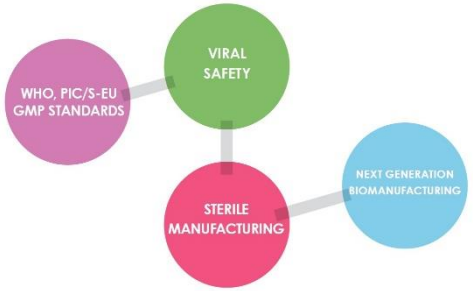

Dinesh Khokal, PhD, Singapore

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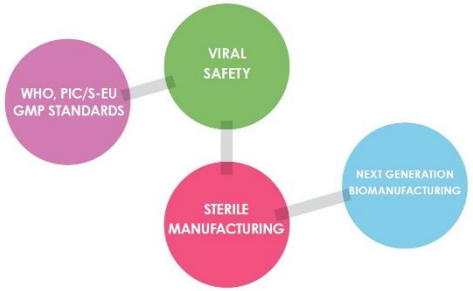
GaBI Educational Workshops

18 December 2019, Hotel Gran Mahakam, Jakarta, Indonesia

2nd ASEAN Educational Workshop on
GMP FOR BIOLOGICALS/BIOSIMILARS



Bioanalytical method validation

Dinesh Khokal, PhD, Singapore
18 December 2019

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BIOANALYTICAL METHOD VALIDATION

2nd ASEAN Educational Workshop on GMP for Biologicals/Biosimilars
Generics and Biosimilars Initiative (GaBI)
18 December 2019, Jakarta, Indonesia

DINESH KHOKAL, PH.D.
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SYNOPSIS

- **Introduction**
- **Method validation parameters**
- **Acceptance criteria for bioanalytical method validation**

BIOANALYTICAL METHOD

- What is a bioanalytical method?
  - A set of procedures used for measuring analyte concentrations in biological samples
- What is Bioanalytical method validation (BMV)
  - Is the process used to establish that a quantitative analytical method is suitable for biochemical applications

BIOANALYTICAL METHOD

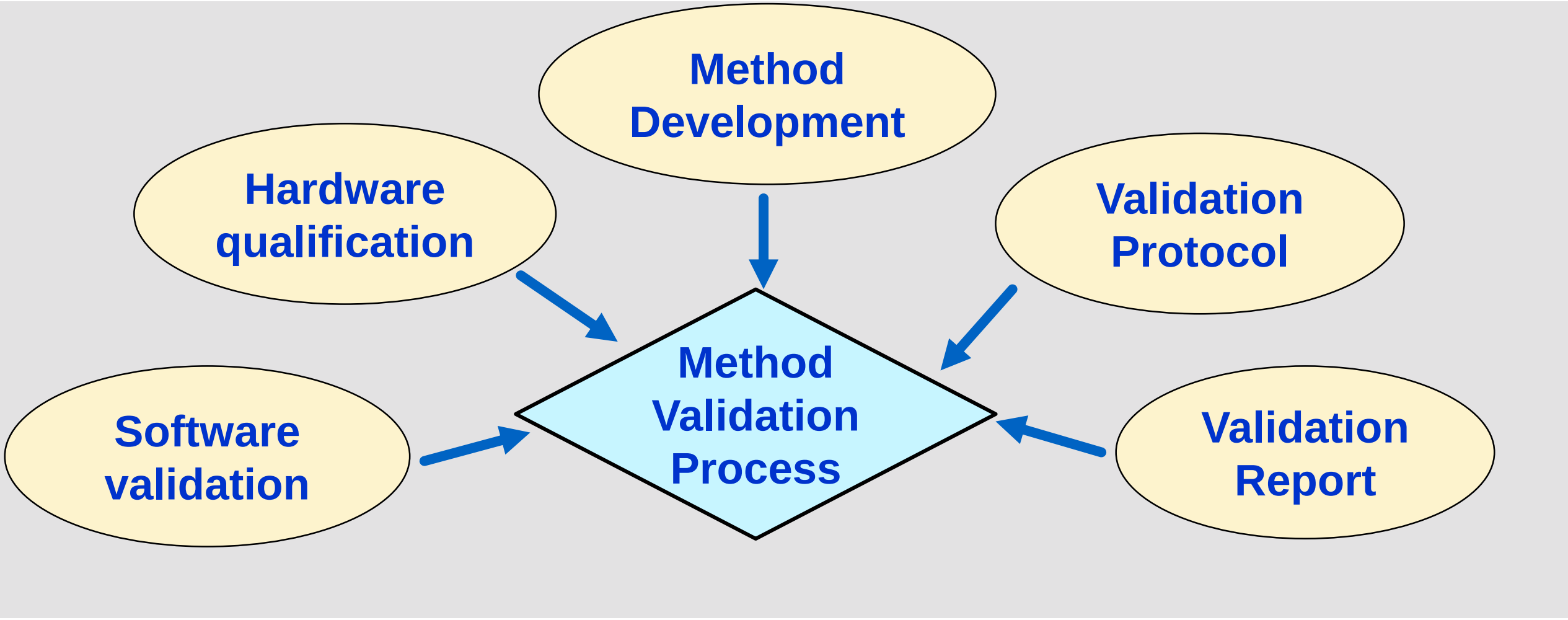
- What are these methods?
  - Quantitative analysis by ligand binding assays (LBAs)
  - Chromatographic methods such as liquid chromatography (LC) or
  - Gas chromatography (GC),
  - Which are typically used in combination with mass spectrometry (MS) detection and occasionally with other detectors

WHY BIOANALYTICAL METHOD VALIDATION?

- To assess the fit-for-purpose and appropriateness for the intended use
- To ensure that the data are reliable
- To provide critical data to support the safety and effectiveness of drugs and biologic products
- Critical for the quantitative evaluation of analytes (i.e., drugs, including biologic products, and their metabolites) and biomarkers in a given biological matrix (e.g. blood, plasma, serum, or urine)

Bioanalytical method validation is essential to ensure the acceptability of assay performance and the reliability of analytical results.

METHOD VALIDATION PROCESS STEPS



BIOANALYTICAL METHOD DEVELOPMENT
THE PURPOSE

BEGIN With END in mind

The purpose is to define:

- the design
- operating conditions
- Limitations, and
- suitability of the method for its intended purpose
- to ensure that the method is optimized for validation

BIOANALYTICAL METHOD DEVELOPMENT WHAT IT INVOLVES?

- Method development involves
  - optimizing the procedures and conditions involved with extracting and detecting the analyte.

BIOANALYTICAL METHOD DEVELOPMENT WHAT IT INCLUDES?

- Optimization of the bioanalytical parameters to ensure that the **method is suitable for validation:**
  - Reference standards
  - Critical reagents
  - Calibration curve
  - Quality control samples (QCs)
  - Selectivity and specificity
  - Sensitivity
  - Accuracy
  - Precision
  - Recovery
  - Stability of the analyte in the matrix

SYNOPSIS

- Introduction
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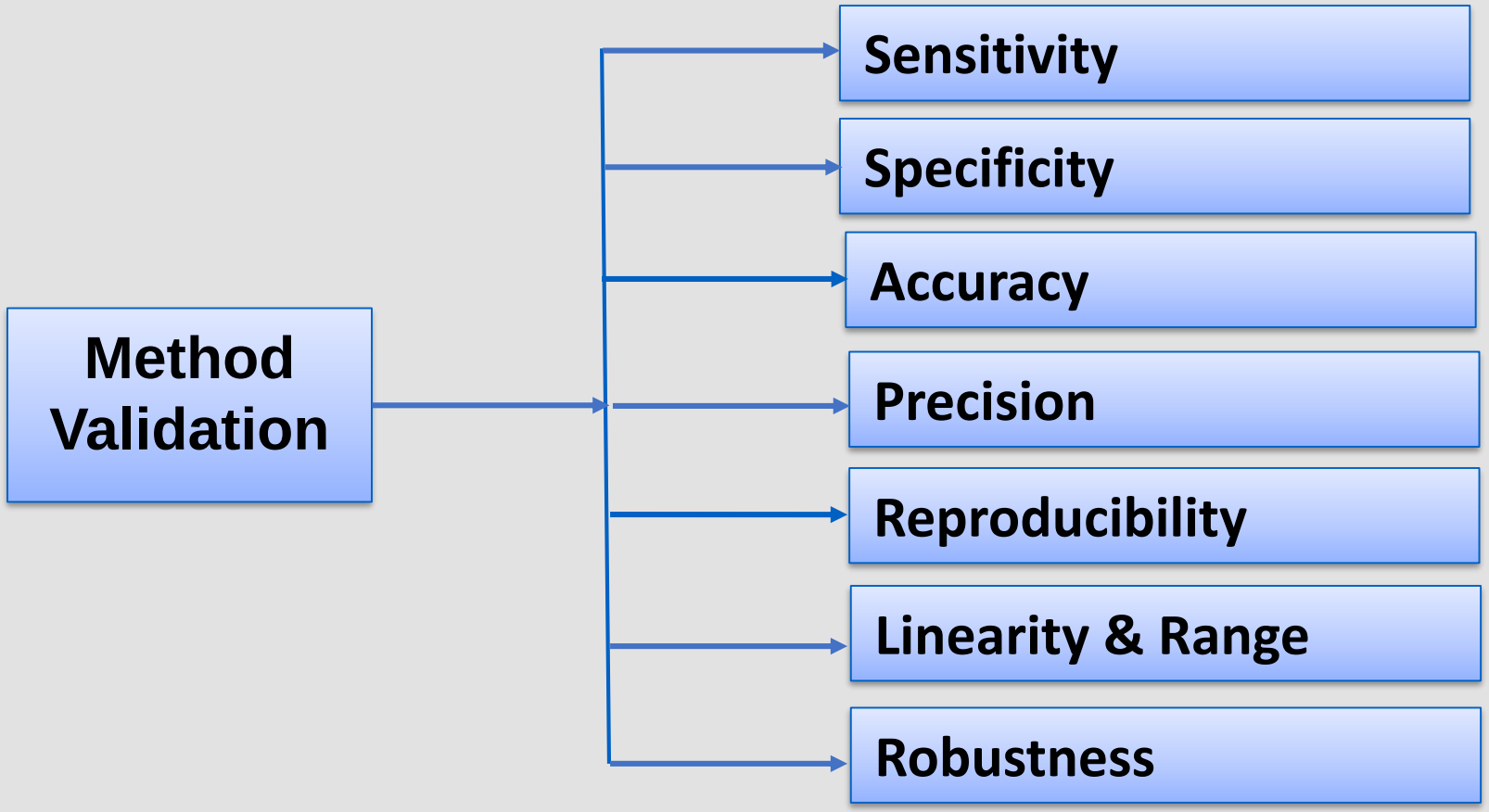
METHOD VALIDATION PARAMETERS



SENSITIVITY

- The lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise
- Defined by
  - The Lower Limit Of Detections (LLOD)
  - Lower Limit Of Quantitation (LLOQ)
- The LLOQ evaluation can be done separately or
- As part of the precision and accuracy assessment for the calibration range.

SPECIFICITY *VS* SELECTIVITY (1)

- Specificity describes the ability of the bioanalytical method to produce a signal only for the analyte of interest and not for other interfering components
  - e.g. LC–MS/MS bioanalytical methods are specific
- Selectivity describes the ability of a method to differentiate analyte of interest from other analytes or endogenous impurities present in samples
  - e.g. HPLC with other detection methods are selective

SPECIFICITY /SELECTIVITY (2)

- The matrix can contain non-specific matrix component such as degrading enzymes, heterophilic antibodies or rheumatoid factor which may interfere with the analyte of interest.
- Specificity/Selectivity is evaluated by spiking blank matrix samples with related molecules at the maximal concentration(s) of the structurally related molecule anticipated in study samples.

SPECIFICITY/SELECTIVITY (3)

- Should analyze blank samples of the appropriate biological matrix individual sources (e.g. plasma) from at least
  - six for Chromatographic Assays (CC)
  - or ten for Ligand Based Assays (LBA)
- Specificity should be evaluated at the low end of an assay where problems occur in most cases
- It is recommended that selectivity is also evaluated at higher analyte concentrations.
- The response of the blank samples should be below the LLOQ in at least 80% of the individual sources.

SPECIFICITY/ SELECTIVITY (4)

- The evaluation of selectivity should demonstrate that no significant response attributable to interfering components is observed at the retention time(s) of the analyte or the Internal Standard (IS) in the blank samples.
- Responses detected and attributable to interfering components
  - should not be more than 20% of the analyte response at the LLOQ and
  - not more than 5% of the IS response in the LLOQ sample for each matrix.

ACCURACY, PRECISION & RECOVERY (1)

- Evaluating the accuracy and precision Involves analyzing replicate QCs at multiple concentrations across the assay range.
- Specifically, should evaluate the performance
  - at the LLOQ
  - low, mid and high QCs and
  - the ULOQ for LBAs
- Experiments for estimating accuracy and precision should include independent runs over several days.
  - a minimum of three for CCs
  - and six for LBAs)

ACCURACY, PRECISION & RECOVERY (2)

- Each Accuracy & Precision run should include
  - a calibration curve and
  - multiple QC concentrations that are analyzed in replicates
  - should determine the accuracy and precision of the method based on the performance of the QC in the A & P runs.
- should optimize the recovery of the analyte to ensure that the extraction is efficient and reproducible.
- Recovery need not be 100 percent, but the extent of the recovery of an analyte and of the ISs should be consistent and reproducible.
- Recovery evaluation is not necessary for LBAs unless sample extraction is involved.

MATRIX EFFECT

- A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix.
- Necessary to evaluate the matrix effect between different independent sources/lots.
- Should be evaluated by analyzing
  - at least 3 replicates of low and high QCs
  - each prepared using matrix from at least 6 different sources/lots.
- The accuracy should be within ±15% of the nominal concentration
- The precision [percent coefficient of variation (%CV)] should not be greater than 15% in all individual matrix sources/lots.

DILUTION INTEGRITY (1)

- Dilution integrity is the assessment of the sample dilution procedure. when required
- To confirm that it does not impact the accuracy and precision of the measured concentration of the analyte.
- The same matrix from the same species used for preparation of the QCs should be used for dilution.
- Dilution QCs should be prepared with analyte concentrations in matrix that are greater than the ULOQ and then diluted with blank matrix.

DILUTION INTEGRITY (2)

- At least 5 replicates per dilution factor should be tested in one run to determine if concentrations are accurately and precisely measured within the calibration range.
- The dilution ratio(s) applied during study sample analysis should be within the range of the dilution ratios evaluated during validation.
- The mean accuracy of the dilution QCs should be within ±15% of the nominal concentration and the precision (%CV) should not exceed 15%.

CALIBRATION CURVE & QUANTITATION RANGE (1)

- The calibration curve demonstrates the relationship between the nominal analyte concentration and the response of the analytical platform to the analyte.
- Calibration standards, prepared by spiking matrix with a known quantity of analyte, span the calibration range and comprise the calibration curve.
- Calibration standards should be prepared in the same biological matrix as the study samples.
- The calibration range is defined
  - by the LLOQ, which is the lowest calibration standard,
  - and the ULOQ, which is the highest calibration standard.
  - There should be one calibration curve for each analyte studied during method validation and for each analytical run.

CALIBRATION CURVE & QUANTITATION RANGE (2)

- Calibration curve should be generated with at least 6 concentration levels of calibration standards, including LLOQ and ULOQ standards, plus a blank sample.
- The blank sample should not be included in the calculation of calibration curve parameters.
- Anchor point samples at concentrations below the LLOQ and above the ULOQ of the calibration curve may also be used to improve curve fitting.
- Minimum of 6 independent runs should be evaluated over several days considering the factors that may contribute to between-run variability.

CALIBRATION CURVE & QUANTITATION RANGE (3)

- The accuracy and precision of back-calculated concentrations of each calibration standard
  - should be within ±25% of the nominal concentration at the LLOQ & 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.

CARRYOVER

- Carry-over is generally not an issue for LBA analyses.
- However, if the assay 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.

Carryover: The appearance of an analyte signal in a sample from a preceding sample.

DILUTION LINEARITY AND HOOK EFFECT (1)

- Due to the narrow assay range in many LBAs, study samples may require dilution in order to achieve analyte concentrations within the range of the assay.
- Dilution linearity is assessed to confirm:
  - (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.

Dilution Linearity: A parameter demonstrating that the method can appropriately analyse samples at a concentration exceeding the ULOQ of the calibration curve without influence of hook effect or prozone effect and that the measured concentrations are not affected by dilution within the calibration range in LBAs.

DILUTION LINEARITY AND HOOK EFFECT (2)

- The same matrix as that of the study sample should be used for preparation of the QCs for dilution.
- Dilution linearity should be demonstrated by generating a dilution QC, i.e., spiking the matrix with an analyte concentration above the ULOQ, analysed undiluted (for hook effect) and diluting this sample (to at least 3 different dilution factors) with blank matrix to a concentration within the calibration range.
- For each dilution factor tested, at least 3 runs should be performed using the number of replicates that will be used in sample analysis.

Hook Effect: Suppression of response due to very high concentrations of a particular analyte. A hook effect may occur in LBAs that use a liquid-phase reaction step for incubating the binding reagents with the analyte. Also referred to as prozone.

DILUTION LINEARITY AND HOOK EFFECT (3)

- The same matrix as that of the study sample should be used for preparation of the QCs for dilution.
- Dilution linearity should be demonstrated by generating a dilution QC, i.e., spiking the matrix with an analyte concentration above the ULOQ,
  - analysed undiluted (for hook effect) and
  - diluting this sample (to at least 3 different dilution factors) with blank matrix to a concentration within the calibration range.
- For each dilution factor tested, at least 3 runs should be performed using the number of replicates that will be used in sample analysis.
- The calculated concentration for each dilution should be within ±20% of the nominal

SYNOPSIS

- Introduction
- Method validation parameters
- **Acceptance criteria for bioanalytical method validation**

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (1)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Sensitivity	• The analyte response at the LLOQ should be ≥ five times the analyte response of the zero calibrator. • The accuracy should be ± 20% of nominal concentration (from ≥ five replicates in at least three runs). • The precision should be ± 20% CV (from ≥ five replicates in at least three runs).	• The accuracy should be ± 25% of the nominal concentration (from ≥ three replicates in at least six runs). • The precision should be ± 25% CV (from ≥ three replicates in at least six runs). • The total error should be ≤ 40%.
Specificity	**Acceptance Criteria:** • See Selectivity below.	**Acceptance Criteria:** • QCs should meet ± 20%, or 25% at the LLOQ and ULOQ.

Source: US FDA Guidance on Bioanalytical Method Validation, May 2018

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (2)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Accuracy and Precision (A & P)	**Accuracy:** Within-run and between runs: • ± 15% of nominal concentrations; except ± 20% at LLOQ. **Precision:** Within-run and between runs: • ± 15% CV, except ± 20% CV at LLOQ	**Accuracy:** Within-run and between runs: • ± 20% of nominal concentrations; except ±25% at LLOQ, ULOQ **Precision:** Within-run and between runs: • ± 20% CV, except ± 25% at LLOQ, ULOQ

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (3)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Selectivity	• Blank and zero calibrators should be free of interference at the retention times of the analyte(s) and the IS. • Spiked samples should be ± 20%LLOQ. • The IS response in the blank should not exceed 5% of the average IS responses of the calibrators and QCs.	• For ≥ 80% of sources, unspiked matrix should be BQL, and spiked samples should be ± 25% at LLOQ, and ± 20% at H QC.
Carryover	• Carryover should not exceed 20% of LLOQ.	• Not applicable

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (4)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Quality Controls (QC)	• Refer to A & P Runs, Other Validation Runs, and Stability Evaluations.	• Refer to A & P Runs, Other Validation Runs, and Stability Evaluations.
Other Validation Runs	• Meet the calibration acceptance criteria • ≥ 67% of QCs should be ± 15% of the nominal (theoretical) values, ≥ 50% of QCs per level should be ± 15% of their nominal concentrations	• Meet the calibration acceptance criteria • ≥ 67% of QCs should be ± 20% of the nominal (theoretical) values, and ≥ 50% of QCs per level should be ± 20% of their nominal concentrations

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (5)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Recovery	**Elements:** • Extracted samples at L, M, and H QC concentrations versus extracts of blanks spiked with the analyte post extraction (at L, M, and H)	**Elements:** • Need to be demonstrated only if extraction is involved
Stability	• The accuracy (% nominal) at each level should be ± 15%.	• The accuracy (% nominal) at each level should be ± 20%.
Dilution	o Accuracy: ± 15% of nominal concentrations o Precision: ± 15% CV	o Accuracy: ± 20% of nominal concentrations o Precision: ± 20% CV

ACCEPTANCE CRITERIA FOR BIOANALYTICAL METHOD VALIDATION (6)

Parameters	Validation Recommendations	
	Chromatographic Assays (CCs)	Ligand Binding Assays (LBAs)
Calibration Curve	• Non-zero calibrators should be ± 15% of nominal (theoretical) concentrations, except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run. • 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run.	• Non-zero calibrators should be ± 20% of nominal (theoretical) concentrations, except at LLOQ and ULOQ where the calibrator should be ± 25% of the nominal concentrations in each validation run. • 75% and a minimum of six non-zero calibrator levels should meet the above criteria in each validation run. • Anchor points should not be included in the curve fit.

FURTHER READINGS

- ***Validation of analytical procedure, Text and Methodology, ICH Q2 (R1), 1994***
- ***Guidance on bioanalytical method validation, EMA, July 2011***
- ***Bioanalytical method validation, Guidance for industry, US FDA, May 2018***
- ***Bioanalytical method validation, ICH M10 (Draft), ICH, Feb 2019***

THANK YOU FOR YOUR ATTENTION

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BACKUP SLIDES

VALIDATION, QUALIFICATION & VERIFICATION

Validation

A validated method is one that for which there is full knowledge of the test's performance and performance assessment has been made to determine reliability and variability.

Protocol for full validation over a long time frame and with many parameters tested

Qualification

A qualified method is one for which there is insufficient knowledge of the test's performance to document full validation.

But a performance assessment has been made to determine reliability and variability

Verification

Verification is definitive term applied to validated methods that typically appear in compendia.

Laboratory need only to demonstrate its ability to perform the test according to stated specifications.

If standards are available from NIST, WHO or Pharmacopeia, these should be used to in test verification

FULL VALIDATION, PARTIAL VALIDATION & CROSS VALIDATION

Full Validation

- A full validation of a bioanalytical method should be performed when establishing a bioanalytical method for quantification of an analyte
- For chromatographic methods a full validation should include the following elements:
  - selectivity, specificity (if necessary), matrix effect, calibration curve (response function), range (lower limit of quantification (LLOQ) to upper limit of quantification (ULOQ)), accuracy, precision, carry-over, dilution integrity, stability and reinjection reproducibility.
- For LBAs the following elements should be evaluated:
  - specificity, selectivity, calibration curve (response function), range (LLOQ to ULOQ), accuracy, precision, carry-over (if necessary), dilution linearity, parallelism (if necessary, conducted during sample analysis) and stability.

FULL VALIDATION, PARTIAL VALIDATION & CROSS VALIDATION

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 are determined according to the extent and nature of the changes made to the method

Cross Validation

- Cross validation is a comparison of validation parameters of two or more bioanalytical methods or techniques that are used to generate data within the same study or across different studies.

METHOD VALIDATION PARAMETERS

Test parameters	Description
Sensitivity	Lower limit of detection (LOD), The lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise
Specificity	Ability to assess unequivocally the analyte in the presence of expected components such as impurities, degraded products and matrix
Precision	Closeness of agreement among measurements obtained from multiple sampling under described conditions
Repeatability	Able to repeat under the same operation conditions, over a short time period
Intermediate precision	Able to obtain the same results within laboratory variations, different days, analysts, equipment, etc.
Reproducibility	Able to obtain the same results among different laboratories variations, different days, analysts, equipment, etc.
Accuracy	Degree of closeness of determined value to the nominal or known true value under prescribed conditions. Accuracy is also sometimes termed trueness.
Quantitation Range	Range of concentration, including upper limit of quantitation (ULOQ) and lower limit of quantitation (LLOQ), that may be reliably and reproducibly quantified through a concentration-response relationship
Linearity	Extent to which the relationship between experimental response value and concentration of the analyte approximates a straight line
Robustness	Ability of the method to deliver accurate, precise results under normal operating-condition variations

VALIDATION PARAMETERS APPLICABLE TO DIFFERENT ANALYTICAL METHODS

Type of analytical method	Recommended validation parameters by ICH	Other factors to be considered
Identity tests	Specificity	Sensitivity
Impurity tests: limit	Specificity, LOD	Accuracy, Range
Impurity test: quantitative	Accuracy, Precision, Specificity, Quantitation limit, Linearity, Range	
Active principle quantitation	Accuracy, Precision, Specificity, Quantitation limit, Linearity, Range	Interfering substance, effect of different matrices e.g., Biological specimens
Potency assays	Accuracy, Intermediate precision, Linearity, Range	Inter-lot precision in biological assays using cells, etc. Sample stability (e.g., Freeze-thaw)