AN OVERALL SUMMARY TABLE FOR A METHOD VALIDATION REPORT*

This table contains fictitious information, which serves illustrative purposes only.

	Results	Hyperlink†	Comments
Methodology	LC/MS/MS	01-SOP-001	
Method Validation Report 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%		
Dilution integrity (specify dilution factors and QC concentrations and matrix 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	Demonstrated for X	**Summary tables**	

Top temperature stability	hours at Y°C	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	
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		

***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 2-EXAMPLE OF INFORMATION FOR REFERENCE STANDARDS FOR METHOD VALIDATION CONDUCTED IN PLASMA MATRIX*

Include information linking the use of specific lots of reference standards for the analyte and internal standard to specific method validation experiments*

This table contains fictitious information, which serves illustrative purposes only.

Reference standard	Retest/expiration date	Lot Numbers	Validation experiment	Dates of Analysis	Evidence of purity (Hyperlink)	Comments
Compound A	MM/DD/YY	RS01	Runs 1-3 (accuracy and precision) Run 3 (selectivity experiment)	MM/DD/YY	001MVR-01/RS01	
Compound A internal standard	MM/DD/YY	RS02	Runs 1-3 (accuracy and precision) Run 3 (selectivity experiment)	MM/DD/YY	001MVR-01/RS02	

*** A similar table would be included in the bioanalytical study report linking the use of reference standards to specific batches or analytical runs.**

TABLE 3-EXAMPLE OF METHOD VALIDATION SUMMARY AND STUDY INFORMATION FOR CLINICAL STUDY XXXXX

This table contains fictitious information, which serves illustrative purposes only.

	Results	Comments
Methodology	LC-MS/MS	
Biological matrix	Human plasma	
Anticoagulant (if applicable)	EDTA	
Calibration curve range	XXX-YYY ng/ml	
Analyte of interest	Compound A	
Internal standard	Compound A internal standard	
Method validation summary		
Method Validation Report Number	MVR-001	
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%	
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%	
Long-term stability	Demonstrated for X days at Y°C	
Freeze-thaw stability	Demonstrated for Y cycles at Z°C	
Study Information		
ISR (include the percentage of samples analyzed)	> 67% of samples acceptable	
Duration from time sample was first drawn to date of last sample analysis (including ISR)	XXX months	
Actual sample storage temperature*	Y°C at AAA Z°C at BBB	

* list the sample storage temperature at each site

TABLE 4-EXAMPLE OF SUMMARY ANALYTICAL RUNS FOR A BIOANALYTICAL STUDY REPORT *

Provide a table summarizing both the failed and accepted runs for each study.

This table contains fictitious information, which serves illustrative purposes only.

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.