REVIEW ARTICLE

BIOANALYTICAL METHOD VALIDATION: A COMPREHENSIVE ASSESSMENT OF VARIOUS REGULATORY GUIDELINES

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ABSTRACT

Bioanalytical methods are used to analyse an analyte in a biological matrix. Bioanalytical method validation is the process of determining the suitability of the given bioanalytical methodology for providing the required analytical data. Validation of the bioanalytical methods demonstrates and ensures that the methods used for the quantification of analyte in biological fluids are reliable, reproducible and suitable for its intended application. Different regulatory agencies like Food and Drug administration (FDA), The National Health Surveillance Agency or Agência Nacional de Vigilância Sanitária (ANVISA), European Medicines Evaluation Agency (EMA), Ministry of Health, Labor and Welfare (MHLW) and International Conference on Harmonization (ICH), provide guidelines for bioanalytical method validation. The present study provides an insight about the history of bioanalytical method validation including the details of various validation parameters and their description as per the different regulatory guidelines. The study also includes the parameters of ligand based assay methods and their description.

Keywords: Bioanalytical methods, validation, regulatory guidelines, ligand based assay

INTRODUCTION

Bio-analysis is the quantitative measurement of the analytes such as drugs and their metabolites in biological fluids or biological systems such as blood, plasma, serum, saliva, tissue extracts, and cerebrospinal fluid etc[1-3]. Bioanalytical method validation is the process of determining the suitability of the given bioanalytical methodology for providing the required analytical data. Validation of the bioanalytical methods demonstrate and ensure that the methods used for the quantification of analyte in biological fluids are reliable, reproducible and suitable for its intended application. The validated bio-analytical methods ensure the criteria of stability, sensitivity, robustness, suitability, reproducibility and reliability for various applications in blood plasma, urine, serum and faeces to satisfy data for regulatory submission requirements as well as research and development[5-9]. Validation of bio-analytical methods is a very important step and it must never be overestimated, as any mistake in the methods may prove to be lethal to human lives.

History of bioanalytical method validation

History of bio-analytical method validation starts with an objective to harmonize the bioanalytical method validation principle by "United States Food and Drug Administration" (USFDA) and "American Association of Pharmaceutical Sciences" (AAP) in 1990. The first USFDA guidance document for industry on bioanalytical method validation was issued as a draft guidance in January 1999; Consequently, USFDA was the first to release the first guideline on bio-analytical method validation in May 2001 and released its new draft guideline in 2013. The USFDA updated their previous guidance recently in May 2018. The "National Health Surveillance Agency" (ANVISA) Brazil, released its first bioanalytical method validation guideline along with analytical validation guidelines in May 2003, which were further revised in 2012. The "European Medicine Agency" (EMA) for European countries also issued its first guideline on bioanalytical method validation in 2011, which came into effect in February 2012. "Ministry of Health, Labour and Welfare" (MHLW) Japan, issued guidelines on bioanalytical method validation in 2013 and issued its draft on Ligand Binding Assay (LBAs) in 2014. ICH (International conference on

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Harmonisation of Technical Requirements for registration of pharmaceuticals for human use) in June 2016 introduced New ICH M10 Guideline on bioanalytical method validation under multidisciplinary guideline. ICH endorsed a concept paper about M10 guideline on 7 October 2016 and the draft guidelines were endorsed on 26 February 2019[10-14].

Even though the scientific basis for evaluation of parameters is same across these guidelines, still there are differences in the acceptance criteria and methodology for few parameters. The chronological development of bioanalytical validation guidelines is depicted in Fig. 1[15-17].

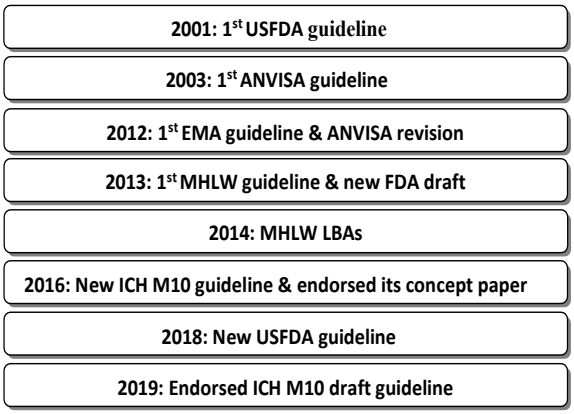

Fig. 1: Flowchart depicting the chronological development of bioanalytical method validation guidelines[4]

Comparison of various international guidelines for bioanalytical method validation

Validation of bioanalytical methods is widely accepted as of utmost importance before the methods are taken into routine use. Various international guidelines and new draft guidelines are issued considering technical up-gradations required from time to time to comply requirements for designing any bioanalytical method and its validation as per the majority of drug authority guidelines across the globe[18-22].

There is a general understanding between various regulatory authorities on the evaluation of validation parameters; but still there are some differences in the methodology and acceptance criteria suggested for bioanalytical method validation. These variations in the guidelines are important for the regulatory submission in the specific region or country which needs to be compared with all aspects parameters to comply the applicable regulatory guidelines to get the required certificate of approval, clinical/ other trials, license to validate a process/ method, for manufacture and analysis of specified product etc. accordingly[23-27].

A common strategy should be planned out before the start of any bioanalytical method development and validation which would cover the minimum and maximum of the acceptance criteria present in the guidelines for each parameter considered for the bioanalytical method validation. As none of the guidelines appear to be restricting to their particular parameters considered, which make it open that additional parameters which are not present in specific guidelines can be performed. Also, the regulatory agencies should be considered for implementing a common guidance for the bio-analytical method validation which will lead to harmonization of the method development and validation of bioanalytical validation worldwide[28-32].

To date, the USFDA, ANVISA, EMA and MHLW guidelines are referred for the bioanalytical method validation. Even though the scientific basis for evaluation of parameters is same across these guidelines, still there are differences in the acceptance criteria and methodology for few parameters[33-38].

There is similarity in USFDA and EMA recommendations on BMV, but they are not identical. The practical conduct of experiment is described more precisely in EMA guidance. USFDA recommendations are more comprehensive. The structure and table represented in the appendix portion of USFDA guidance is very helpful. The way of reporting a concentration below LLOQ is now fortunately and correctly replaced by the definition below LLOQ (BQL). In case of BMV guidance on various parameters the EMA does not consider the parameter "Recovery" at all. The comparative study of various regulatory guidelines in reference to various analytical method validation parameters is depicted in Tables I to VII[39-44].

Selectivity: In validation, the terms selectivity and specificity are used interchangeably. Both the terms have same meaning with a little difference between them. And it is important to understand this difference. The term specificity is defined as the ability of a method to indicate unequivocally the presence of specific analyte of interest but does not produce any signal for other interfering components in the sample which may be expected to be present. These interfering components might include impurity, degradants, matrix etc. whereas; selectivity is defined as the ability of a method to differentiate and resolve the various components of the sample and detect the analyte of interest quantitatively[45-50]. The comparison of selectivity in various regulatory guidelines is depicted in Table I.

Table I: Comparison of various guidelines for validation parameter "Selectivity"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	It is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.	Blank analyte samples of the appropriate biological matrix from at least six individual sources are considered.	At the retention times of the analyte(s) and the Internal Standard (IS) the Blank and zero calibrators should be free of interference. • Spiked samples should be within range of ± 20% of the LLOQ. • IS response in the blank should not exceed 5% of the average IS responses of calibrators & QCs
ANVISA guidelines (2012)[10]	Capability of the method to distinguish and quantify the analyte and IS in the presence of other components in the sample.	• Biological matrix samples collected from 6 individual sources must be analyzed. • It includes 4 normal samples, a lipemic sample and a haemolysed sample. • In case of whole blood, 5 standards and 1 lipemic sample is recommended.	The interfering peak response at the retention time should be lower than 20% of the response of LLOQ samples and lower than 5% of the IS response.
MHLW guidelines (2013)[11]	Capability of an analytical method to measure and differentiate the analyte and the IS in the presence of other components in sample.	• Evaluation of selectivity is done using blank matrix samples collected from at least 6 individual sources. • The absence of each analyte and IS should have to be confirmed.	The criteria of the acceptance asserts that the response of interfering components should not be higher than 20% of the response of LLOQ for analyte and not higher than 5% for the IS.
EMA guidelines (2012)[12]	Capability of the bioanalytical method to measure and differentiate the analyte(s) of interest and IS in the presence of components which may be expected to be present in the sample.	• Selectivity is evaluated using at least 6 individual sources of blank matrix samples. • Use of less number of sources than specified is acceptable in case of rare matrices.	The acceptance criteria says that the response of interfering component should be less than 20% response of the LLOQ for the analyte and 5% of the IS response.
ICH guidelines (2019)[14]	Selectivity is ability of an analytical method to differentiate and measure analyte in presence of potential interfering substances in blank biological matrix.	• Selectivity is evaluated using blank samples (matrix samples processed without addition of an analyte or IS) obtained from at least 6 individual sources/ lots (non- haemolysed and non-lipaemic). • Use of fewer sources may be acceptable in the case of rare matrices. • Selectivity for the IS should also be evaluated.	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.

Table II: Comparison of various guidelines for validation parameter "Accuracy"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	The nearness/ closeness of the mean test results obtained by the method to the actual value (concentration) of the analyte during the analyte test run.	Accuracy should be determined and established by applying at least three independent A & P runs, four QC levels per run (LLOQ, L, M, H QC), and ≥ five replicates per QC level.	The accuracy run should meet the calibration curve acceptance criteria and include the LLOQ calibrator. • This run has no QC acceptance criteria except LLOQ calibrator. Accuracy: Within-run and between runs: • ± 15% of nominal concentrations; except • ± 20% at LLOQ
ANVISA guidelines (2012)[10]	Accuracy is defined as the degree of match between the individual test results obtained and a value accepted as reference.	Accuracy must be determined using 5 replicates in at least 5 concentrations in each run of the test. And it must be determined in the same analytical test run (i.e., intra accuracy) and in at least three different test runs (i.e., inter-run).	The acceptance criteria assert that the deviation should not exceed 15%, except for the quantification limit for which values ≤20% are allowed
MHLW guidelines (2013)[11]	Accuracy is defined as the degree of closeness between analyte concentration obtained by the method and its theoretical concentration.	• Within-each run the accuracy should be evaluated by using at least 5 replicates at each concentration level in a single run. • Between-each run the accuracy should be evaluated by analysis in at least 3 different analytical test runs.	The acceptance criteria assert that the mean accuracy at each concentration level should be within 15%, except at the LLOQ, where it should be within 20%.
EMA guidelines (2012)[12]	The closeness of the value determined by the method to the nominal concentration of the analyte (which is expressed in percentage).	• Within-each run the accuracy is evaluated by using minimum of 5 replicates- samples per concentration level at minimum of 4 concentration levels in a single run. • Between-each test run the accuracy should be determined by using at least 3 test-runs analyzed on different days.	The acceptance criteria assert that the mean concentration should be within 15%, except for the LLOQ which should be within 20% of the nominal value.
ICH guidelines (2019)[14]	The degree of closeness of the measured value to the nominal or known true value under prescribed conditions (or as measured by a particular method). In this document accuracy is expressed as percent relative error of the nominal value.	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 2 days.	Over all accuracy at each concentration level should be within ±15% of the nominal concentration, except at the LLOQ, where it should be within ±20%. Precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Accuracy: Accuracy of a bioanalytical method is defined as the degree of closeness between observed test result concentration and the true concentration of the analyte present in the sample. The accuracy is also termed as trueness of the method[51-56]. The comparison of accuracy in various regulatory guidelines is depicted in Table II.

Precision: Precision of a bioanalytical method is defined as the degree of closeness of individual test results of an analyte obtained from multiple sampling of the same homogenous sample under the same desired conditions[57-64] . The comparison of precision in various regulatory guidelines is depicted in Table III.

Table III: Comparison of various guidelines for validation parameter "Precision"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	The closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.	The precision should be determined and established with at least three independent A&P runs, four QC levels per run (LLOQ, L, M, H QC), and ≥ five replicates per QC level.	The precision run should meet the calibration curve acceptance criteria and include the LLOQ calibrator. This run has no QC acceptance criteria except LLOQ calibrator. Precision: Within-run and between runs: ± 15% CV, Except ± 20% CV at LLOQ.
ANVISA guidelines (2012)[10]	Precision is defined as the closeness of the test results obtained by repeated measurement of multiple aliquots from a single source matrix.	Precision is measured using at least 5 replicates in at least 5 concentrations in single run (intra- precision) and at least 3 different runs (inter- precision).	The criteria of acceptance assert that the relative standard deviation or %CV should be below 15%, except for LLOQ, which should not exceed 20%.
MHLW guidelines (2013)[11]	Precision is defined as the variation between individual concentration determined in repeated measurements	Precision is measured using at least 5 replicates (intra- precision) and 3 runs (inter- precision), at 4 different concentrations.	% CV should not exceed 15%, except for LLOQ, where it should not exceed 20%.
EMA guidelines (2012)[12]	Precision is defined as the closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variance	Precision is measured using 5n samples at 3 concentrations in a single run (intra) and 3 runs for 3 concentrations on at least two different days	The %CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.
ICH guidelines (2019)[14]	The closeness of agreement (i.e., degree of scatter) among a series of measurements. Precision is expressed as coefficient of variation (CV) or the relative standard deviation (RSD) expressed as a percentage.	Within-run precision should be evaluated by analysing at least 5 replicates at each QC concentration level in each analytical run. Between-run precision should be evaluated by analysing each QC concentration level in at least 3 analytical runs over at least 2 days.	Overall accuracy at each concentration level should be within ±15% of the nominal concentration except at the LLOQ, where it should be within ±20%. The precision (%CV) of the concentrations determined at each level should not exceed 15%, except at the LLOQ, where it should not exceed 20%.

Recovery: It is the extraction efficiency by analyte detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector quantitative response obtained for the true concentration of the analyte in solvent[65-72]. The comparison of recovery in various regulatory guidelines is depicted in Table IV.

Calibration curve/linearity: Calibration curve demonstrates a relationship between the response of the instrument (i.e., signal produced by the instrument for the analyte) and the known concentration of the analyte present in the sample[73-78] . The comparison of calibration curve/ linearity in various regulatory guidelines is depicted in Table V.

Table IV: Comparison of various guidelines for validation parameter "Recovery"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	It is the extraction efficiency by analyte detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector quantitative response obtained for the true concentration of the analyte in the solvent.	Established by extracting the samples at L, M, and H QC concentrations versus extracts of blanks spiked/ added with the analyte post extraction (at L, M, and H).	Not mentioned
ANVISA guidelines (2012)[10]	The recovery measures the efficiency of the extraction procedure of an analytical method within a variation limit.	The process of the recovery is performed by comparing the analytical results of samples extracted from biomatrix at three concentrations (low, medium and high), with unextracted standards representing 100% recovery.	Acceptance criteria considers the recovery % near 100% are desirable, nevertheless lower values are also accepted, provided the recovery is precise and accurate.
MHLW guidelines (2013)[11]	Recovery is the measure of the efficiency at which an analytical method recovers the analyte through the sample- processing steps.	Recovery is determined by comparing the analyte response in a biological sample spiked [added] with the analyte and processed, with the response in a biological blank sample that is processed and then spiked with the analyte.	Recovery demonstrates reproducibility, rather than showing a higher recovery rate of the analyte.
EMA guidelines (2012)[12]	Not mentioned	Not mentioned	Not mentioned
ICH guidelines (2019)[14]	The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method.	Not mentioned	Not mentioned

Table V: Comparison of various guidelines for validation parameter "Calibration curve/linearity"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	The calibration curve reveals the relationship between response of the instrument and the known concentrations of the analyte.	The calibration curve should incorporate a blank (no analyte, no IS), a zero calibrator (blank plus IS), and at least six, non-zero calibrator levels covering the quantization range, including LLOQ in every run. • All blanks and calibrators used should be in the same matrix as the study samples. • The concentration- response relationship should satisfy and fit with the simplest regression model.	Non-zero calibrators (calibration standard) should be ± 15% of nominal (theoretical) concentration, except at LLOQ where the calibrator should be ± 20% of the nominal concentrations in each validation run and the minimum of six non-zero calibrator levels should meet the above criteria in each validation run.
ANVISA guidelines (2012)[10]	Calibration curve/ linearity represent relationship between the response of the instrument and the known concentration of the analyte to be calibrated.	The calibration method should include the analysis of a blank sample, zero sample and at least 6 non-zero samples including LLOQ, containing drug standard and IS.	Acceptance- criteria consider that the LLOQ response should be ≥5 times the response to blank response and precision should be below 20% of the CV and accuracy within ±20%, ULOQ should have precision below 15% of CV and accuracy within ±15% of nominal concentration For calibration curve, the standard should be below 15% of nominal concentration, except for LLOQ where calibrator should not deviate by 20% and 75% of non-zero including LLOQ should be within limit.
MHLW guidelines (2013)[11]	Calibration curve demonstrates relationship between theoretical concentration and response of analyte shown by instrument performing calibration.	The calibration method should include a blank sample, a zero sample, and at least 6 concentration levels of calibration standards, including an LLOQ sample.	Acceptance criteria consider that the deviation should be less than or equal to 20% and 15% in relation to the nominal concentration of LLOQ and other concentrations respectively. At least 4 of the 6 concentrations must comply including LLOQ and ULOQ, the R2 must be equal to or higher than 0.98.
EMA guidelines (2012)[12]	Calibration curve is relationship which can simply and adequately describe response of instrument with regard to concentration of analyte in sample.	The calibration method should consider a minimum of 6 concentration levels, along with a blank sample and a zero sample in replicate.	The acceptance criteria assert that the accuracy of back calculated concentrations of each calibration standard should be within ± 20% deviation of the theoretical concentration at the LLOQ, or ±15% deviation at all the other levels. At least 75% of the calibration

			standards, with a minimum of 6 levels, including the LLOQ and the highest levels, should meet the above criteria.
ICH guidelines (2019)[14]	Calibration curve demonstrates relationship between nominal analyte concentration and response of analytical platform to analyte.	A calibration curve should be generated with a blank sample, a zero sample (blank sample spiked with IS), and at least 6 concentration levels ofcalibration standards, including the LLOQ and the ULOQ. Preparation of calibration standards should be done in the same biological matrix as study samples.	The accuracy of back-calculated concentration of each calibration standard should be within ±20% of nominal concentration at LLOQ and within ±15% at all other levels. At least 75% of calibration standard with a minimum of 6 calibration standard levels should meet above criteria. In case that replicates are used, criteria (within ±15% or ±20% for LLOQ) should also be fulfilled for at least 50% of calibration standards tested per concentration level.

Sensitivity/ Detection limit: It represents that lowest amount or concentration of the analyte of interest present in the sample that can be detected by the instrument but not necessarily quantified. And this detection limit shows the sensitivity of an instrument[79-86] . The comparison of sensitivity/ detection limit in various regulatory guidelines is depicted in Table VI.

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

- Freeze-thaw stability: Freeze-thaw stability is done to investigate the effect of repeated freezing and thawing on the stability of the analyte of interest and ensures integrity of the drug.

Table VI: Comparison of various guidelines for validation parameter "Sensitivity/Detection limit"

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	It is the lowest amount of an analyte present in the sample which can be quantified with acceptable precision and accuracy.	The lowest non zero standard on the calibration curve defines the sensitivity (LLOQ).	The criteria of sensitivity analysis for analyte response at LLOQ should be ≥ five times the analyte response of the zero calibrator. • The accuracy should be ± 20% of nominal concentration (from ≥ 5 replicates in at least 3 runs). • The precision should be ± 20% CV (from ≥ 5 replicates in at 3 runs).
ANVISA guidelines (2012)[10]	Sensitivity is defined as lowest concentration of an analyte that the bioanalytical procedure can distinguish reliably from background in test sample.	For sensitivity testing, at least 5 determination should be carried out at LLOQ. Establish detection limit by analysing solutions of known and decreasing concentrations of drug up to detectable level.	The acceptance criteria requires that the ratio of 5:1 between signal to noise should be obtained and response to LLOQ should be at least 5 times greater than interference in blank samples. Peak should be identifiable with precision of 20% and accuracy of 80–120%.

MHLW guidelines (2013)[11]	Sensitivity is defined as the lowest concentration of an analyte at which the analyte can be quantified with reliable accuracy and precision in the sample.	To test sensitivity LLOQ should be adapted to expected concentration range in the study.	The acceptance criteria require that the analyte response at the LLOQ should be at least 5 times the response of that in a blank sample. Mean accuracy and precision at LLOQ should be within ± 20% deviation of the nominal concentration and not more than 20%, respectively.
EMA guidelines (2012)[12]	Sensitivity is defined as the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision during test.	To test sensitivity LLOQ should be adapted to expected concentration in study and LLOQ should be established using minimum of 5 determination	The acceptance criteria requires that the analyte signal should be at least 5 times the signal of a blank sample and the accuracy at LLOQ should be within 80– 120% with precision ≤20%.
ICH guidelines (2019)[14]	Sensitivity is defined as lowest amount of an analyte in sample, which can be measured with acceptable accuracy and precision.	Not mentioned	Not mentioned

- Bench-top stability (short-term stability): Short-term stability, which is also referred to as a process or bench-top stability, is evaluated to confirm whether there is any degradation or instability of samples during the preparation/extraction steps prior to analysis.
- Long-term stability: Long-term stability is designed to confirm the stability of analyte in the test system matrix covering the length of time from sample collection to sample analysis.
- Stock solution stability: The period over which the solutions will be used as the stability of the stock solutions is independent of the stability of RS from which the stock solution is prepared and thus, it is not advisable to assign an expiration date that is matching with the RS (Reference standard). So it becomes important to study the stability of stock solution as a part of assay validation[86-91].

The comparison of stability in various regulatory guidelines is depicted in Table VII.

Table VII: Comparison of various guidelines for validation parameter "Stability"

Guidelines	Definition	Method	Post preparative stability	Acceptance criteria
USFDA Draft guidelines/ recommendations (2018)[6]	The stability is defined as the chemical stability of an analyte in a given matrix under specific conditions for given time intervals.	**Freeze thaw stability-** at least 3 replicates at L and HQC concentrations performed .	Not mentioned	For acceptance the accuracy (% nominal) at each level should be ± 15%.
		Bench top stability- at least 3 replicates at L and HQC concentrations performed.		
		Long term stability- at least 3 replicates at L and HQC concentration performed.		
		Stock solution stability - at least 3 replicates at L and HQC concentration performed		

ANVISA guidelines (2012)[10]	Stability is parameter aimed at determining if an analyte is remaining chemically unchanged in matrix in specific condition, at time interval of storage condition	**Freeze thaw stability-** Stability should be assessed after 3 freeze-thaw cycles of the test sample using 3 test samples of HQC and LQC after storage for 12 h	The drug stability must be evaluated in the processed sample including IS at the same conditions and for a period of time longer than the duration of the analytical run using at least 3 samples of HQC and LQC & results are to be compared with recently analyzed samples.	Stability test samples are considered to be stable when there is no deviation higher than 15% of the average concentration obtained from nominal value with the exception of the LLOQ, for which a deviation of up to 20% is acceptable.
		Bench top stability - Stability test should be performed using at least 3 replicate samples of HQC and LQC at room temperature for 4–24 h should be analyzed.		
		Long term stability - At least 3 samples of HQC & LQC are used after storage of sample that exceeds time interval between collection of first sample and analysis of last sample.		
		Stock solution stability - It should be performed at room temperature after at least 6 h of preparation and results are compared with recently prepared solutions.		
MHLW guidelines (2013)[11]	The chemical or biological stability of an analyte in a given matrix under specific conditions over given time intervals.	**Freeze thaw stability -** Stability test should be performed using 3 replicates per concentration of sample i.e. HQC and LQC in same condition as used for the study sample after freeze-thaw cycles.	The stability is evaluated by at least 3 replicates per concentration levels of QC samples before and after storage duration.	For testing the stability the mean accuracy in the measurement at each level of stability test should be within ±15% deviation of the theoretical concentration. If any other criteria are more appropriate then that can also be used.
		Bench top stability - The stability test should be evaluated using 3 replicates per concentration of HQC & LQC samples with QC samples before and after storage.		
		Long term stability - It should be performed on the samples that have been stored for a time that is longer than the actual storage period.		
		Stock solution stability - Stock solution evaluation is performed by at least 3 replicates at each concentration levels of HQC and LQC.		

EMA guidelines (2012)[12]	The chemical or biological stability of an analyte in a given matrix under specific conditions over given time intervals.	**Freeze thaw stability -** Stability test is performed after freeze-thaw cycles which should be equal to or greater than study samples **Bench top stability -** It recommend to evaluate the stability using at least triplicates of LQC and HQC. **Long term stability -** The QC samples should be stored under the same conditions as the study samples and analyzed afterwards. **Stock solution stability –** It recommend bracketing approach can be used for the study of stability of stock and working solution.	The stability of the processed sample at room temperature or under the storage conditions to be used during the study. On-Instrument/ Auto sampler stability of the processed sample at injector or Auto sampler temperature.	The mean accuracy in measurement at each level should be within ±15% deviation of the theoretical [standard reference] concentration.
ICH guidelines (2019)[14]	Not mentioned	**Freeze thaw stability** - Stability of analyte should be assessed after multiple cycles of freezing & thawing. Stability evaluated using LQC and HQC. Minimum 3 stability QCs should be prepared & analysed per concentration level/storage condition/time point. **Bench top stability -** Stability evaluated using low & high concentration Stability QCs. Aliquots of low & high stability QCs are analysed at time zero & after applied storage conditions that are to be evaluated. 3 stability QCs should be prepared & analysed per concentration Level & kept on bench top at same temperature & for at least same duration as study samples. **Long term stability -** The long-term stability of the analyte in matrix stored in the freezer should be established. Low and high stability QCs should be stored in the freezer under the same storage conditions & at least for the same duration as study samples.	The stability of processed samples, including the time until completion of analysis (in autosampler/ instrument), should be determined. Stability of the processed sample at the storage conditions to be used during the analysis of study samples (dry extract or in the injection phase) on instrument / auto sampler stability of the processed sample at injector or auto sampler temperature.	The back- calculated concentrations of calibration standards should be within ±15% of nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standard concentrations, with a minimum of six concentration levels, should fulfil these criteria.

		Stock solution stability - Stability of the stock solutions of the analyte and IS should be determined under storage conditions used during the analysis of study samples by using the lowest and the highest concentrations of these solutions. They are 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.		

Ligand binding assay (LBAs): It is a very well accepted industrial method done for the quantification of antibodies, amino acids, proteins, DNA, ribonucleic acids, biosimilars and other macromolecules in pharmacokinetic, toxicokinetic, pharmacodynamic, and immunogenicity studies during preclinical and clinical development. In LBAs, a therapeutic monoclonal antibody is considered to be the ligand, while the binding molecules is usually a target protein, or an anti-idiotypic antibody directed against the therapeutic antibody[92-97].

In a majority of these assays, the utilization of antigen-antibody reaction is done. i.e., Enzyme Immunoassay (EIA).The ligand binding assay is mainly done for the identification and the quantification of macromolecules such as peptides and proteins as well as low molecular weight drugs and also for the measurement of concentration of drugs present in the biological samples obtained in toxicokinetic studies and clinical trials. Various regulatory guidelines are issued for the validation of ligand binding assay. A single guideline for both small and large drug molecules is provided by USFDA and EMA whereas MHLW has provided a separate guidance for the LBA validation in 2014. These assays are mostly run without prior separation of the analyte of interest due to their inherent characteristics and complex structure of the macromolecules which makes the extraction process problematic. Such assays do not measure the macromolecule directly but indirectly measure the binding reaction with reagents employed in the assay for which the analytical test is planned[98-104].

Comparison of ligand binding assay guidelines updated by various international regulatory authorities

Various regulatory guidelines are issued for the validation of ligand binding assay (LBA). A single guideline for both small and large drug molecules is provided by USFDA, EMA and ICH whereas MHLW has provided a separate guidance for the LBA validation (in 2014)[104]. Comparison of ligand binding assay guidelines updated by various international regulatory authorities is depicted in Table VIII to X[98-104].

Table VIII: LBA validation 'Specificity' binding ability to target analyte only

Guidelines	Definition	Method	Acceptance criteria
USFDA Draft guidelines (2013)[7]	Not specified	Not specified	Not specified
USFDA guidelines recommendation (2018)[6]	It is evaluated by spiking blank matrix samples with related molecules at maximal concentrate of structurally related molecule study samples.	The lowest nonzero standard on the calibration curve defines the sensitivity (LLOQ).	Accuracy should be ± 25% of the nominal concentration (from ≥ three replicates in at least six runs). Precision should be ± 25% CV (from ≥ three replicates in at least six runs). Total error: Should be ≤ 40%.

Guidelines	Definition	Method	Acceptance criteria
MHLW guidelines (2013)[11]	It is defined as the ability of an analytical method to detect and differentiate the analyte from other substances, including its related substances.	The method of evaluating specificity of LBA is evaluated by using blanks samples, the blank samples are spiked [added] with the related substance at concentrations anticipated [presumed/ probable values] in the study samples and QC samples with analyte concentration near to LLOQ and ULOQ.	Assay results for blank samples and blank samples spiked with related substance should be below LLOQ. Accuracy in measurements of QC samples spiked with related substance should demonstrate an accuracy of within ±20% of theoretical concentration or within ±25% of theoretical concentration at LLOQ and ULOQ of related probable-interfering substance
EMA guidelines (2012)[12]	Specificity of the binding reagent is defined as its ability to bind solely to the analyte of the interest	The specificity of LBA should be tested with QC samples by adding increasing concentration of available "related molecules" or drugs expected to be concomitantly administered and measuring the accuracy of macromolecule of interest at both LLOQ and ULOQ.	Assay acceptance criteria of QC samples are that, its value should be within 25% of the nominal - [expected/ standard/ theoretically calculated] concentration values
ICH guidelines (2019)[14]	Ability of an analytical method to detect analyte from other substances, including related substances (e.g., substances that are structurally similar, metabolites, isomer or impurities).	Specificity is evaluated by spiking blank matrix samples with related molecules at the maximal concentration (s) of the structurally related molecule anticipated in study samples.	The response of blank samples spiked with related molecules should be below the LLOQ. The accuracy of the target analyte in presence of related molecules should be within ±25% of the nominal values.

Table IX: LBA validation "Selectivity"

Guidelines	Definition	Method	Acceptance criteria
USFDA draft guidelines (2013)[7]	Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.	Selectivity is evaluated by comparing CC in biological fluids with calibrators in buffer using at least 10 sources of blank matrix and parallelism of diluted study samples should be evaluated to detect matrix effect.	Not specified
USFDA guidelines recommendation (2018)[6]	Selectivity is the ability of the method to detect and differentiate the analyte of interest in the presence of other "unrelated compounds" (non-specific interference) in sample matrix.	It is done by investigating parallelism (for endogenous products). The analysis of blank samples in the matrix is done by using ≥ 10 individual sources.	≥ 80% of sources, unspiked matrix should be BQL, and spiked samples should be ± 25% at LLOQ, & ±20% at H QC.

Guidelines	Definition	Method	Acceptance criteria
MHLW guidelines (2013)[11]	Selectivity is the analytical binding ability to detect and differentiate the analyte in presence of other components in the analyte sample to be tested for validating the selectivity.	The test sample is evaluated by using blank samples obtained from 10 individual sources and at or near LLOQ prepared from individual blank samples less than 10 sources are acceptable when matrix is limitedly available.	At least 80% of the blank samples should be below LLOQ & at least 80% of the near-LLOQ Q C samples should demonstrate an accuracy of within ±20% of theoretical concentration or within ±25% at the LLOQ
EMA guidelines (2012)[12]	Selectivity is the ability to measure the analyte of interest in the presence of unrelated compounds in the matrix.	The sample is tested by spiking at least 10 sources including lipemic & haemolysed sample matrix at or near LLOQ When interference is concentration dependent, it is essential to determine minimum concentration where interference occurs during test both LLOQ & ULOQ.	Accuracy should be within 20% (25% at the LLOQ) of the nominal spiked concentration in at least 80% of the matrices evaluated.
ICH guidelines (2019)[14]	Selectivity is the ability to measure the analyte of interest in the presence of unrelated compounds in the matrix.	Blank samples obtained from at least 10 individual sources and by spiking the individual blank matrices at the LLOQ and at high QC level are evaluated. Response of blank samples should be below the LLOQ in at least 80% of individual sources.	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.

Table X: LBA validation "Accuracy, precision and recovery"

Guidelines	Method	Acceptance criteria
USFDA draft guidelines (2013)[7]	The accuracy and precision are determined by replicate analysis of samples containing known amount of analyte using minimum of 5 repeat determinations per concentration and a minimum of 3 concentrations in range of probable/ expected study sample concentration. Recovery is applicable for the LBA that employ sample extraction, it is the measured concentration relative to the and known amount of analyte added to the matrix at 3 concentrations	For considering acceptance criteria of accuracy and precision, mean value should be within 20% of actual value except at LLOQ, where it should not deviate by more than 25%. For recovery, no specific criteria are provided, but generally 100% recovery is required.

USFDA guidelines recommendation (2018)[6]	Accuracy and precision should be evaluated using at least six independent accuracy and precision runs, five QC levels per run (LLOQ, L, M, H, ULOQ (QC), and ≥ 3 replicates per QC level.	Acceptance criteria for accuracy: Within-run & between runs: • ± 20% of nominal concentrations; except ±25% at LLOQ and ULOQ. Acceptance criteria for precision: Within-run and between runs: • ± 20% CV, except ± 25% at LLOQ and ULOQ. Total Error: QCs should be ±30%, except at LLOQ, ULOQ ±40%.
MHLW guidelines (2013)[11]	The parameters accuracy and precision are assessed by QC samples with a minimum of 5 different concentrations (LLOQ, low- mid- high QCs, ULOQ) within the calibration range of analyte of interest. Accuracy and precision should be evaluated by repeating the analysis in at least 6 analytical runs.	Accuracy and precision are considered valid when, the mean within-run and between-run accuracy at each concentration level should be within 20% deviation of the theoretical [nominal] concentration, except at the LLOQ and ULOQ, where it should be below 25%. Considering total error at each level should not exceed 30%, except at LLOQ and ULOQ, where it should not exceed 40% level.
EMA guidelines (2012)[12]	For determining the accuracy and precision, at least 5 different concentration QC samples should be taken and the measurement should be made across at least 6 repeat/ independent assay runs over several days. The mean concentration should be within 20% of the nominal [standard/mean] value at each concentration level for within-run and between-run accuracy.	The mean concentration should be within 20% of the nominal value at each concentration level for within- run and between-run accuracy. With-in run and between-run precision should not exceed 20% (25% at LLOQ & ULOQ). The total error should not exceed 30% (40% at LLOQ & ULOQ).
ICH guidelines (2019)[14]	Accuracy and precision should be determined by analysing the QCs within each run (within-run) and in different runs (between - run). Accuracy and precision should be evaluated using runs and data. Accuracy and precision should be determined by analysing at least 3 replicates per run at each QC concentration level (LLOQ, low, medium, high, ULOQ) in at least 6 runs over 2 or more days.	The overall within-run and between-run accuracy at each concentration level should be within ±20% of the nominal values, except for the LLOQ and ULOQ, which should be within ±25% of nominal value. Within-run & between-run precision of the QC concentrations determined at each Level should not exceed 20%, except at the LLOQ & ULOQ, where it should not exceed 25%. Total error (i.e., sum of absolute value of the errors in accuracy (%) & precision (%)) should be evaluated. The total error should not exceed 30% (40% at LLOQ & ULOQ).

CONCLUSION

Validation of bio-analytical method is vital before routine use of bio-analytical method. For bio-analytical method validation, various guidelines have been issued by major regulatory authorities, like- United States Food and Drug Administration (USFDA) of United States, European Medical agency (EMA) of Europe, National Health Surveillance Agency (ANVISA) of Brazil, Ministry of Health and Labour Welfare (MHLW) of Japan, and others are updated time to time. The variation, similarities and comparison exist in acceptance criteria and methodology though they have general agreements. Till date, the USFDA, ANVISA, EMA and MHLW guidelines are referred for the bioanalytical method validation. Even though the scientific basis for evaluation of parameters is same across these guidelines, still there are differences in the acceptance criteria and methodology for few parameters. There is similarity in USFDA and EMA guidelines but they are not identical. The practical conduct of experiment is described more precisely in EMA guidance. USFDA recommendations are more comprehensive. There are differences in validation parameters. International Council for Harmonization combines advantages of guidelines to resolve differences in terminologies and reducing the efforts to comply various guidelines.

In comparison to the 2013 draft guidance document the present 2018 guidance document has not been changed significantly, in general, the major change the present guidance document underwent was the wording of the different guidance sections. In the assistance on full validation of bioanalytical methods is where the new USFDA 2018 guidance document presents consistency with other guidance documents such as the EMA guidance document on validation of bioanalytical methods.

In analytical method validation, the evaluation of complex biological matrices such as serum, plasma or other body fluids, were not included where variations among individuals can be quite large. Therefore a guideline for this specific need was established separately. In addition, regulatory guidelines include stability aspects, matrix effect, ligand binding assays (LBAs) etc. for bio-analytical method validation compliance. In 2013, Ministry of Health, Labour and Welfare (MHLW), Japan issued its draft guidance for low molecular weight drugs and bioanalytical method (Ligand binding assay) validation in pharmaceutical development. Various regulatory guidelines are issued for the validation of ligand binding assay (LBA). A single guideline for both small and large drug molecules is provided by USFDA, EMA and ICH whereas MHLW has provided a separate guidance for the LBA validation (in 2014).

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