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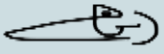
Signature		**Signature**	
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Acronyms

BA/BE	Bioavailability/Bioequivalence
C max	Maximum Concentration
CoA	Certificate of Analysis
DMM	Dried Matrix Method
eCTD	electronic Common Technical Document
EDTA	Ethylenediaminetetraacetic Acid
ICH	The International Conference on Harmonization.
IS	Internal Standard
ISR	Incurred Sample Reanalysis
LBA	Ligand Binding Assay
LC	Liquid Chromatography
LLOQ	Lower Limit of Quantification
MRD	Minimum Required Dilution
MS	Mass Spectrometry
QC	Quality Control
QCs	Quality Control Sample
SOP	Standard Operating Procedure
ULOQ	Upper Limit of Quantification
V/V	Volume in Volume

Definitions

The Guideline uses definitions harmonized with European Medicine Agency Good Pharmacovigilance Practice (EMA GVP), International Council for Harmonization (ICH), Council for International Organizations of Medical Sciences (CIOMS), Upsala Monitoring Center (UMC) and World Health Organizations (WHO) terminology. For any definition not included in this section, users should refer to EMA GVP Glossary. ICH guidelines, CIOMS, UMC and WHO technical documents.

Analyte	A specific chemical moiety being measured, including an intact drug, a biomolecule or its derivative or a metabolite in a biological matrix.
Analytical Run (also referred to as "Run")	A complete set of analytical and study samples with appropriate number of calibration standards and QCs for their validation. Several runs may be completed in one day or one run may take several days to complete.
Anchor Calibration Standards/Anchor Points	Spiked samples set at concentrations below the LLOQ or above the ULOQ of the calibration curve and analyzed to improve curve fitting in LBAs
Batch (for Reference Standards and Reagents)	A specific quantity of material produced in a process or series of processes so that it is expected to be homogeneous within specified limits. Also referred to as "Lot".
Biological Drugs	Drugs that are made by living organisms or cells (e.g., therapeutic proteins).
Biological Matrix	A biological material including, but not limited to, blood, serum, plasma and urine
Binding Reagent	A reagent that binds to the analyte in LBA-based bioanalytical methods.
Blank Sample	A sample of a biological matrix to which no analyte, no IS and no additional-alternative matrix or buffer has been added.

Interfering Substance	A substance that is present in the matrix that may affect the quantification of an analyte.
Internal Standard (IS)	A structurally similar analogue or stable isotope labelled compound added to calibration standards, QCs and study samples at a known and constant concentration to facilitate quantification of the target analyte.
Ligand Binding Assay (LBA)	A method to analyze an analyte of interest using reagents that specifically bind to the analyte. The analyte is detected using reagents labelled with e.g., an enzyme, radioisotope, fluorophore or chromophore. Reactions are carried out in microtiter plates, test tubes, disks, etc.
Matrix Effect	The direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample.
Minimum Required Dilution (MRD)	The initial dilution factor by which biological samples are diluted with buffer solution for the analysis by LBAs. The MRD may not necessarily be the ultimate dilution but should be identical for all samples including calibration standards and QCs. However, samples may require further dilution.
Nominal Concentration	Theoretical or expected concentration
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
Partial Validation	Validation based on evaluation of selected validation parameters. Applicable to methods that were changed after full validation.
Processed Sample	The final sample that has been subjected to various manipulations (e.g., extraction, dilution, concentration).

Quality Control Sample (QC)	A biological matrix spiked with a known quantity of analyte that is used to monitor the performance of a bioanalytical method and assess the integrity and validity of the results of the unknown samples analyzed in an individual batch or run
Reference Standard	A well-characterized substance of known purity and identity used to prepare calibration and quality control samples
Reintegration	Change of the original integration of a chromatographic peak
Replicate	One of several determinations or measurements of a sample, calibration standards or QC.

CHAPTER ONE

Introduction

This guideline sets the requirements for validating bioanalytical methods and conducting study sample analysis. It provides clear expectations to ensure that all quantitative measurements of drugs, metabolites, and biomarkers in biological matrices are reliable, reproducible, and scientifically justified. It outlines the standards necessary to generate data suitable for regulatory submissions and to ensure consistency across laboratories and studies.

Purpose

The purpose of this guideline is to ensure that all bioanalytical methods used in regulatory-relevant studies are scientifically sound, validated, and consistently applied. It aims to promote the generation of high-quality data that support the assessment of safety, efficacy, and quality of medicinal products throughout development and post-marketing phases. Comprehensive requirements and additional guidance are provided in the ICH M10 Guideline on Bioanalytical Method Validation and Study Sample Analysis.

Scope

This guideline applies to all quantitative bioanalytical methods used to measure drugs, metabolites, and biomarkers in biological matrices for nonclinical and clinical studies submitted for regulatory review. The document is intended for applicants for marketing authorizations, pharmaceutical companies and manufacturers, and contract research organizations (CROs) involved in developing, validating, or applying bioanalytical methods.

Structure

This is the first version of this guideline and is organized into four chapters. CHAPTER ONE covers the Introduction, Purpose, Scope, and Structure. CHAPTER TWO outlines the detailed procedures and methods. CHAPTER THREE defines responsibilities in relation to this guideline. CHAPTER FOUR includes the document history and version control and references.

CHAPTER TWO

2. Procedures & Methods:

2.1 Method development

The main objective of method development is to define the method design, operating conditions, limitations, and suitability for validation. Applicants should, where feasible, understand the analyte's physicochemical properties, metabolism, blood/plasma distribution, protein binding, and any relevant prior analytical methods.

Development should define procedures and conditions for reliable analyte quantification, including reference standards, critical reagents, calibration curves, QCs, selectivity, specificity, sensitivity, accuracy, precision, recovery, stability, and MRD where applicable.

Extensive documentation is not required during development; however, any method changes made after development, especially those affecting sample analysis, should be justified and documented. Validation then confirms suitability for study sample analysis

2.2 Method validation

2.2.1 Full validation

Bioanalytical method validation confirms that an assay reliably measures analyte concentrations in biological matrices. Full validation is required for new methods, published methods adopted for regulatory use, or commercial kits repurposed for drug development.

When multiple analytes are measured, such as drugs, metabolites, enantiomers, or isomers, each analyte must meet the applicable validation and analytical requirements.

For chromatographic methods, full validation should generally assess selectivity, specificity, matrix effects, calibration curve and range, accuracy, precision, carry-over, dilution integrity, stability, and reinjection reproducibility, unless scientifically justified otherwise.

For ligand-binding assays, validation should generally assess specificity, selectivity, calibration curve, range, accuracy, precision, carry-over, dilution linearity, stability, and, where suitable samples are available, parallelism.

Validation should reflect actual sample analysis. The validation matrix should match the study matrix, including anticoagulants and additives. Scientifically justified surrogate matrices may be used when the study matrix is rare or difficult to obtain.

Matrix differences within the same species are generally not significant. The bioanalytical method and validation procedures should be predefined in a protocol, study plan, report, laboratory notebook, or SOP.

2.2.2 Partial validation

Modifications to a fully validated analytical method may be evaluated by partial validation. Partial validation can range from as little as one accuracy and precision determination to a nearly full validation. The items in a partial validation should be determined according to the extent and nature of the changes made to the method.

2.2.3 Cross validation

Cross validation is required to demonstrate how the reported data are related when multiple bioanalytical methods and/or multiple bioanalytical laboratories are involved.

2.3 Chromatography

2.3.1 Reference standards

Calibration standards and QCs are prepared by spiking blank biological matrix with analyte solutions from reference standards. They should use separate stock solutions unless one stock solution is verified for accurate preparation and stability. An appropriate internal standard should be added to calibration standards, QCs, and study samples. If no internal standard is used, scientific justification is required.

Reference standards and internal standards should be well characterized, traceable, and suitable for the method because their quality can affect analytical results and study conclusions.

Reference standards may be compendial, commercial, or fully characterized in-house/external materials. A CoA or equivalent record should document identity, purity, storage, retest/expiry date, and batch information.

A CoA is not required for the IS if its suitability has been demonstrated—for example, by showing that the analyte or any impurities do not cause analytical interference.

For MS methods, a stable isotope-labelled analyte is recommended as the internal standard where feasible. Its isotope purity, absence of exchange, and potential unlabeled analyte contribution should be evaluated.

Stock and working solutions should be prepared only from reference standards that remain within their documented stability period, as stated in the CoA (expiration or retest date).

2.3.2 Validation

2.3.2.1 Selectivity

Selectivity is the method's ability to measure the analyte without interference from blank matrix components.

Selectivity should be assessed using blank samples from at least six individual sources/lots, unless fewer are justified for rare matrices. Internal standard selectivity should also be evaluated.

Interference at the analyte or IS retention time should not exceed 20% of the analyte response at the LLOQ or 5% of the IS response in the LLOQ sample.

For the investigation of selectivity in lipemic matrices at least one source of matrix should be used. To be scientifically meaningful, the matrix used for these tests should be representative as much as possible of the expected study samples. A naturally lipemic matrix with abnormally high levels of triglycerides should be obtained from donors. Although it is recommended to use lipemic matrix from donors, if this is difficult to obtain, matrix can be spiked with triglycerides even though it may not be representative of study samples. However, if the drug impacts lipid metabolism or if the intended patient population is hyperlipidemic, the use of spiked samples is discouraged. This evaluation is not necessary for nonclinical studies unless the drug impacts lipid metabolism or is administered in a particular animal strain that is hyperlipidemic.

For the investigation of selectivity in hemolyzed matrices at least one source of matrix should be used. Hemolyzed matrices should be obtained by spiking matrix with hemolyzed whole blood (at least 2% V/V) to generate a visibly detectable hemolyzed sample.

2.3.2.2 Specificity

Specificity is the ability to distinguish the analyte from related substances, including metabolites, isomers, impurities, degradation products, and relevant concomitant medications.

When related substances are expected, their impact should be assessed during validation or using pre-dose study samples. For LC-MS methods, assessment may include molecular-weight comparison and chromatographic separation.

Interfering responses should not exceed 20% of the analyte response at the LLOQ or 5% of the IS response.

The possibility of back-conversion of a metabolite into the parent analyte during the successive steps of the analysis (including extraction procedures or in the MS source) should also be evaluated when relevant (e.g., potentially unstable metabolites such as ester analytes to ester/acidic metabolites, unstable N-oxides or glucuronide metabolites, lactone-ring structures). It is acknowledged that this evaluation will not be possible in the early stages of drug development of a new chemical entity when the metabolism is not yet evaluated. However, it is expected that this issue should be investigated, and partial validation performed if needed. The extent of back-conversion, if any, should be established and the impact on the study results should be discussed in the Bioanalytical Report.

2.3.2.3 Matrix effect

Matrix effect is an altered analyte response caused by matrix components and should be evaluated across independent matrix sources/lots during validation.

Matrix effect should be assessed using at least three replicates of low and high QCs prepared from at least six matrix sources/lots. Accuracy should be within ±15% and precision should not exceed 15%; fewer sources may be justified for rare matrices.

The matrix effect should also be evaluated in relevant patient populations or special populations (e.g., hepatically impaired or renally impaired) when available. An additional evaluation of the matrix effect is recommended using hemolyzed or lipemic matrix samples during method validation on a case-by- case basis, especially when these conditions are expected to occur within the study.

2.3.2.4 Calibration curve and range

The calibration curve defines the relationship between nominal analyte concentration and analytical response. It should be prepared in the same biological matrix as study samples and span the range from LLOQ to ULOQ, with one curve per analyte and analytical run.

Each calibration curve should include a blank sample, a zero sample, and at least six calibration levels, including the LLOQ and ULOQ.

The regression model, weighting, and transformation should be predefined and determined during validation. Blank and zero samples should not be used to calculate the regression equation.

Calibration parameters and back-calculated concentrations should be reported. At least three independent validation runs over several days should be acceptable. Accuracy should be within ±20%

at the LLOQ and ±15% at other levels, with at least 75% of standards and at least six levels meeting criteria in the case that replicates are used, the criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of the calibration standards tested per concentration level. In the case that a calibration standard does not comply with these criteria, this calibration standard sample should be rejected, and the calibration curve without this calibration standard should be re-evaluated, including regression analysis. For accuracy and precision runs, if all replicates of the LLOQ or the ULOQ calibration standard in a run are rejected, then the run should be rejected, the possible source of the failure should be determined and the method revised, if necessary. If the next validation run also fails, then the method should be revised before restarting validation.

The calibration curve should be prepared using freshly spiked calibration standards in at least one assessment. Subsequently, frozen calibration standards can be used within their defined period of stability.

2.3.2.5 Accuracy and precision

A. Preparation of quality control sample

QCs should mimic study samples and be prepared, stored, and analyzed under anticipated study conditions to confirm method validity.

Calibration standards and QCs should use independent stock solutions unless a shared stock solution has verified accuracy and stability. Blank matrix should be free of interference and matrix effects.

Accuracy and precision QCs should include at least four levels: LLOQ, low QC, medium QC, and high QC.

For non-accuracy and precision validation runs, low, medium and high QCs may be analyzed in duplicate. These QCs, along with the calibration standards, will provide the basis for the acceptance or rejection of the run.

B. Evaluation of accuracy and precision

Accuracy and precision should be assessed within runs and between runs using the same data.

Within-run accuracy and precision should be evaluated by analysing at least 5 replicates at each QC concentration level in each analytical run. Between-run accuracy and precision should be evaluated by analysing each QC concentration level in at least 3 analytical runs over at least two days. To enable the evaluation of any trends over time within one run, it is recommended to demonstrate accuracy and precision of the QCs over at least one of the runs in a size equivalent to a prospective analytical run of study samples. Reported method validation data and the determination of accuracy and

precision should include all results obtained, including individual QCs outside of the acceptance criteria, except those cases where errors are obvious and documented. Within-run accuracy and precision data should be reported for each run. If the within-run accuracy or precision criteria are not met in all runs, an overall estimate of within-run accuracy and precision for each QC level should be calculated. Between- run (intermediate) precision and accuracy should be calculated by combining the data from all runs.

The calibration curves for these assessments should be prepared using freshly spiked calibration standards in at least one run. If freshly spiked calibration standards are not used in the other runs, stability of the frozen calibration standards should be demonstrated.

Accuracy should be within ±15% of nominal concentration, except ±20% at the LLOQ. Precision should not exceed 15%, except 20% at the LLOQ. For non-accuracy and precision runs, at least two-thirds of total QCs and at least 50% at each level should meet ±15% criteria.

2.3.2.6 Carry-over

Carry-over is residual analyte from a previous sample that affects a subsequent measurement.

Carry-over should be minimized during development and evaluated during validation by analyzing blanks after the ULOQ standard. Carry-over should not exceed 20% of the analyte response at the LLOQ or 5% of the IS response. If unavoidable, mitigation procedures must be validated and applied.

2.3.2.7 Dilution integrity

Dilution integrity confirms that sample dilution does not affect accuracy or precision. The same matrix species used for QCs should be used for dilution.

Dilution QCs should exceed the ULOQ and be diluted with blank matrix. At least five replicates per dilution factor should be tested. Mean accuracy should be within ±15% and precision should not exceed 15%.

In the cases of rare matrices, use of a surrogate matrix for dilution may be acceptable. It should be demonstrated that this does not affect precision and accuracy.

2.3.2.8 Stability

Stability studies should confirm that sample preparation, processing, analysis, and storage do not affect analyte concentration.

Stability conditions should reflect study sample storage, handling, matrix, anticoagulant, and container conditions. Published literature alone is not sufficient. Validated storage periods should cover or exceed study sample storage periods.

Stability of the analyte in the matrix is evaluated using low and high concentration QCs. Aliquots of the low and high QCs are analyzed at time zero and after the applied storage conditions that are to be evaluated. One bulk QC should be prepared at each concentration level. For each concentration tested, the bulk sample should be divided into a minimum of 3 aliquots that will be stored, stressed and analyzed.

The QCs should be analyzed against a calibration curve, obtained from freshly spiked calibration standards in a run with its corresponding freshly spiked QCs or QCs for which stability has been proven. The mean concentration at each QC level should be within ±15% of the nominal concentration. If the concentrations of the study samples are consistently higher than the ULOQ of the calibration range, the concentration of the high QC should be adjusted to reflect these higher concentrations. It is recognized that this may not be possible in nonclinical studies due to solubility limitations.

For fixed dose combination products and specifically labelled drug regimens, the freeze-thaw, bench-top and long-term stability tests of an analyte in matrix should be conducted with the matrix spiked with all of the dosed compounds.

The following stability assessments should be considered, as applicable:

A. Stability of the analyte in matrix

Freeze-thaw stability in matrix

To assess the impact of repeatedly removing samples from frozen storage, the stability of the analyte should be assessed after multiple cycles of freezing and thawing. Low and high QCs should be thawed and analyzed according to the same procedures as the study samples. QCs should be kept frozen for at least 12 hours between the thawing cycles. QCs for freeze-thaw stability should be assessed using freshly prepared calibration standards and QCs, or QCs for which stability has been proven. The number of freeze-thaw cycles validated should equal or exceed that of the freeze-thaw cycles undergone by the study samples, but a minimum of three cycles should be conducted.

Bench-top (short-term) stability in matrix

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

Low and high QCs should be thawed in the same manner as the study samples and kept on the bench top at the same temperature and for at least the same duration as the study samples.

The total time on the bench top should be concurrent; it is not acceptable to use additive exposure to bench top conditions (i.e., time from each freeze-thaw evaluation should not be added up).

Long-term stability in matrix

The long-term stability of the analyte in matrix stored in the freezer should be established. Low and high QCs should be stored in the freezer under the same storage conditions and at least for the same duration as the study samples.

For chemical drugs, the stability at one temperature (e.g., -20°C) to can be extrapolated to lower temperatures (e.g., -70/-80°C).

For biological drugs, a bracketing approach can be applied, e.g., in the case that the stability has been demonstrated at -70/-80°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored.

B. Stability of the analyte in processed samples

The stability of processed samples, including the time until completion of analysis (in the autosampler/instrument), should be determined. For example:

• Stability of the processed sample under the storage conditions to be used during the analysis of study samples (dry extract or in the injection phase)

• On-instrument/autosampler stability of the processed sample at injector or autosampler temperature.

The total time that a processed sample is stored must be concurrent (i.e., autosampler and other storage times cannot be added together).

C. Stability of the analyte and IS in stock and working solutions

The stability of the stock and working solutions of the analyte and IS should be determined under the storage conditions used during the analysis of study samples by using the lowest and the highest concentrations of these solutions. They should be assessed using the response of the detector.

Stability of the stock and working solutions should be tested with an appropriate dilution, taking into consideration the linearity and measuring range of the detector. If the stability varies with concentration, then the stability of all concentrations of the stock and working solutions needs to be assessed. If no isotopic exchange occurs for the stable isotopically-labelled IS under the same storage

conditions as the analyte for which the stability is demonstrated, then no additional stability determinations for the IS are necessary. If the reference standard expires, or it is past the retest date, the stability of the stock solutions made previously with this lot of reference standard are defined by the expiration or retest date established for the stock solution. The practice of making stock and working solutions from reference standards solely for extending the expiry date for the use of the reference standard is not acceptable.

In addition, the following test should be performed if applicable:

D. Stability of the analyte in whole blood

Sufficient attention should be paid to the stability of the analyte in the sampled matrix (blood) directly after collection from subjects and prior to preparation for storage to ensure that the concentrations obtained by the analytical method reflect the concentrations of the analyte in the subject's blood at the time of sample collection.

If the matrix used is plasma, the stability of the analyte in blood should be evaluated during method development (e.g., using an exploratory method in blood) or during method validation. The results should be provided in the Validation Report.

2.3.2.9 Reinjection reproducibility

Reinjection reproducibility should be evaluated when processed samples may need reinjection due to instrument interruption or equipment failure.

Assessment should include reinjection of a run containing calibration standards and at least five replicates of low, medium, and high QCs after storage. Precision and accuracy of reinjected QCs support processed-sample viability.

The results should be included in the Validation Report or provided in the Bioanalytical Report of the study where it was conducted.

2.4 Study sample analysis

Study samples may be analyzed after validation, although some parameters such as long-term stability may be completed later. Before regulatory submission, validation must be complete and samples, QCs, and calibration standards must be processed according to the validated method. System suitability, if used, should be predefined and should not use subject samples.

2.4.1 Analytical run

An analytical run includes a blank sample, zero sample, at least six calibration levels, QCs at low, medium, and high levels in duplicate or at least 5% of study samples, and the study samples. QCs should bracket study samples and support run accuracy and precision. Study samples should always be bracketed by QCs.

Calibration standards and QCs should be prepared independently unless stock solution accuracy and stability are verified. Samples should preferably be processed as a single batch; if multiple batches are unavoidable, each batch should include low, medium, and high QCs.

For comparative BA/BE studies, it is advisable to analyze all samples of one subject together in one analytical run to reduce variability.

The impact of any carry-over that occurs during study sample analysis should be assessed and reported. If carry-over is detected, its impact on the measured concentrations should be mitigated (e.g., non-randomization of study samples, injection of blank samples after samples with an expected high concentration) or the validity of the reported concentrations should be justified in the Bioanalytical Report.

2.4.2 Acceptance criteria for an analytical run

Acceptance or rejection criteria should be predefined in the protocol, study plan, or SOP. For runs with multiple batches, criteria apply to both the full run and individual batches.

Calibration standards in a failed batch cannot be used to support the acceptance of other batches within the analytical run.

Back-calculated calibration standards should be within ±15% of nominal concentration, except ±20% at the LLOQ. At least 75% of standards, including at least six concentration levels, should meet criteria.

Does not meet the criteria, this calibration standard should be rejected and the calibration curve without this calibration standard should be re-evaluated and a new regression analysis performed.

If the rejected calibration standard is the LLOQ, the new lower limit for this analytical run is the next lowest acceptable calibration standard of the calibration curve. This new lower limit calibration standard will retain its original acceptance criteria (i.e., ±15%). If the highest calibration standard is rejected, the ULOQ for this analytical run is the next acceptable highest calibration standard of the calibration curve. The revised calibration range should cover at least 3 QC concentration levels (low,

medium and high). Study samples outside of the revised range should be reanalyzed. If replicate calibration standards are used and only one of the LLOQ or ULOQ standards fails, the calibration range is unchanged.

At least two-thirds of total QCs and at least 50% at each concentration level should be within ±15% of nominal values. If not, the run should be rejected and affected samples reanalyzed, unless reinjection is justified by an assignable technical cause.

Analytical runs containing samples that are diluted and reanalyzed should include dilution QCs to verify the accuracy and precision of the dilution method during study sample analysis. The concentration of the dilution QCs should exceed that of the study samples being diluted (or of the ULOQ) and they should be diluted using the same dilution factor. If multiple dilution factors are used in one analytical run, then dilution QCs need only be diluted by the highest and lowest dilution factors. The within-run acceptance criteria of the dilution QC(s) will only affect the acceptance of the diluted study samples and not the outcome of the analytical run.

When several analytes are assayed simultaneously, there should be one calibration curve for each analyte studied. If an analytical run is acceptable for one analyte but has to be rejected for another analyte, the data for the accepted analyte should be used. The determination of the rejected analyte requires re-extraction and analysis only for the analyte that is reanalyzed. Only data for this reanalyzed analyte needs to be reported.

The back-calculated concentrations of the calibration standards and QCs of passed and accepted runs should be reported. The overall (between-run) accuracy and precision of the QCs of all accepted runs should be calculated at each concentration level and reported in the analytical report. If the overall mean accuracy and/or precision fails the 15% criterion, an investigation to determine the cause of the deviation should be conducted. In the case of comparative BA/BE studies, it may result in the rejection of the data.

2.4.3 Calibration range

If study sample concentrations are expected within a narrow range, the calibration range and QC concentrations should be adjusted to reflect the expected concentrations.

If sample concentrations cluster unexpectedly at one end of the curve, analysis should stop and the calibration range or QC levels should be revised before continuing. Reanalysis of previously analyzed samples is not required solely because of the revision.

The same applies if a large number of the analyte concentrations of the study samples are above the ULOQ. The calibration curve range should be changed, if possible, and QC(s) added or their concentrations modified. If it is not possible to change the calibration curve range or the number of samples with a concentration above the ULOQ is not large, samples should be diluted according to the validated dilution method.

At least 2 QC levels should fall within the range of concentrations measured in study samples. If the calibration curve range is changed, the bioanalytical method should be revalidated (partial validation) to verify the response function and to ensure accuracy and precision.

2.4.4 Reanalysis of study samples

Reasons for study sample reanalysis, number of replicates, and criteria for selecting the reported value should be predefined before analysis begins.

The number and percentage of reanalyzed samples should be reported and discussed in the Bioanalytical Report. For comparative BA/BE studies, rejected-run values should be reported separately.

Some examples of reasons for study sample reanalysis are:

- Rejection of an analytical run because the run failed the acceptance criteria with regard to accuracy of the calibration standards and/or the precision and accuracy of the QCs
- IS response significantly different from the response for the calibration standards and QCs (as pre- defined in an SOP)
- The concentration obtained is above the ULOQ
- The concentration observed is below the revised LLOQ in runs where the lowest calibration standard has been rejected from a calibration curve, resulting in a higher LLOQ compared with other runs
- Improper sample injection or malfunction of equipment
- The diluted study sample is below the LLOQ
- Identification of quantifiable analyte levels in pre-dose samples, control or placebo samples
- Poor chromatography (as pre-defined in an SOP)

For comparative BA/BE studies, reanalysis of study samples for a PK reason (e.g., a sample concentration does not fit with the expected profile) is not acceptable, as it may bias the study result.

Reanalyzed samples should be identified with the initial value, reason for reanalysis, repeat values, accepted value, and justification. A summary by reason should also be included. Non-reportable results may require a single reanalysis; confirmatory cases require replicates if sample volume allows.

The safety of trial subjects should take precedence over any other aspect of the trial. Consequently, there may be other circumstances when it is necessary to reanalyze specific study samples for the purpose of a safety investigation.

2.4.5 Reinjection of study samples

Reinjection of processed samples can be made in the case of equipment failure if reinjection reproducibility has been demonstrated during validation or provided in the Bioanalytical Report where it was conducted. Reinjection of a full analytical run or of individual calibration standards or QCs simply because the calibration standards or QCs failed, without any identified analytical cause, is not acceptable.

2.4.6 Integration of chromatograms

Chromatogram integration and reintegration procedures should be predefined. Deviations, reintegration lists, reasons, original/reintegrated chromatograms, and initial/repeat results should be retained and reported where required.

2.5 Ligand binding assays

2.5.1 Key reagents

A. Reference standard

For LBAs, reference standards should be well characterized and documented. Where possible, calibration and QC reference material should come from the same drug substance batch used in nonclinical or clinical dosing. If the reference batch changes, comparability should be assessed before use.

B. Critical reagents

Critical reagents, such as binding proteins, aptamers, antibodies, conjugates, and enzymatic reagents, directly affect assay performance and should be identified, defined, and quality assured.

Critical reagent documentation should include identity, source, batch/lot number, purity where applicable, concentration where applicable, stability or retest date, and storage conditions.

applicable) and stability/retest date/storage conditions (refer to Table 1). Additional characteristics may be warranted.

Critical reagent lifecycle management should ensure consistency between original and new batches. Minor changes may require limited comparative assessment, while major changes may require additional validation.

Retest dates and validation parameters should be documented in order to support the extension or replacement of the critical reagent. Stability testing of the reagents should be based upon the performance in the bioanalytical method and upon general guidance for reagent storage conditions. It can be extended beyond the expiry date from the supplier. The performance parameters should be documented in order to support the extension or replacement of the critical reagent

2.6 Validation

LBA assay format, including the number of wells per sample, should be specified in the protocol, study plan, or SOP and used consistently during validation and sample analysis. Reportable concentrations should be calculated according to predefined procedures.

2.6.1 Specificity

LBA specificity assesses cross-reactivity with structurally related molecules or concomitant medications. Related molecules should be tested at expected maximum concentrations, and target analyte accuracy at the LLOQ and ULOQ should be within ±25%.

In the event of non-specificity, the impact on the method should be evaluated by spiking increasing concentrations of interfering molecules in blank matrix and measuring the accuracy of the target analyte at the LLOQ and ULOQ. It is essential to determine the minimum concentration of the related molecule where interference occurs. Appropriate mitigation during sample analysis should be employed, e.g., it may be necessary to adjust the LLOQ/ULOQ accordingly or consider a new method.

During method development and early method validation, these “related molecules” are frequently not available. Additional evaluation of specificity may be conducted after the original validation is completed.

2.6.2 Selectivity

LBA selectivity assesses the ability to detect analyte in the presence of non-specific matrix components such as enzymes, heterophilic antibodies, or rheumatoid factor.

Selectivity should be evaluated using at least 10 blank sources spiked at the LLOQ and high QC level, unless fewer are justified for rare matrices. At least 80% of blank sources should show responses below the LLOQ.

The accuracy should be within ±25% at the LLOQ and within ±20% at the high QC level of the nominal concentration in at least 80% of the individual sources evaluated.

Selectivity should be evaluated in lipemic samples and hemolyzed samples. For lipemic and hemolyzed samples, tests can be evaluated once using a single source of matrix.

Selectivity should be assessed in samples from relevant patient populations (e.g., renally or hepatically impaired patients, inflammatory or immuno-oncology patients if applicable). In the case of relevant patient populations, there should be at least five individual patients.

2.6.3 Calibration curve and range

For LBAs, the calibration curve should be prepared in the same biological matrix as study samples where possible and should span the LLOQ to ULOQ. Surrogate matrix use should be scientifically justified.

The curve should include at least six calibration levels, including LLOQ and ULOQ, plus a blank. Anchor points may be used to improve curve fitting but are not part of the quantifiable range.

A minimum of 6 independent runs should be evaluated over several days considering the factors that may contribute to between-run variability.

The accuracy and precision of back-calculated concentrations of each calibration standard should be within ±25% of the nominal concentration at the LLOQ and ULOQ, and within ±20% at all other levels. At least 75% of the calibration standards excluding anchor points, and a minimum of 6 concentration levels of calibration standards, including the LLOQ and ULOQ, should meet the above criteria. The anchor points do not require acceptance criteria since they are beyond the quantifiable range of the curve.

Back-calculated LBA standards should be within ±25% at the LLOQ and ULOQ and ±20% at other levels. At least 75% of non-anchor standards, including at least six levels, should meet criteria.

2.6.4 Accuracy and precision

2.6.4.1 Preparation of quality control samples

LBA QCs should mimic study samples and be prepared, stored, and analyzed under anticipated study conditions.

QC preparation should be independent from calibration standards unless shared stock accuracy and stability are verified. At least five QC levels should be used: LLOQ, low, medium, high, and ULOQ.

For non-accuracy and precision validation runs, low, medium and high QCs may be analyzed in duplicate. These QCs, along with the calibration standards, will provide the basis for the acceptance or rejection of the run.

2.6.4.2 Evaluation of accuracy and precision

LBA accuracy and precision should be assessed within and between runs using the same data.

At least three replicates per QC level should be analyzed in at least six runs over two or more days. All valid results should be reported unless exclusions are clearly documented.

Overall accuracy and precision should be within ±20%, except ±25% at the LLOQ and ULOQ. Precision should not exceed 20%, except 25% at the LLOQ and ULOQ.

For non-accuracy and precision validation runs, at least 2/3 of the total QCs and at least 50% at each concentration level should be within ±20% of the nominal values.

Furthermore, the total error (i.e., sum of absolute values of the errors in accuracy (%) and precision (%)) should be evaluated. The total error should not exceed 30% (40% at LLOQ and ULOQ).

2.6.5 Carry-over

Carry-over is generally not an issue for LBA analyses. However, if the analytical platform is prone to carry-over, the potential of carry-over should be investigated by placing blank samples after the calibration standard at the ULOQ. The response of blank samples should be below the LLOQ.

2.6.6 Dilution linearity and hook effect

Dilution linearity confirms that dilution does not affect measured concentrations and that high concentrations above the ULOQ are not affected by hook effect.

(i) that measured concentrations are not affected by dilution within the calibration range and (ii) that sample concentrations above the ULOQ of a calibration curve are not impacted by hook effect (i.e., a signal suppression caused by high concentrations of the analyte), whereby yielding an erroneous result.

The same matrix as that of the study sample should be used for preparation of the QCs for dilution.

Dilution linearity should be demonstrated using a QC above the ULOQ, analyzed undiluted and at least three dilution factors, using replicate dilution series consistent with planned sample analysis.

The calculated mean concentration for each dilution should be within ±20% of the nominal concentration after correction for dilution and the precision should not exceed 20%.

The dilution factor(s) applied during study sample analysis should be within the range of dilution factors evaluated during validation.

2.6.7 Stability

LBA stability studies should confirm that preparation, processing, analysis, and storage do not affect analyte concentration.

The storage and analytical conditions applied to the stability tests, such as the sample storage times and temperatures, sample matrix, anticoagulant, and container materials should reflect those used for the study samples. Reference to data published in the literature is not considered sufficient. Validation of storage periods should be performed on QCs that have been stored for a time that is equal to or longer than the study sample storage periods.

Stability of the analyte in the studied matrix should be evaluated using low and high concentration QCs. Aliquots of the low and high QCs are analyzed at time zero and after the applied storage conditions that are to be evaluated. One bulk QC should be prepared at each concentration level. For each concentration tested, the bulk sample should be divided into a minimum of three aliquots that will be stored, stressed and analyzed.

The QCs are analyzed against a calibration curve, obtained from freshly spiked calibration standards in a run with its corresponding freshly spiked QCs or QCs for which stability has been proven. While the use of freshly prepared calibration standards and QCs is the preferred approach, it is recognized that in some cases, for macromolecules, it may be necessary to freeze them overnight. In such cases, valid

justification should be provided and freeze-thaw stability demonstrated. QCs should be kept frozen for at least 12 hours between the thawing cycles. The mean concentration at each QC level should be within ±20% of the nominal concentration.

Since sample dilution may be required for many LBA methods due to a narrow calibration range, the concentrations of the study samples may be consistently higher than the ULOQ of the calibration curve. If this is the case, the concentration of the QCs should be adjusted, considering the applied sample dilution, to represent the actual sample concentration range.

For fixed dose combination products and specifically labelled drug regimens, the freeze-thaw, bench-top and long-term stability tests of an analyte in matrix should be conducted with the matrix spiked with all of the dosed compounds, on a case-by-case basis.

The investigation of stability should cover bench top (short-term) stability at room temperature or sample preparation temperature and freeze-thaw stability. In addition, long-term stability should be studied.

For chemical drugs, the stability at one temperature (e.g., -20°C) can be extrapolated to lower temperatures (e.g., -70/-80°C).

For biological drugs, a bracketing approach can be applied, e.g., in the case that the stability has been demonstrated at -70/-80°C and at -20°C, then it is not necessary to investigate the stability at temperatures in between those two points at which study samples will be stored.

2.7 Study sample analysis

LBA study samples may be analyzed after validation, although long-term stability may be completed later. Before regulatory submission, validation must be complete and samples, QCs, and standards must follow the validated method.

2.7.1 Analytical run

An LBA analytical run includes a blank, at least six calibration levels, QCs at low, medium, and high levels in two sets or at least 5% of study samples, and study samples. QCs should bracket study samples.

Most often microtiter plates are used for LBAs. An analytical run may comprise of one or more plate(s). Typically, each plate contains an individual set of calibration standards and QCs. If each plate contains its own calibration standards and QCs then each plate should be assessed on its own. However, for some platforms the sample capacity may be limited. In this case, sets of calibration standards may be placed on the first and the last plate, but QCs should be placed on every single plate. QCs should be placed at least at the beginning (before) and at the end (after) of the study samples of each plate. The QCs on each plate and each calibration curve should fulfil the acceptance criteria for an analytical run. For the calculation of concentrations, the calibration standards should be combined to conduct one regression analysis. If the combined calibration curve does not pass the acceptance criteria the whole run fails.

2.7.2 Acceptance criteria for an analytical run

LBA run acceptance or rejection criteria should be predefined in the protocol, study plan, or SOP. For multi-batch runs, criteria apply to both the run and individual batches.

Calibration standards in a failed batch cannot be used to support the acceptance of other batches within the analytical run.

Back-calculated calibration standards should be within ±20% of nominal concentration, except ±25% at the LLOQ and ULOQ.

±25%. At least 75% of the calibration standards, with a minimum of 6 concentration levels, should fulfil this criterion. This requirement does not apply to anchor calibration standards. If more than 6 calibration standards are used and one of the calibration standards does not meet these criteria, this calibration standard should be rejected and the calibration curve without this calibration standard should be re-evaluated and a new regression analysis performed.

If the rejected calibration standard is the LLOQ, the new lower limit for this analytical run is the next lowest acceptable calibration standard of the calibration curve. If the highest calibration standard is rejected, the new upper limit for this analytical run is the next acceptable highest calibration standard of the calibration curve. The new lower and upper limit calibration standard will retain their original acceptance criteria (i.e., □20%). The revised calibration range should cover all QCs (low, medium and high). The study samples outside of the revised assay range should be reanalyzed.

Each run should contain at least 3 levels of QCs (low, medium and high). During study sample analysis, the calibration standards and QCs should mimic the analysis of the study sample with regard to the number of wells used per study sample. At least 2/3 of the QCs and 50% at each concentration level should be within 20% of the nominal value at each concentration level. Exceptions to these criteria should be justified and predefined in the SOP or protocol.

The overall mean accuracy and precision of the QCs of all accepted runs should be calculated at each concentration level and reported in the analytical report. In the case that the overall mean accuracy and/or precision exceeds 20%, additional investigations should be conducted to determine the cause(s) of this deviation. In the case of comparative BA/BE studies, it may result in the rejection of the data.

2.7.3 Calibration range

At least two QC levels should fall within the concentration range measured in study samples. If unexpected clustering occurs, analysis should stop and the calibration range or QC levels should be revised before continuing.

2.7.4 Reanalysis of study samples

Reasons for LBA study sample reanalysis, number of reanalyzes, and criteria for selecting the reported value should be predefined before sample analysis begins.

The number of samples (and percentage of total number of samples) that have been reanalyzed should be reported and discussed in the Bioanalytical Report. For comparative BA/BE studies, a separate table should report values from rejected runs.

Some examples of reasons for study sample reanalysis are:

- Rejection of an analytical run because the run failed the acceptance criteria with regard to accuracy of the calibration standards and/or the precision and accuracy of the QCs,
- The concentration obtained is above the ULOQ
- The concentration obtained is below the LLOQ in runs where the lowest calibration standard has been rejected from a calibration curve, resulting in a higher LLOQ compared with other runs
- Malfunction of equipment
- The diluted sample is below the LLOQ
- Identification of quantifiable analyte levels in pre-dose samples, control or placebo samples.
- When samples are analyzed in more than one well and non-reportable values are obtained due to one replicate failing the pre-defined acceptance criteria (e.g., excessive variability between wells, one replicate being above the ULOQ or below the LLOQ).

For comparative BA/BE studies, reanalysis of study samples for a PK reason (e.g., a sample concentration does not fit with the expected profile) is not acceptable, as it may bias the study result.

Reanalyzed samples should be identified with the initial result, reason, repeat values, final accepted value, and justification. A summary by reason should also be provided. Non-reportable results may require one reanalysis; confirmatory cases require multiple determinations if sample volume allows.

The safety of trial subjects should take precedence over any other aspect of the trial. Consequently, there may be other circumstances when it is necessary to reanalyze specific study samples for the purpose of an investigation.

2.8 Incurred sample reanalysis (ISR)

ISR verifies the reliability of reported sample concentrations when study samples may behave differently from calibration standards and QCs because of binding, back-conversion, inhomogeneity, concomitant medication, or study-specific biological components.

ISR should be performed at least in the following situations:

- For nonclinical studies within the scope of this guideline, ISR should, in general, be performed at least once per species.
- All pivotal comparative BA/BE studies
- First clinical trial in subjects
- Pivotal early patient trial(s), once per patient population
- First or pivotal trial in patients with impaired hepatic and/or renal function

ISR is performed by reanalyzing a predefined subset of study samples in separate runs on different days using the same bioanalytical method.

For studies with up to 1,000 samples, at least 10% should be reanalyzed. For larger studies, reanalyze 10% of the first 1,000 samples plus 5% of samples above 1,000. Selection criteria should be predefined and cover key concentration ranges, including near Cmax and elimination phase.

(100) plus 5% of the number of samples that exceed 1000 samples should be assessed. Objective criteria for choosing the subset of study samples for ISR should be predefined in the protocol, study plan or an SOP. While the subjects/animals should be picked as randomly as possible from the dosed study population, adequate coverage of the concentration profile is important. Therefore, it is recommended that the samples for ISR be chosen around the maximum concentration (Cmax) and some in the elimination phase. Additionally, the samples chosen should be representative of the whole study.

Samples should not be pooled, as pooling may limit anomalous findings. ISR samples and QCs should be processed and analyzed in the same manner as in the original analysis. ISR should be performed within the stability window of the analyte, but not on the same day as the original analysis.

The percent difference between the initial concentration and the concentration measured during the repeat analysis should be calculated in relation to their mean value using the following equation:

$$\% \text{ difference} = \frac{\text{repeat value} - \text{initial value}}{\text{mean value}} x\ 100$$

Acceptance criteria are ±20% for at least two-thirds of chromatographic repeats and ±30% for at least two-thirds of LBA repeats.

If the overall ISR results fail the acceptance criteria, an investigation should be conducted and the causes remediated. There should be an SOP that directs how investigations are triggered and conducted. If an investigation does not identify the cause of the failure, the potential impact of an ISR failure on study validity should also be provided in the Bioanalytical Report. If ISR meets the acceptance criteria yet shows large or systemic differences between results for multiple samples, this may indicate analytical issues and it is advisable to investigate this further.

Examples of trends that are of concern may include:

- All ISR samples from one subject fail
- All ISR samples from one run fail

All aspects of ISR evaluations should be documented to allow reconstruction of the study and any investigations. Individual samples that are quite different from the original value (e.g., > 50%, "flyers") should not trigger reanalysis of the original sample and do not need to be investigated. ISR sample data should not replace the original study sample data.

2.9 Partial and cross validation

2.9.1 Partial validation

Partial validation evaluates changes to a fully validated method and may range from one accuracy/precision assessment to nearly full validation. Previously established stability may not need repeating after facility transfer if justified.

For chromatographic methods, typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following situations:

- Analytical site change using same method (i.e., bioanalytical method transfers between laboratories)
- A change in analytical method (e.g., change in detection systems, platform)

- A change in sample processing procedures
- A change in sample volume (e.g., the smaller volume of pediatric samples)
- Changes to the calibration concentration range
- A change in anticoagulant (but not changes in the counter-ion) in biological fluids (e.g., heparin to ethylenediaminetetraacetic acid (EDTA))
- Change from one matrix within a species to another (e.g., switching from human plasma to serum or cerebrospinal fluid) or changes to the species within the matrix (e.g., switching from rat plasma to mouse plasma)
- A change in storage conditions

For LBAs, typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following situations:

- Changes in LBA critical reagents (e.g., lot-to-lot changes)
- Changes in MRD
- A change in storage conditions
- Changes to the calibration concentration range
- A change in analytical method (e.g., change in detection systems, platform)
- Analytical site change using same method (i.e., bioanalytical method transfers between laboratories)
- A change in sample preparation
- A change in anticoagulant (but not changes in the counter-ion) in biological fluids (e.g., heparin to ethylenediaminetetraacetic acid (EDTA))

The parameters of the partial validations should meet the full validation criteria. If these criteria are not satisfied, additional investigation and validation is warranted.

2.9.2 Cross validation

Cross validation demonstrates comparability when multiple bioanalytical methods or laboratories generate data for the same analyte.

Cross validation is required under the following situations:

- Data are obtained from different fully validated methods within a study.
- Data are obtained within a study from different laboratories with the same bioanalytical method.
- Data are obtained from different fully validated methods across studies that are going to be combined or compared to support special dosing regimens, or regulatory decisions regarding safety, efficacy and labelling.

If data are obtained from different fully validated methods, and these data are not to be combined across studies, cross validation is not generally required.

Cross validation should be performed in advance of study samples being analysed, if possible.

Cross validation should compare the same QCs and, where available, study samples spanning the concentration range using both methods or laboratories.

Bias can be assessed by Bland-Altman plots or Deming regression. Other methods appropriate for assessing agreement between two methods (e.g., concordance correlation coefficient) may be used too. Alternatively, the concentration vs. time curves for study samples could be plotted for samples analyzed by each method to assess bias.

The use of multiple bioanalytical methods for the measurement of the same analyte in the conduct of one comparative BA/BE study is strongly discouraged.

2.10 Additional considerations

2.10.1 Methods for analytes that are also endogenous molecules

Endogenous analytes may be difficult to quantify because the method may not distinguish therapeutic and endogenous molecules, and endogenous levels may vary by population, disease, time, or treatment.

If available, biological matrix to prepare calibration standards and QCs should be the same as the study samples (i.e., authentic biological matrix) and it should be free of matrix effect and interference. The endogenous concentration in the biological matrix chosen should be low enough to obtain an adequate signal-to-noise ratio (e.g., <20% of the LLOQ).

If interference-free matrix is unavailable, concentrations may be determined using surrogate matrix, surrogate analyte, background subtraction, or standard addition approaches.

A. Surrogate matrix approach:

The matrix for the calibration standards is substituted by a surrogate matrix. Surrogate matrices can vary widely in complexity from simple buffers or artificial matrices that try to mimic the authentic one, to stripped matrices or matrices from other species.

B. Surrogate analyte approach:

Stable-isotope labelled analytes are used as surrogate standards in mass spectrometric methods to construct the calibration curve for the quantification of endogenous analytes. In this approach, it is assumed that the physicochemical properties of the authentic and surrogate analytes are the same with the exception of molecular weight. However, isotope standards may differ in retention time and MS sensitivity, therefore, before application of this approach, the ratio of the MS responses (i.e., the response factor) of the labelled to unlabeled analyte should be close to unity and remain constant over the entire calibration range. If the response factor does not comply with these requirements, it should be incorporated into the regression equation of the calibration curve.

C. Background subtraction approach:

The concentration of the endogenous analyte observed in a pooled/representative matrix is subtracted from the concentration observed in the spiked standards, subsequently the net differences are used to construct the calibration curve.

When the background concentrations are lowered by dilution of the blank matrices before spiking with the standards (e.g., if a lower LLOQ is required) the composition of the matrices in the study samples and the calibration standards is different, which may cause different recoveries and matrix effects.

These differences should be considered when validating the method.

D. Standard addition approach:

The standard addition approach is only applicable for analytical platforms with linear responses. Typically, the standard addition method is used to determine the concentration of the endogenous analyte in the authentic matrix to be used for preparation of standards and QCs. However, this approach can be employed for determination of study samples as well. In this approach, every study sample is divided into aliquots of equal volume. All aliquots, but one, are separately spiked with

known and varying amounts of the analyte standards to construct a calibration curve for either the authentic blank matrix or every study sample (e.g., with 3 to 5 points). The endogenous blank concentration or the study sample concentration is then determined as the negative x-intercept of the standard calibration curve prepared in that particular study sample.

Validation of an analytical method for an analyte that is also an endogenous compound will require the following considerations, in addition to the validation shown in sections 2.

2.10.1.1 Quality control samples for methods for analytes that are also endogenous molecules

For endogenous analytes, QC preparation should account for endogenous concentrations and represent expected study sample concentrations.

The QCs should resemble study samples and should be prepared in the same matrix. In principle, all QC concentrations used for validation should be aliquots of the authentic biological matrix unspoked (endogenous QC with a concentration between LLOQ and low QC, if possible) and spiked with known amounts of the authentic analyte (low QC, middle QC and high QC). In spiked samples (e.g., LLOQ, low QC), the added amount should be enough to provide concentrations that are three-fold higher or statistically different from the endogenous concentration. If options such as multiple lots, alternative vendors and matrices of special populations that might contain a lower concentration of the analyte continue to yield matrices in which the endogenous levels are so high that it is not possible to prepare the low QC in the authentic matrix, diluted (surrogate) matrix may be used.

2.10.1.2 Selectivity, recovery and matrix effects for methods for analytes that are also endogenous molecules

The assessment of selectivity is complicated by the absence of interference-free matrix. For chromatography, peak purity should be investigated as part of method validation by analyzing matrices obtained from several donors (at least six normal blanks, one hemolyzed blank and one lipemic blank) using a discriminative detection system (e.g., tandem mass spectrometry (MS/MS)). Other approaches, if justified by scientific principles, may also be considered.

For the Standard Addition and Background Subtraction Approaches, as the same biological matrix and analyte are used for study samples and calibration standards, the same recovery and matrix effect occurs in the study samples and the calibration standards. However, if the endogenous component were not completely identical (e.g., recombinant proteins) the potential difference in recovery should be assessed in a parallelism test. For the Surrogate Matrix and Surrogate Analyte Approaches, the matrix effect and the extraction recovery may differ between calibration standards and study samples.

Matrix effect should be evaluated to ensure that it does not impact accuracy and precision, mainly at LLOQ level.

- If the Surrogate Matrix Approach is used, the impact of the different matrix effect and recovery in both the surrogate and authentic matrix should be assessed. This should be investigated in an experiment using QCs spiked with analyte in the matrix, the endogenous matrix only and spiked analyte in the surrogate matrix alone against the surrogate calibration curve.
- If the Surrogate Analyte Approach is used in mass spectrometry chromatographic methods, the impact of the different matrix effect and recovery between surrogate and authentic endogenous analytes should be evaluated. This should be investigated in an experiment using QCs spiked with analyte in the matrix, the endogenous matrix only and spiked surrogate analyte in the matrix against the surrogate calibration curve.
- In certain cases, dilution of the QCs with surrogate matrix may be necessary (e.g., for background subtraction methods) where the endogenous level is high and the LLOQ needs to be reduced. In these cases, the recovery and matrix effect experiments should be repeated with authentic biological matrices with endogenous concentrations between LLOQ and low QC, if available.

The composition of the biological matrix might affect method performance, it is necessary to investigate matrices from at least six (chromatographic methods)/ten (LBA) different donors, except in the Standard Addition Approach, where each sample is analyzed with its own calibration curve.

2.10.1.3 Parallelism for methods for analytes that are also endogenous molecules

Parallelism assures that observed changes in response per given changes in analyte concentrations are equivalent for the surrogate and the authentic biological matrix across the range of the method.

Parallelism should be evaluated in the Surrogate Matrix and Surrogate Analyte Approaches, taking into account that parallelism is assessed differently in LBA and chromatographic methods.

2.10.1.4 Accuracy and precision for methods for analytes that are also endogenous molecules

In case of using a surrogate matrix or analyte approaches, the assessment of accuracy and precision should be performed by analyzing the QCs against the surrogate calibration curve.

The concentration of the endogenous molecule in the blank matrix may be determined and subtracted from the total concentrations observed in the spiked samples. Accuracy is recommended to be

calculated using the following formula when QCs are spiked with the authentic analyte in the matrix containing endogenous levels of the analyte:

$$\text{Aaccuracy (\%)} = 100 \times \frac{(\text{Measured concentration of spiked sample} - \text{endogenous concentration})}{\text{Spiked concentration}}$$

Only the precision can be determined from the analysis of each unspoked/endogenous QC.

2.10.1.5 Stability for methods for analytes that are also endogenous molecules

To mimic study samples as much as possible, stability experiments should be investigated with the authentic analyte in the authentic biological matrix and with unspoked/endogenous QCs (blank matrix with endogenous molecule) as well as spiked low QC and high QCs.

However, if a surrogate matrix is used for calibration standards, stability should also be demonstrated for the analyte in the surrogate matrix, as this could differ from stability in the authentic biological matrix.

2.11 Parallelism

Parallelism assesses whether serially diluted study samples remain parallel to the calibration curve. It should be evaluated or justified, particularly for LBAs where matrix interference is suspected. A high-concentration sample should be diluted to at least three levels, and back-calculated concentrations should not exceed 30% CV.

2.12 Recovery

Recovery should be evaluated for extraction-based methods by comparing processed spiked samples with extracted blank samples spiked after processing. Recovery need not be 100% but should be consistent for analyte and IS across concentrations.

Biological blank sample that is processed and then spiked with the analyte. Recovery of the analyte does not need to be 100%, but the extent of recovery of an analyte and of the IS (if used) should be consistent. Recovery experiments are recommended to be performed by comparing the analytical results for extracted samples at multiple concentrations, typically three concentrations (low, medium and high).

2.13 Minimum required dilution

MRD is the dilution factor used in LBA samples to reduce background or matrix interference. It should be identical for calibration standards, QCs, and samples, defined during development, and documented in the Validation Report. Changes require partial validation. MRD should be defined in the Validation Report of the analytical method.

2.14 Commercial and diagnostic kits

This guideline does not apply to diagnostic kits intended for point-of-care diagnosis, including companion or complementary diagnostic kits.

If a commercial or research-use kit is repurposed for drug concentration measurement, its validation should be assessed against the standards in this guideline. Validation considerations for kit assays include, but are not limited to, the following:

- If the reference standard in the kit differs from that of the study samples, testing should evaluate differences in assay performance of the kit reagents. The specificity, accuracy, precision and stability of the kit assay should be demonstrated under actual conditions of use in the facility conducting the sample analysis. 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.
- 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.
- Calibration standards and QCs should be prepared in the same matrix as the study samples. Kits with calibration standards and QCs prepared in a matrix different from the study samples should be justified and appropriate experiments should be performed.
- If multiple kit assay lots are used within a study, lot-to-lot variability and comparability should be addressed for any critical reagents included in the kits.
- If a kit using multiple assay plates is employed, sufficient replicate QCs should be used on each plate to monitor the accuracy of the assay. Acceptance criteria should be established for the individual plates and for the overall analytical run.

2.15 New or alternative technologies

If a new or alternative technology is used from the start of drug development, cross validation with an existing technology is not required.

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 those obtained with another platform. Therefore, when a new or alternative analytical platform is replacing a previous platform used in the development of a drug it is important that the potential differences are well understood. The data generated from the previous platform/technology should be cross validated to that of the new or alternative platform/technology. Seeking feedback from the regulatory authorities is encouraged early in drug development. The use of two methods or technologies within a comparative BA/BE study is strongly discouraged.

The use of new technology in regulated bioanalysis should be supported by acceptance criteria established a priori based on method development and verified in validation.

2.15.1 Dried matrix methods

Dried matrix methods offer micro sampling advantages but require additional validation beyond standard LC-MS or LBA validation, including assessment of hematocrit, sample homogeneity, extraction, and ISR sampling requirements.

- Hematocrit (especially for spotting of whole blood into cards)
- Sample homogeneity (especially for sub-punch of the sample on the card/device)
- Extraction of the sample from the dried matrix
- DMM sample collection for ISR
    - Care should be taken to ensure sufficient sample volumes or numbers of replicates are retained for ISR
    - Should be assessed by multiple punches of the sample or samples should be taken in duplicate.

When DMM is used for clinical or nonclinical studies in addition to typical liquid approaches (e.g., liquid plasma samples) in the same studies, these two methods should be cross validated.

2.16 Documentation

SOPs and accurate records are essential for validated analytical methods. Validation data should be documented and available for audit and inspection. Table 1 outlines documentation expected for regulatory submission and inspection.

Documentation should allow reconstruction of the study and include source data, protocols, reports, operational and environmental records, and correspondence between involved parties.

Records may be paper or electronic but should be contemporaneous, preserve original data, and document the reason for any change or reprocessing.

2.16.1 Summary information

Summary information should include the following items in of the Common Technical Document (CTD; or electronic CTD, eCTD)) or reports:

- A summary of methods used for each study should be included. Each summary should provide the method title, method identification code, the assay type, the Bioanalytical Report code, effective date of the method, and the associated Validation Report codes.
- A summary table of all the relevant Validation Reports should be provided for each analyte, including Partial Validation and Cross Validation Reports. The table should include the method identification code, the type of method, the reason for the new method or additional validation (e.g., to lower the limit of quantification). Changes made to the method should be clearly identified.
- A summary table cross-referencing multiple identification codes should be provided when a method has different codes for the method, the Validation Reports and the Bioanalytical Reports.
- Discussion of method changes (e.g., evolution of methods, reason(s) for revisions, unique aspects)
- For comparative BA/BE studies, a list of regulatory site inspections including dates and outcomes for each analytical site if conducted over the last three years, and one year post study completion.

CHAPTER THREE

Responsibilities:

Applicants for Marketing Authorizations	• Submit complete BE study data. • Select appropriate reference product. • Ensure study design meets guideline requirements. • Address regulatory queries.
Pharmaceutical Companies / Manufacturers	• Develop and manufacture the generic product under GMP • Ensure batch consistency for BE studies • Provide formulation and quality documentation • Oversee outsourced activities (CROs)
Contract Research Organizations (CROs)	• Conduct BE studies under GCP/GLP • Ensure ethical approvals • Use validated analytical methods • Maintain accurate data and complete study reports
Health Care Policy Makers	• Create policies supporting safe, effective, affordable generics • Strengthen regulatory capacity • Promote public trust in generic medicines
DSC BE Assessors	• Scientifically evaluate BE submissions • Review analytical, clinical, and statistical data • Ensure guideline compliance • Prepare assessment reports and recommendations
DSC CROs inspectors	• Verify compliance with guidelines while conducting inspections • Issue approvals or requests for additional information

CHAPTER FOUR

Document History and Version Control

Version	Description	Review Date
1	Initial Release	August 2026
2		
3		

References:

International Council for Harmonization (ICH) (2022), *ICH guideline M10 bioanalytical method validation and study sample analysis.*

World Health Organization (WHO) (2025), *WHO guidelines on bioanalytical method validation and study sample analysis, (TRS 1060) Annex 6, 2025.*

Glossary of ICH terms and definitions *Compiled by CIOMS from the International Council for Harmonization (ICH)'s Version 8, 5 June 2025.*

Annex:

Appendix 1:

Table 1: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Chromatographic system suitability	• Dates, times, and samples used for suitability testing	• Not applicable	• Not applicable
Synopsis **overview of method evolution**	• History/evolution of methods (e.g., to explain revisions, unique aspects with supportive data, if available)	• Not applicable	• Not applicable
Reference standards	• CoA or equivalent alternative to ensure quality (including purity), stability/expiration/retest date(s), batch number, and manufacturer or source • Records of receipt, use, and storage conditions. • If expired, recertified CoA, or retest of quality and identity with retest dates	• A copy of the CoA or equivalent alternative including batch/lot number, source, quality (including purity), storage conditions, and expiration/retest date, or table with this information. • If expired, quality and stability at the time of use and retest dates and retested values.	• A copy of the CoA or equivalent alternative including batch /lot number, source, quality (including purity), storage conditions, and expiration/retest date or a table with this information. • If expired, quality and stability at the time of use and retest dates and retested values.
Internal standard	• IS quality or demonstration of suitability • Records of receipt, use, and storage conditions	• Name of reagent or standard • Origin	• Name of reagent or standard • Origin

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Critical reagents	• Name of reagent • Batch/Lot number • Source/Origin • Concentration, if applicable • Retest date (expiry date) • Storage conditions	• Name of reagent • Batch/Lot number • Source/Origin • Concentration, if applicable • Retest date (expiry date) • Storage conditions	• Name of reagent • Batch/Lot number • Source/Origin • Concentration, if applicable • Retest date (expiry date) • Storage conditions
Stock/Working solutions	• Record of preparation, and use of stock/working solutions • Storage location and condition	• Notation that solutions were used within stability period • Stock/working solution stability • Storage conditions	• Notation that solutions were used within stability period • Stock/working solution stability † • Storage conditions†
Blank matrix	• Records of matrix descriptions, lot numbers, receipt dates, storage conditions, and source/supplier	• Description, lot number, receipt dates	• Description, lot number, receipt dates††
Calibration standards and QCs	• Records and date of preparation • Record of storage temperature (e.g., log of in/out dates, analyst, temperatures, and freezer(s))	• Description of preparation including matrix • Batch number, preparation dates and stability period • Storage conditions (temperatures, • Dates, duration, etc.)	• Description of preparation† • Preparation dates and stability period • Storage conditions†

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Standard Operating Procedures (SOPs; Procedures)	Procedures for all aspects of analysis, such as: • Method/procedure (validation/analytical) • Acceptance criteria (e.g., run, calibration curve, QCs) • Instrumentation • Reanalysis • ISR • Record of changes to SOP (change, date, reason, etc.)	• A detailed description of the method procedures	• A list of procedures/analytical protocols used for the method
Sample tracking	• Study sample receipt, and condition on receipt • Records that indicate how samples were transported and received. Sample inventory and reasons for missing samples • Location of storage (e.g., freezer unit) • Tracking logs of QCs, calibration standards, and study samples • Freezer logs for QCs, calibration standards, and study samples entry and exit	• Not applicable	For all studies • Dates of receipt of shipments number of samples • Sample condition on receipt • Analytical site storage condition and location • Storage: total duration from sample collection to analysis • List of any deviations from planned storage conditions, and potential impact <u>Additionally, for Comparative BA/BE Studies</u> <u>also include:</u> • The subject ID

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*

Analysis	• Documentation and data for system suitability checks for chromatography
	• Instrument use log, including dates of analysis for each run
	• Sample extraction logs including documentation of processing of calibration standards, QCs, and study samples for each run, including dates of extraction
	• Identity of QCs and calibration standard lots, and study samples in each run
	• Documentation of instrument settings and maintenance
	• Laboratory information management system (LIMS)
	• Validation information, including documentation and data for:
	  ○ Selectivity, specificity, sensitivity, precision and accuracy, carry-over, dilution, recovery, matrix effect
	  ○ Bench-top, freeze-thaw, long-term, extract, and stock solution stability
	  ○ Cross/partial validations, if applicable
		For all studies:
		• Table of all runs (including failed runs), and analysis dates
		• Table of calibration standard concentration and response functions results (calibration curve parameters) of all accepted runs with accuracy and precision.
		• Table of within- and between- run QC results and calibration standards (from accuracy and precision runs). Values outside the acceptance criteria should be clearly marked.
		• Include total error for LBA methods
		• Data on selectivity, specificity, dilution linearity and sensitivity (LLOQ), carry-over, recovery. Bench-top, freeze-thaw, long-term, extract, and stock solution stability
		• Partial/cross-validation, if applicable
		• Append separate report for additional validation, if any
		Additionally, for comparative BA/BE studies also include:
		• Instrument ID for each run-in comparative BA/BE study †
		• 100% of run summary table of accepted and failed runs
			For all studies:
			• Table of all runs, status (accepted and failed), reason for failure, and analysis dates.
			• Table of calibration standard concentration and response function results (calibration curve parameters) of all accepted runs with accuracy and precision.
			• Table of QCs results of all accepted runs with overall (between-run) accuracy and precision results of the QCs and between- run accuracy and precision results from accepted runs.
			• Table of reinjected runs with results from reinjected runs and reason(s) for reinjection
			• QCs graphs trend analysis encouraged
			• Study concentration results table.
			Additionally, for comparative BA/BE studies also include:
			• Instrument ID for each run-in comparative BA/BE study†
			• ARE response plots for each analytical run, including failed runs
			• 100% of run summary table of accepted and failed runs

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Chromatograms and reintegration	• Electronic audit trail: • 100% e-chromatograms of original and reintegration from accepted and fail runs • Reason for reintegration • Mode of reintegration 100% of run summary tables of accepted and failed runs, including calibration curve, regression, weighting function, analyte and IS response and retention time, response ratio, integration type	**For all studies:** • Representative chromatograms (original and reintegration) • Reason for reintegration • Chromatograms may be submitted as a supplement **Additionally, for comparative BA/BE studies also include** • 100% chromatograms of original and reintegration from accepted and fail runs. • 100% of run summary table of accepted and failed runs	**For all studies:** • Chromatograms may be submitted as a supplement • For studies other than comparative BA/BE, randomly selected chromatograms from 5% of samples submitted in application dossiers • Reason for reintegration • Identification and discussion of chromatograms with manual reintegration • SOP for reintegration, as applicable **Additionally, for comparative BA/BE studies also include** • 100% of chromatograms. • Original and reintegrated chromatograms and initial and repeat integration results • 100% of run summary table of accepted and failed runs

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Deviations from procedures	• Contemporaneous documentation of deviations/unexpected events • Investigation of unexpected events • Impact assessment	• 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
Reanalysis/Repeat analysis	• Procedures for conducting reanalysis/repeat analysis (define reasons for reanalysis, etc.) • Retain 100% of repeat/reanalyzed data • Contemporaneous records of reason for repeats	• Not applicable	**For all studies:** • Table of sample IDs, reason for repeat analysis, original and repeat analysis values, reason for reported values, run IDs **Additionally, for comparative BA/BE studies also include** • for comparative BA/BE studies, values from rejected runs should be included in a separate table.
ISR	• Procedure for ISR • ISR data: Run IDs, run summary sheets, chromatograms or other electronic instrument data files • Document ISR failure investigations, if any	• Not applicable	• ISR data table (original and reanalysis values and run IDs, percent difference, percent passed) • ISR failure investigations, if any††

Table 1 continued: Documentation and Reporting

Items	Documentation at the analytical site	Validation report*	Bioanalytical report*
Communication	• Between involved parties (Applicant, contract • research organizations, and consultants) related to study/method	• Not applicable	• Not applicable
Audits and inspections	• Evidence of audit and inspections	• Not applicable / Refer to Section 2.16 for summary information to include in the eCTD	• Not applicable / Refer to Section 2.16 for summary information to include in the eCTD

*The applicant is expected to maintain data at the analytical site to support summary data submitted in validation and bioanalytical reports. validation and bioanalytical reports should be submitted in the application.

† May append or link from validation report.

††Submit either in validation report or in bioanalytical Rep