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Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry☆

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ABSTRACT

The concepts, importance, and application of bioanalytical method validation have been discussed for a long time and validation of bioanalytical methods is widely accepted as pivotal before they are taken into routine use. United States Food and Drug Administration (USFDA) guidelines issued in 2001 have been referred for every guideline released ever since; may it be European Medical Agency (EMA) Europe, National Health Surveillance Agency (ANVISA) Brazil, Ministry of Health and Labour Welfare (MHLW) Japan or any other guideline in reference to bioanalytical method validation. After 12 years, USFDA released its new draft guideline for comments in 2013, which covers the latest parameters or topics encountered in bioanalytical method validation and approached towards the harmonization of bioanalytical method validation across the globe. Even though the regulatory agencies have general agreement, significant variations exist in acceptance criteria and methodology. The present review highlights the variations, similarities and comparison between bioanalytical method validation guidelines issued by major regulatory authorities worldwide. Additionally, other evaluation parameters such as matrix effect, incurred sample reanalysis including other stability aspects have been discussed to provide an ease of access for designing a bioanalytical method and its validation complying with the majority of drug authority guidelines.

1. Introduction

The word "Bioanalytics" refers to the analysis of the desired analyte in biological fluids. In the present pharmaceutical industry, the bioanalytical methods are playing a crucial role in the quantitative determination of low molecular weight drug molecules and macromolecules. The quantitative determination leads to the evaluation and interpretation of pharmacokinetic, bioavailability, drug–drug interaction, bioequivalence and compatibility studies. Validation of any analytical method ensures that the developed method is reproducible, stable, sensitive, robust, suitable and reliable for its application in blood, plasma, urine, serum and faeces analysis. Bioanalytical validation ensures the high-quality data for regulatory submission as well as for the drug discovery and development. The history of bioanalytical method validation was identified globally in 1990, in a workshop sponsored by United States Food and Drug Administration (USFDA) and American Association of Pharmaceutical Sciences (AAPS) with an objective to harmonize the method validation principles. USFDA released the first guideline for the bioanalytical method validation in May 2001. National Health Surveillance Agency (ANVISA), Brazil released its first bioanalytical guidelines in May 2003 in combination with analytical validation guidelines, which were further amended in May 2012. European Medicines Agency (EMA, European authority) issued its guidelines which became effective since February 2012. Ministry of Health, Labour and Welfare (MHLW), Japan in 2013 issued its draft guidance for low molecular weight drugs and bioanalytical method (ligand binding assay) validation in pharmaceutical development. Although there is a general understanding between regulatory authorities worldwide on the evaluation of validation parameters, still there are some differences in the methodology and acceptance criteria employed for bioanalytical method validation. These variations in the guidelines are important for the regulatory submission in the specific region or country. The present review discusses the

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most widely followed guidelines for bioanalytical method validation along with their acceptance criteria on different parameters.

1.1. Introduction to the guidelines

USFDA (2001) bioanalytical method validation guidelines provide assistance to sponsors for Investigational New Drug (IND) Application, New Drug Application (NDA), Abbreviated New Drug Application (ANDAs) and supplements for development and validation of bioanalytical methods used in clinical pharmacology, bioavailability and bioequivalence studies. The guideline is also applicable to the bioanalytical methods used for non-clinical pharmacology/toxicology studies and preclinical studies in blood, serum, plasma, urine, tissues and skin samples by using gas chromatography (GC), high-pressure liquid chromatography (HPLC), and combination of GC and LC with mass spectroscopy (MS) such as GC–MS, GC–MS/MS, LC–MS and LC–MS/MS. However, in the case of veterinary drug approval, the guidance is applicable only to blood and urine samples. The draft guidance issued in 2013 extended its scope for Biological Licence Application (BLAs) and provided assistance for developing bioanalytical method validation information for biomarker concentration evaluation [1]. The EMA guidance applies to bioanalysis in animal toxicological studies and all phases of clinical trials, along with providing validation recommendation for ligand binding assay. EMA kept the methods used for the quantitative determination of biomarkers in the assessment of pharmacodynamic endpoints out of its scope [2]. However, if anyone looks closely at the guidelines, then one can easily understand that the guidelines are basically based on the Good Laboratory Practices (GLP), but still USFDA guidance lacks the recommendation for the performance of bioanalytical method validation in compliance with GLP, whereas EMA has recommended performing bioanalytical methods used in non-clinical pharmaco-toxicological studies in agreement with the principles of GLP, but not mandatorily [1–3]. Likewise, ANVISA is also limited to the bioanalytical methods using GC, HPLC and their combination with MS used for the quantitative determination of drugs in blood, serum, plasma and urine. ANVISA extended its applicability to other matrices also which has been lacking in the other guidelines [4]. Its amendment has been published in 2012, which extended its scope and provided new criteria for validation of analytical procedure [5]. Japan's MHLW provided a draft for two separate guidelines for small and large molecular weight drugs, respectively and the guidelines are not restricted to a specific detector or analytical technique unlike earlier guidelines [6]. Full validation is recommended by all the regulatory authorities for the new chemical entity and when the method is applied for the first time whether or not mentioned in the literature and also when the metabolites are added to an existing assay for quantification [1–5]. Additionally, EMA and MHLW guidelines recommend for full validation for each species and matrix which is seen lacking in the USFDA guidance [2,6]. Partial validation is generally conducted with an aim to demonstrate the maintenance of the performance and reliability of the method when minor changes are made to a validated bioanalytical method which includes but not limited to transferring of method to another laboratory; change in equipment, calibration range, limited sample volume, another matrix or species; change in anticoagulant, sample processing procedure etc. The changes should be notified, and also, the partial validation should be justified [1,7,8]. Cross-validation is required for the validation parameters when two or more bioanalytical methods are used to generate data within the same study from different methods or different laboratories and across different studies. EMA guides the accuracy to be within ±15% and may be wider, if justified for QC samples and the difference between the two values should be within ±20% of the mean for at least 67% of the repeats for study samples [2]. MHLW too kept the same criteria with additional consideration to intra-and inter-laboratory precision [6]. USFDA and ANVISA lack detailed information or acceptance criteria for cross-validation [1,3–5].

2. Chromatographic methods

2.1. Reference standards

USFDA draft guidance (2013) in addition to USFDA (2001) guidance recommends the characterization of Internal Standard (IS) and analyte including the certificate of analysis and in case of expiration of IS or reference standard (RS), the stock solution made from the expired lot should not be used unless purity is re-established [1,3]. EMA does not recommend the requirement of a certificate of analysis for IS-certified standard, as long as it's suitability for use is demonstrated [2]. MHLW recommends the demonstration of lack of analytical interference with the analyte before the use of IS [6]. ANVISA recommends the use of IS and RS which is made official by Pharmacopeia or any other code and in the absence of reference standard, studies using secondary standard will be admitted provided with certification is proved [4].

2.2. Bioanalytical method validation

2.2.1. Selectivity

Specificity and selectivity are two interchangeable terms used in validation. In spite of both terms having same literal meaning, it is important to understand the difference. Specificity describes the ability of the bioanalytical method to produce a signal only for the analyte of interest and not for other interfering components. Whereas, selectivity describes the ability of a method to differentiate analyte of interest from other analytes or endogenous impurities present in samples [9,10]. While LC–MS/MS bioanalytical methods are considered to be specific and HPLC with other detection methods are considered selective [10]. Generally, the procedure followed for the evaluation of selectivity is to compare the response of an analyte in the biological sample at the lower limit of quantification (LLOQ) with blank matrix sample. It is recommended to take blank matrix from at least six different sources and compare it with the spiked LLOQ in the matrix. ANVISA initially added the requirement of proving specificity in four normal, one haemolysed and one lipemic biological matrix which was further limited to matrices other than whole blood, for which five standard and a lipemic sample are recommended [4,5]. There is a lack of acceptance criteria in USFDA guidance, but other guidelines have provided their acceptance criteria which have been stated in Table 1. Additionally, ANVISA also suggested that if one or more samples show interference above the limits, new samples of at least six other different sources should be tested and if one or more samples of the second group show interference above the limits, the method should be changed in order to eliminate it [4,5]. EMA additionally suggested investigating the extent of interference caused by the metabolites of the drugs, interferences from degradation products and interferences from possible co-administered medications. Also, the possibility of back-conversion of a metabolite into parent during the successive steps of analysis should be evaluated when relevant and in the case when it is difficult to obtain the metabolites of interest, the back-conversion can be checked by applying incurred sample reanalysis [2].

2.2.2. Accuracy, precision and recovery

Accuracy of any bioanalytical method depends on the closeness between observed and true value of concentration, expressed either as % bias or % nominal, determined using quality control samples prepared at concentration levels covering the dynamic range of the method i.e. lower limit of quantification (LLOQ), low quality

Table 1
Comparison of validation parameter "Selectivity/Specificity" [1–6].

Selectivity	USFDA guidelines for BMV (2001)	USFDA draft guidelines for BMV (2013)	ANVISA guidelines for BMV (2003, 2012)	MHLW guidelines for BMV (2013)	EMA guidelines for BMV (2011)
Definition	Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample	Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample	Ability of the method to distinguish and quantify the analyte and IS in the presence of other sample components	Ability of an analytical method to measure and differentiate the analyte and the IS in the presence of other components in samples	Ability 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
Method	Analysis of blank samples of appropriate biological matrix (plasma, urine or other matrix) obtained from at least six sources	Analysis of blank samples of appropriate biological matrix (plasma, urine or other matrix) obtained from at least six sources	Samples of the biological matrix obtained from six individual must be analyzed, including four normal samples, a lipemic sample and a haemolysed sample. In case of whole blood, five standards and a lipemic sample is recommended	Evaluated using blank matrix samples obtained from at least 6 individual sources, the absence of each analyte and IS should be confirmed	Selectivity should be provided using at least 6 individual sources of matrix, use of fewer sources is acceptable in case of rare matrices
Acceptance criteria	Not Specified	Not Specified	Response of interfering peak at the retention time should be lower than 20% of the response of LLOQ samples and lower than 5% of the IS response	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	Response of interfering component should be less than 20% response of the LLOQ for the analyte and 5% of the IS response

control (LQC), middle quality control (MQC), and high quality control (HQC). LLOQ is prepared at the lower limit of quantification; LQC is above LLOQ but not more than 3 times of LLOQ. MQC is prepared in the midway of dynamic range, ideally should be mean of LQC and HQC. The HQC samples are prepared closer to the upper limit of quantification (ULOQ) and should not be more than 80–85% of ULOQ. Each accuracy batch usually contains one blank sample, one zero sample and calibration standards i.e. LLOQ and ULOQ in duplicates followed by five or six sets of quality control samples (LLOQ, LQC, MQC and HQC). The accuracy should be determined on a single day and multiple days. Precision of any bioanalytical method is its ability to produce reproducible results between series of measurements from homogenous samples, expressed as % coefficient of variation (%CV) or % relative standard deviation (%RSD) which can be calculated using following formula:

$$\%\text{RSD} = \frac{\text{SD}}{\text{Mean concentration}} \times 100$$

The guidelines define accuracy and precision in their words as stated in Table 2. While USFDA lacks in elaborating the experimental conduct of between-run accuracy and precision, other regulatory agencies have clearly mentioned the use of at least 3 runs which are compared in Table 2. Recovery of a bioanalytical method measures the efficiency of the extraction procedure within a variation limit. 100% recovery is desirable, but acceptability depends upon guidelines issued by various drug regulatory agencies. Higher recovery indicates efficient extraction procedure, higher sensitivity and accuracy of the bioanalytical method. Higher recovery or the extraction efficiency can be achieved by optimization of pH, extraction procedures and combination of extraction solvents. The pH is used by the nature of drug and its binding efficiency with the matrix or plasma proteins and also the chemical properties of the drug. Based on the chemical properties of the molecule and the matrix, different extraction procedures are applied which mainly include protein precipitation, liquid–liquid extraction and solid phase extraction. Recovery can be classified into absolute and relative recoveries [11]. Relative recovery is determined by the comparison of analyte response obtained from extracted biological samples to that of spiked analyte in the extracted blank matrix at the same concentration. Absolute recovery is determined by comparing analyte response obtained in extracted samples with analyte response in neat aqueous solutions prepared at the same concentration. The relative recovery represents the 'matrix effects' and its importance during method development will be highlighted in the upcoming subsections [10]. The absolute recovery of the analyte during method validation should be determined at least at three concentration levels viz., LQC, MQC and HQC, respectively using, at least, five replicates of each. Once the absolute recoveries have been determined, overall recovery and %CV of all absolute recoveries should be calculated and the overall %CV less than 20% indicate the consistency and reproducibility of recoveries over the dynamic range. The recovery for IS should be determined at its working concentration. While EMA does not define or provide any recommendation regarding recovery, other guidelines suggest determining the recovery in study samples or assay of the analyte. USFDA defines recovery as the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. In contrast to the earlier USFDA guidance document, the draft guideline also recommends that the recovery experiments should be performed in comparison with unextracted standards that represent 100% recovery [1,3]. EMA does not consider recovery at all [2]. The comparison in methodology and acceptance criteria is mentioned in Table 2.

2.2.3. Calibration/linearity curve

The linearity or concentration–response of an analytical method refers to the ability of the method to produce a signal, which is either directly or by mathematical transformation, proportional to the concentration of the analyte present in the sample. It is generally recommended to analyse a minimum of six calibration concentration levels, a blank sample (matrix sample processed

Table 2
Comparison of validation parameter "Accuracy, Precision and Recovery"[1–6].

	USFDA guidelines for BMV (2001)	USFDA draft guidelines for BMV (2013)	ANVISA guidelines for BMV (2003, 2012)	MHLW guidelines for BMV (2013)	EMA guidelines for BMV (2011)
Accuracy					
Definition	The closeness of mean test results obtained by the method to the actual value (concentration) of the analyte	The closeness of mean test results obtained by the method to the actual value (concentration) of the analyte	Represents the degree of match between the individual results found and a value accepted as reference	The degree of closeness between analyte concentration determined by the method and its theoretical concentration	The closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage)
Method	Measured using a minimum of 5 determinations per concentration and a minimum of 3 concentrations in the range of expected concentration is recommended.	Measured using a minimum of 5 determinations per concentration and a minimum of 3 concentrations in the range of expected concentration is recommended	Must be determined with 5 replicates in at least 5 concentrations in each run and must be determined in the same analytical run (intra accuracy) and in at least three different runs (inter-run)	Within-run accuracy should be evaluated by at least 5 replicates at each concentration level in a single run. Between-run accuracy should be evaluated by analysis in at least 3 analytical runs	Within-run accuracy is evaluated by a minimum of 5 samples per level at minimum of 4 concentration levels in a single run. Between run should be evaluated from at least three runs analyzed on different days
Acceptance criteria	The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%	The mean value should be within 15% of the nominal value except at LLOQ, where it should not deviate by more than 20%	The deviation should not exceed 15%, except for the quantification limit for which values $\leq$20% are allowed	The mean accuracy at each concentration level should be within 15%, except at the LLOQ, where it should be within 20%	The mean concentration should be within 15%, except for the LLOQ which should be within 20% of the nominal value
Precision					
Definition	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 closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix	Closeness of the results obtained by repeated measurement of multiple aliquots from a single source matrix	Variation between individual concentrations determined in repeated measurements	The closeness of repeated individual measures of analyte. Precision is expressed as the coefficient of variance (%CV).
Method	Measured using a minimum of 5 determinations per concentration and at minimum of 3 concentrations	Measured using a minimum of 5 determinations per concentration and at minimum of 3 concentrations	Measured using at least 5 replicates in at least 5 concentrations in single run (intra-precision) and at least 3 different runs (inter-precision)	Measured using at least 5 replicates (intra-precision) and three runs (inter- precision), at 4 different concentrations	Measured using 5 samples at 3 concentrations in a single run (intra) and 3 runs for 3 concentrations on at least two different days (inter)
Acceptance criteria	At each concentration level precision should not exceed 15% of the%CV except for the LLOQ, where it should not exceed 20% of the CV	At each concentration level precision should not exceed 15% of the%CV except for the LLOQ, where it should not exceed 20% of the CV	Relative standard deviation (RSD) or%CV should be below 15%, except for LLOQ, which should not exceed 20%	At each level, %CV should not exceed 15%, except for LLOQ, where it should not exceed 20%	%CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%
Recovery					
Definition	The detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent	The detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in solvent	Measures the efficiency of the extraction procedure of an analytical method within a variation limit	Measure of the efficiency at which an analytical method recovers the analyte through the sample-processing steps	Not defined
Method	Performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery	Performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery	Performed by comparing the analytical results of samples extracted from biomatrix at three concentrations (low, medium and high), with unextracted standards representing 100% recovery	Determined by comparing the analyte response in a biological sample spiked with the analyte and processed, with the response in a biological blank sample that is processed and then spiked with the analyte	Not defined
Acceptance criteria	Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and IS should be consistent, precise, and reproducible	Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and IS should be consistent, precise, and reproducible	Recovery percentages near 100% are desirable, nevertheless lower values are accepted, provided the recovery is precise and accurate	Demonstrate the reproducibility, rather than to show a higher recovery rate	Not defined

Table 3
Comparison of validation parameter "Calibration Curve/Linearity Standard"[1–6].

Linearity	USFDA guidelines for BMV (2001)	USFDA draft guidelines for BMV (2013)	ANVISA guidelines for BMV (2003, 2012)	MHLW guidelines for BMV (2013)	EMA guidelines for BMV (2011)
Definition	Relationship between instrument response and known concentrations of the analyte	Relationship between instrument response and known concentrations of the analyte	Represents the relationship between the response of the instrument and the known concentration of the analyte	A calibration curve demonstrates the relationship between a theoretical concentration and a response of an analyte	Relationship which can simply and adequately describe the response of the instrument with regard to the concentration of analyte
Method	Should consist of a blank sample, a zero sample, and 6–8 non-zero samples covering the expected range, including LLOQ	Should consist of a blank sample, a zero sample and at least 6 non-zero covering the expected range, including LLOQ	Should include the analysis of a blank sample, zero sample and at least 6 non-zero samples including LLOQ, containing drug standard and IS	Should contain a blank sample, a zero sample, and at least 6 concentration levels of calibration standards, including an LLOQ sample	A minimum of 6 concentration levels, along with a blank sample and a zero sample in replicate
Acceptance criteria	LLOQ: 5 times the response compared to blank response and with a precision of 20% and accuracy of 80–120% Calibration curve: 20% deviation of the LLOQ from nominal concentration and 15% deviation of standards other than LLOQ from nominal concentration	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 the 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	Deviation 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 R^2 must be equal to or higher than 0.98.	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	The back calculated concentrations of the calibration standards should be within ±15% of the nominal value, except for the LLOQ for which it should be within ±20%. At least 75% of the calibration standards, with a minimum of six calibration standard levels, must fulfil this criterion. In case replicates are used, the criteria should also be fulfilled for at least 50% of the calibration standards tested per concentration level

without drug standard and internal standard) and a zero sample (matrix sample processed with internal standard) in the same biological matrix as the samples in the study by spiking the matrix with known amount of analyte. Once the response (Y) is obtained at various concentration levels (X) of calibration standards, a least square regression analysis is performed assuming the errors in Y are normally distributed around a mean value of zero. Once the least regression equation is obtained, statistical tests such as *t*-test can be used as an indicative of the accuracy of the method [10,12]. An increase or decrease in error variance with concentration represents heteroscedasticity and a weighted linear regression based on the relative error (%RE) at all concentration levels is preferred over ordinary regression [10,13]. ANVISA recommends the use of the simple mathematical model i.e. linear model for the evaluation and in case any non-linear model is proposed, and then it must to show mathematically that the linear model is not appropriate, and the model should include at least 8 samples of different concentrations on the calibration curve (CC) [5]. The assay range in bioanalytical methods is higher in comparison to other analytical methods. Hence, accuracy at lower points is either over or under the predicted [10,13]. Even though the bioanalytical methods exhibit a linear relationship between concentration and response, complex quadratic equations can be chosen when the range of the bioanalytical method is extremely high. The LLOQ should be at least 10% of the expected maximum concentration point in the concentration time profile (C_{max}), and ULOQ should be at least 2 times the expected C_{max} value. Regression coefficient (R^2) > 0.98 is generally sufficient, but it has been highlighted that statistical tests such as lack-of-fit may enhance confidence in the selected mathematical models as R^2 is a poor indicator of linearity [14]. The procedure for rejection of calibration standard is only highlighted in EMA guidelines; the comparison of methodology and acceptance criteria is mentioned in Table 3.

2.2.4. Sensitivity/detection limit

Sensitivity or detection limit of a bioanalytical method is the lowest concentration of analyte which can be determined with acceptable accuracy and precision [10]. In contrast to the earlier guidance, USFDA provided a definition of sensitivity in its draft guidance. The sensitivity of a bioanalytical method is expressed at the LLOQ, which is defined as the lowest concentration of an analyte at which the analyte can be quantified with reliable accuracy and precision. In case of instrumental method, the detection limit can be estimated based on the ratio of 3 times the noise of the baseline using the following equation:

$$\text{Detection Limit} = \frac{\text{SD} \times 3}{\text{Slope of calibration curve}}$$

where, SD is the standard deviation of the intercept with the Y axis of at least 3 calibration curves containing concentrations of the drug close to the presumed quantification limit.

The quantitation limit is expressed as concentration of analyte in sample which can be represented by the following equation:

$$\text{Quantitation Limit} = \frac{\text{SD} \times 10}{\text{Slope of calibration curve}}$$

The comparison in definition, methodology and acceptance criteria from different regulatory agencies is discussed in Table 4.

Table 4
Comparison of validation parameter "Sensitivity/Detection Limit/Lower limit of quantification"[1–6].

Sensitivity/Detection Limit/LLOQ	USFDA guidelines for BMV (2001)	USFDA draft guidelines for BMV (2013)	ANVISA guidelines for BMV (2012)	MHLW guidelines for BMV (2013)	EMA guidelines for BMV (2011)
Definition	The lowest amount of an analyte in a sample that can be quantatively determined with suitable precision and accuracy	Lowest analyte concentration that can be measured with acceptable accuracy and precision	Lowest concentration of an analyte that the bioanalytical procedure can distinguish reliably from the background	Lowest concentration of an analyte at which the analyte can be quantified with reliable accuracy and precision	Lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision
Method	Establish LLOQ using at least five samples independent of standards and determine%CV or confidence interval	Establish LLOQ using at least five samples independent of standards and determine%CV or confidence interval	At least five determinations should be carried out at LLOQ. Establish detection limit by analysing solutions of known and decreasing concentrations of the drug up to detectable level	LLOQ should be adapted to expected concentration in the study	LLOQ should be adapted to expected concentration in the study and LLOQ should be established using a minimum of five determinations
Acceptance criteria	Should be at least 5 times the response compared to blank response. LLOQ analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120%	Should be at least 5 times the response compared to blank response. LLOQ analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120%	Ratio of 5:1 between signal to noise should be obtained and response to LLOQ should be at least 5 times greater than the interference in blank samples. Peak should be identifiable with precision of 20% and accuracy of 80–120%	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	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%

2.2.5. Stability

Stability has always been considered as an important parameter during analysis both chemically and biologically (the chemical stability of a drug is a function of its physicochemical properties), the stability in method validation should reflect situations which are likely to be encountered during study sample handling and analysis. The main aim of evaluating stability in method validation is to detect any degradation of the analyte of interest during the entire period of sample collection, storage, extraction and analysis. It is recommended to confirm stability at each and every step of sample preparation and analysis, as well as in the conditions used for the long term storage. It should be noted that stability in a particular matrix and container should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after short-term storage, long-term storage, and after going through freeze and thaw cycles. Regulatory authorities recommend freeze-thaw stability, short-term temperature stability or bench top stability, long-term stability, stock solution stability and post-operative stability to be performed using a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free biological matrix. Stability samples should be compared with freshly made calibrators and freshly made standard solutions, at least three replicates of each of the low and high concentrations of analyte should be assessed. In the case of working solutions of the analyte and IS the stability of stock should be evaluated at room temperature over a short period and intended storage conditions for long periods. Stability of stock of working standard should be assessed by comparing with dilutions prepared freshly. In spite of the requirement of stability studies in bioanalytical method validation, EMA guidelines lack any specific procedure, but USFDA and ANVISA have provided a specific methodology. Also, no specifications are provided for acceptance of stock or working solution stability, 85–115% accuracy in comparison to freshly prepared solutions is deemed to be acceptable by all the regulatory bodies.

2.2.5.1. Freeze-thaw stability. Freeze-thaw stability studies are conducted to investigate the influence of repeated freezing and thawing on the stability of the analyte of interest and to ensure the integrity of the drug. In some cases, it has been seen that freezing of samples can cause loss of analyte due to various reasons such as adsorption of an analyte to precipitated plasma proteins, crystallisation from urine or other reasons [15]. From practical standpoint also, it is often necessary to subject samples to multiple freeze-thaw cycles as it may include failed analytical runs or the use of incorrect dilution factors [16]. The comparison between the freeze-thaw stability among different guidelines is discussed in Table 5.

2.2.5.2. Bench-top or 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 [16]. It is advisable to cover the entire time taken for sample work-up and evaluation, upto 6 h is considered to be sufficient for most of the sample preparation procedure, but some guidelines recommend covering 2–24 h stability [10,16]. All the guidelines recommend covering, at least three samples at room temperature or the same condition which are used for the sample preparation during analysis. The detailed comparison between these guidelines is mentioned in Table 5.

2.2.5.3. Long-term stability. Long-term stability assessment is designed to confirm the stability of analyte in the test system matrix covering the length of time from sample collection to sample analysis [16]. The main objective of performing long-term stability is to examine whether the analyte is stable in the biological matrix and whether any matrix degradation occurs which may interfere with the analytical method performance [15,17]. It is critical that quantitation of stability samples should be made against freshly spiked calibration standards. It is recommended that the matrix standards against which frozen and then thawed samples are quantitated should not have been previously frozen unless stability under those storage conditions has been demonstrated. The

Table 5
Comparison of validation parameter "Stability"[1–6].

Stability	USFDA guidelines for BMV (2001)	USFDA draft guidelines for BMV (2013)	ANVISA guidelines for BMV (2012)	MHLW guidelines for BMV (2013)	EMA guidelines for BMV (2011)
Method					
Freeze-Thaw stability	Performed using 3 aliquots at HQC and LQC at intended temperature for 24 h after 3 freeze-thaw cycles	Stability should be assessed for a minimum of three freeze-thaw cycles	Stability should be assessed after 3 freeze-thaw cycles using a minimum of 3 samples of HQC and LQC after storage for 12 h	Performed using 3 replicates per concentration i.e. HQC and LQC in the same condition as used for the study sample after freeze-thaw cycles	Performed after freeze-thaw cycles which should be equal to or greater than the freeze/thaw cycles intended for the study samples
Short-Term temperature stability (Bench Top stability)	Three aliquots of each of the HQC and LQC should be thawed at room temperature and kept for 4–24 h before analysis	Should be designed and conducted to cover the laboratory handling conditions that are expected for study samples	At least three replicate samples of HQC and LQC at room temperature for 4–24 h should be analyzed	Stability is evaluated using 3 replicates per concentration of HQC and LQC samples with QC samples before and after storage	Evaluate the stability using at least triplicates of LQC and HQC
Long Term stability	Determined by storing three aliquots each of HQC and LQC under the same conditions intended for study samples, concentration of stability samples to be compared with the mean of back-calculated values of the standards from 1st day of long-term stability testing	Storage time in a long-term stability evaluation should be equal or exceed the time between the date of first and date of last sample analysis	At least three samples of HQC and LQC are used after the storage of sample that exceeds the time interval between collection of first sample and analysis of last sample	Should be performed on the samples that have been stored for a time that is longer than the actual storage period	QC samples should be stored under the same conditions as the study samples and analyzed
Stock solution stability	Stability of stock solution of drug and IS should be evaluated at room temperature for at least 6 h after the completion of storage time and compared with the instrument response of freshly prepared solutions	Stability of the stock solution and IS should be evaluated and in case when stock solution is in different state or in different buffer composition from the certified RS, the stability data should be generated to justify the duration of stock solution storage stability	Stability of the drug and IS should be performed at room temperature after at least 6 h of preparation and results are compared with recently prepared solutions	Evaluation is performed by at least 3 replicates at each concentration levels of HQC and LQC	Bracketing approach can be used for the study of stability of stock and working solution
Acceptance criteria					
Post-preparative stability	Stability of the drug and IS should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards which includes the stability of samples for the resident time in the Autosampler	The stability of processed samples, including the resident time in the Autosampler, should be determined	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 is evaluated by at least 3 replicates per concentration levels of QC samples before and after storage	Stability of the processed sample at room temperature or under the storage conditions to be used during the study. On-Instrument/Autosampler stability of the processed sample at injector or Autosampler temperature
Acceptance criteria	Not specified	Stability sample results should be within 15% of nominal concentrations	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	The mean accuracy in the measurement at each level should be within ±15% deviation of the theoretical concentration. If any other criteria are more appropriate then that can also be used	The mean accuracy in the measurement at each level should be within ±15% deviation of the theoretical concentration

time period allowed to elapse between assessments may be variable. Usually the first few assessments are made on a daily basis and once the stability is demonstrated, the time period between assessments may be extended to weekly, and then monthly basis or even less frequently [16]. It is recommended to keep the duration of long-term stability more than the time between the first sample collection and last sample analysis [10]. However, there is no specific recommendation in the guidelines regarding the experimental design and the time points at which evaluation of stability is to be performed, but it has been advised to perform the long-term stability on three different occasions to ensure enough stability data before performing the study [10,18]. As per EMA guidelines, the bracketing technique is acceptable for small molecules i.e. it is not necessary to investigate the stability of small molecule in between if the stability is proven for any molecule at −70 and −20 °C, but for large molecules such as peptides and proteins, the stability at each temperature should be studied [2]. ANVISA and USFDA have similar guidance providing the information regarding the storage time, the quantity of samples and comparison of samples to the average of the values previously calculated on the first day of the test [1,3,4]. In spite of similar methodology, there are some variations in the guidelines, which have been detailed in Table 5.

2.2.5.4. Stock solution stability. The study samples are typically quantitated against matrix standard samples which are prepared by spiking stock solutions of the analyte in the biological matrix and these stock solutions are commonly made in aqueous buffers, organic solvents or mixtures, so it becomes important to study the stability of stock solution as a part of assay validation. It is important to generate the stock solution stability data to justify 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 [16]. Stock solution stability is performed by preparing a fresh solution from the RS and comparing the absolute response of the fresh results with that of the stored solution and the stability of the standard solutions of the analyte and IS should be evaluated to cover the time interval from preparation until use and the stability testing conditions should be same such as light or dark, temperature, solvent and container in which the study is to be performed [15,16]. In the case of large molecules such as peptides, it is advisable and recommended that the stock solution stability should be performed from the same lot of reference standard that was used to prepare the aged stock solution [15,16]. In the case when a stable isotope is used as IS, it is not necessary to study the solution stability if it is demonstrated that no isotope exchange reactions occur under the same conditions [2,4,5]. While EMA lacks in providing any specific procedure or methodology to be followed for the evaluation of stock or internal standard solution stability but recommends bracketing approach for the stability at each concentration levels [2], the comparison within different guidelines has been mentioned in Table 5.

2.2.5.5. Post-preparative stability. Also referred as processed sample stability, is the stability of analyte and IS after completion of sample preparation. It is further classified into; (i) sample extract reanalysis (ii) on-instrument stability and (iii) extract stability.

2.2.5.5.1. Sample extract reanalysis. It can also be referred to as reinjection reproducibility and is performed to determine the possibility of reinjection/reanalysing processed samples in the event of interruption of initial analysis due to any reason such as instrument failure. It can be performed by keeping a set of matrix standards which have been kept for the initial analysis or the processed samples for the time period that assess sample extract reanalysis stability (typically 24–72 h) and reanalysed; the obtained results are calculated using both the standard curve derived from the initial analysis of the standards as well as that derived from the reanalysed standards.

2.2.5.5.2. On-instrument stability. The main aim of performing on-instrument stability is due to the reason that the standards are analyzed at different time points when compared to the study samples as the matrix standard, QC samples, and the study samples are analyzed in either a serial or in a parallel manner. Assuming that the study samples are bracketed by QC samples during their analysis, data are generated with each batch of samples to demonstrate on-instrument stability [16].

2.2.5.5.3. Extract stability. In extract stability, the stability of stored sample extracts is assessed by their analysis against freshly prepared matrix standard extracts. Generally, the processed sample stability analysis should cover at least the maximum time required for the completion of the sample workup until completion of the measurements, allowing extra time to cover possible delay and the conditions of light and temperature at which the investigation is conducted. The extract stability study should be conducted under similar conditions as those of the conditions anticipated during the sample analysis [17,19]. It has been suggested in the various literatures to demonstrate stability after 24, 36 and 48 h so that in the case of incomplete assay due to any instrumental error, the reanalysis can be performed on the next day [17,19]. In the guidelines, the main focus has been kept on the stability of reconstituted sample and the stability of the reconstituted sample under autosampler conditions, also considering the resident time in the autosampler. There have been variations in the acceptance criteria for stability studies from different regulatory authorities worldwide, and this lack of harmonization has raised confusion among bioanalytical scientists for evaluation of post-preparative stability during method validation. In order to generate a consensus, this post-preparative stability has been discussed extensively in various conferences, meetings, and workshops [10].

2.2.6. Carryover effect

Carryover is seen as a major problem that influences the accuracy and precision of any bioanalytical method and is also of great importance in LC–MS/MS based bioanalytical methods, where the dynamic range is very high. Generally, it is caused due to residual analyte from the high concentration sample analyzed previously in the run which may also affect the subsequent samples depending on the concentration of previous samples. The carryover effect can also be seen randomly, in the case when the eluting residue from column affects the samples coming later during analysis, so the extent of carryover should be investigated during method development and should be eliminated or minimised. Carryover can be reduced by optimizing the composition of washing solvent and autosampler needle flushing volume in such a way to remove traces of the residual drug that may stick in the needle. In the case of removal of the basic analyte, the addition of formic acid to washing solvent is recommended along with an increase in the percentage of organic solvent in flushing solvent.

If the carryover is due to the column, then a change in mobile phase is recommended, by increasing the ratio of strong solvent or by altering the pH, altering the flow rate which will have an effect on the solubility of the analyte. Along with measures mentioned above, it should be ensured that the samples that are known to have high concentrations are never analyzed directly before the samples with very low concentrations. Carryover effect can also be counteracted by using an extra blank sample or by diluting the samples into a limited calibration range. All these approaches are being used in various laboratories and industries worldwide to counteract carryover effect in HPLC and LC–MS/MS analysis. In spite of having high importance, it is a surprise that USFDA initial guidelines and ANVISA guidelines do not provide any guidance regarding carry-over effect while the new draft USFDA guidance,

includes some information but still lacks methodology and limitations. New draft USFDA guidance states that the carryover effect should be assessed and monitored during analysis and if it occurs, it should be mitigated or reduced [1,3–5]. In contrast EMA and MHLW provide detailed information regarding the carryover effect. Both guidelines specify that the carryover effect should be addressed and minimised during method development, and validation. The carryover effect should be assessed by injecting blank samples after a high concentration sample or calibration standard at the ULOQ. As per EMA and MHWL, the carryover in the blank samples following the high concentration standard should not be greater than 20% of the LLOQ and 5% for the IS. Both guidelines suggest putting up with appropriate measures to tackle the carryover issues so that it does not affect the accuracy and precision, which could include the injection of blank samples after samples with an expected high concentration, before the analysis of the next study samples [2,6].

2.2.7. Dilution integrity

Dilution integrity is performed to evaluate the capability of the method to reliably quantify concentrations above ULOQ or in case of partial sample volume. It is performed by diluting the QC samples (with higher analyte concentrations, at least two times the ULOQ) with blank matrix depending on the expected concentration levels during the study. USFDA recommends the evaluation of dilution integrity during method validation and ANVISA recommends extending the calibration curve (CC) range or diluting the samples when concentration above ULOQ are encountered during sample analysis. However, the USFDA initial, draft guidance and ANVISA failed to provide the experimental procedure and acceptance criteria [1,3–5]. EMA specifies that if applicable, the dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix (at least five determinations per dilution factor). Accuracy and precision should be within the set criteria. Dilution integrity should cover the dilution applied to the study samples [2]. MHLW states that the dilution integrity should be evaluated by at least 5 replicates per dilution factor after diluting a sample with a blank matrix to bring the analyte concentration within the calibration range. The dilution factors should be selected by considering in the measurements of diluted samples should be within ±15% deviation of the theoretical concentration. EMA suggest that the evaluation of the dilution integrity should be covered by partial validation and the use of another matrix may be acceptable, as long as it has been demonstrated that this does not affect accuracy and precision.

2.2.8. Matrix effect

It is defined as the direct or indirect alteration or interference in response which may be due to the presence of unintended analytes or other interfering substances in the sample. Generally, it is caused by the matrix components, concomitant medications and metabolites eluting along with analyte, thereby influencing the abundance of analyte in MS source by affecting the ability of analyte in solution phase to get transferred into gas phase as charged ions and neutralizing the charged gas phase analyte ions by charge transfer or charge stripping mechanism [10,20]. Matrix effect can also be caused when molecules co-eluting with the compounds of interest alter the ionization efficiency of the electrospray interface. Matrix effects are unseen in the chromatograms but have a deleterious effect on accuracy, precision and sensitivity of the method due to which it is necessary to critically evaluate matrix effect during bioanalytical method development. A minimal matrix effect can significantly affect the performance of the method during sample analysis due to the subject to subject variation of the biological matrix and might be different from the blank lots used for spiking calibration curve and QC samples during method validation. Usually, the matrix effect is assessed either by post extraction addition method or the post-column infusion method. Various articles are present over the past describing the procedures for evaluation and elimination of matrix effect in method development and validation [21].

In the post-column infusion method, simultaneous injection of an extracted blank matrix sample is made onto a constantly infused high concentration analyte solution and the changes in the instrument response for the analyte are continuously monitored over a period of time. It is a qualitative approach and does not provide a quantitative understanding of the extent of matrix effects as it can only identify chromatographic regions which are susceptible to matrix effects. This will allow the analyst to modify the retention time of the analyte so that it does not elute in susceptible zones due to which this approach is considered time-consuming and needs significant chromatographic parameter optimization particularly if multiple analytes have to be monitored.

In post extraction spiking method, the matrix effect is assessed by comparing the analyte response in the neat aqueous sample *versus* analyte spiked in extracted blank matrix sample at the same concentration. This method is found useful and allows quantitative assessment of matrix effects for all analytes including the IS and is widely accepted.

Matrix effect can be evaluated by spiking analyte at two concentration levels (low and high QC) in six different lots of blank matrix and reading against freshly prepared CC [10]. The matrix effect is calculated by matrix factor (MF). Matrix factor can be calculated by the following formula:

$$\text{Matrix Effect} = \frac{\text{Analyte(or IS)response in spiked blank extract}}{\text{Analyte(or IS)response in neat solution}}$$

If the value of matrix factor equals to 1, then it denotes that there is no matrix effect, if it is less than one then it indicates suppression, and if more than 1 then it indicates enhancement of the analyte response [10]. If the matrix factor indicates enhancement or suppression, then the method should be modified to nullify it. Also, the IS normalized MF can be calculated using following formula:

$$\text{Internal Standard normalized Matrix Effect} = \frac{\text{Matrix effect for analyte}}{\text{Matrix effect for Internal Standard}}$$

If the value of IS normalized MF is equal to 1, then it shows the acceptability of the method with the same extent of matrix effect for the analyte and IS. During method development, the analyte is spiked at two concentrations and IS at working concentration in six different lots of blank matrix. The %CV of the IS normalized MF should not be more than 15% for demonstrating the absence of matrix effect. There is no specification for IS normalized MF for acceptance of a method, but literature and data from various labs show 0.80–1.20 as the acceptable limit.

Along with the approaches mentioned above, there are other approaches which also have been employed. One of these methods includes the calculation of %CV of slopes of the calibration curves obtained using six different lots of blank matrix. This method may be easy, but there are controversies over the evaluation in six batches as it may not be indicative of testing samples from hundreds of subjects. Moreover, the variability of extraction and matrix effects might contribute to the total variability of assay results. But this approach is found highly efficient in detecting the matrix effects for LC–MS/MS based bioanalytical methods. There are various approaches which have been suggested by Matuszewski et al. and Kollipara et al. to overcome the matrix effects by appropriate design of the bioanalytical method which include: development of method under efficient chromatographic conditions to ensure analyte peak is well separated from impurities; improving the sample

extraction method, and use of pure, stable isotope labelled IS to ensure the same extent of ionization efficiency and recovery of analyte [10,22]. It is also highlighted that the stable isotope labelled IS may not always account for matrix effect of analyte particularly if the difference in average molecular weight between the analyte and IS is more than 5, resulting in a change in retention time [23]. It should be noted that any change in the ionization mode, extraction method and chromatographic conditions at any stage of the methods' life cycle, might alter the extent of matrix effect and hence should be always evaluated. ANVISA does not present any guidelines for the evaluation of matrix effects in its guidance, while USFDA guidelines suggest that the matrix effect should be investigated to ensure that precision, selectivity and sensitivity will not be compromised [1]. The USFDA draft guidance recommends taking appropriate steps to ensure the lack of matrix effects throughout the application of the method and the matrix effect on ion suppression or enhancement or extraction efficiency should be addressed. The draft guidelines also included that the calibration curve in biological fluids should be compared with calibrators in the buffer to detect matrix effects using at least ten sources of the blank matrix along with other examples mentioned in the earlier guidelines. EMA suggests that for each analyte and IS, the MF and the IS normalized MF should be calculated for each lot of matrix. The %CV of the IS normalized MF calculated from the 6 lots of matrix should not be greater than 15%, and the determination should be performed at a low and a high level of concentration. EMA also provides an alternative approach in the case of on-line sample preparation, the variability of the response from lot to lot should be assessed by analysing at least 6 lots of matrix, spiked at a low and a high level of concentration. EMA suggests that the validation report should include the peak areas of the analyte and IS, and the calculated concentration for each individual sample and the overall %CV for the concentration should not be greater than 15%. In case it is difficult to obtain matrix, then less than 6 different lots of matrix can be used with justification, but matrix effect should be still investigated. In case when the excipients are known to have matrix effect are used in the formulations then matrix effect should be studied with matrix containing the excipient, in addition to blank matrix, the matrix used for this kind of evaluation should be obtained from subjects receiving excipients, unless it has been demonstrated that the excipient is not metabolised or transformed *in vivo*. The effect of the excipients can be studied by the determination of MF or by a dilution of a high concentration study sample with a blank matrix not containing the excipient. EMA also focused and recommended to investigate the matrix effect on other samples in addition to the normal matrix. Also, if the samples from any special population are to be analyzed, then it has been recommended to study matrix effect using a matrix of the same population. MHLW like EMA also provides an alternative method for evaluation, by analysing QC samples, each prepared using matrix from at least 6 different sources and the precision determined concentration should not be greater than 15%. MHLW also suggests the use of less than 6 sources in case of limited availability of matrix [1–6].

2.2.9. Analysis of study samples

It is recommended that the samples should be analyzed only after the validation of the bioanalytical method and should be completed within the time period for which the stability data has been reported. It may also be important to evaluate the performance of the method depending on the time lag between actual validation and study sample analysis. It is also recommended to evaluate the validity of the bioanalytical method during study sample in each analytical run by using the calibration standards and QC samples. As per EMA, an analytical run consists of the blank sample, a zero sample, calibration standards at a minimum of 6 concentration levels, at least 3 levels of QC samples in duplicate and study samples to be analyzed. All samples should be processed and extracted as one single batch of samples in an order in which they are intended to be submitted or analyzed. Analysing samples which have been prepared separately as several batches should be avoided in a single run. The acceptance criteria should be pre-established in a standard operating procedure (SOP) or in a study plan which should be defined for the complete analytical run or for the separate batches in the run. MHLW recommends the use of biological samples obtained from the pharmacokinetic studies and clinical trials to be analyzed using conditions that have been validated along with a blank sample, a zero sample, calibration standards at a minimum of 6 concentration levels and QC samples. MHLW also recommends evaluating the validity of the bioanalytical method using the CC and QC samples, while in studies that serve pharmacokinetic data as a primary endpoint, reproducibility should be confirmed for each study per matrix by performing incurred sample reanalysis. USFDA guidelines issued in 2001, state the same criteria as mentioned in other guidelines, also mentions that the biological samples can be analyzed with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data [1–6].

2.2.9.1. Calibration curve. If the analyte concentrations in the study samples are anticipated to be in a narrow range, then EMA recommends to either narrow the CC range and adapt the concentration of QC samples or add new QC samples at different concentration levels as appropriate, in order to reflect the concentrations of the study samples. Similarly, if the sample concentrations are above ULOQ, then the CC range should be extended to cover the anticipated concentrations, and at least 2 QC samples should be in the range of the study sample concentrations. In both the above situations the method should be revalidated partially to verify the response function and to ensure accuracy and precision [2]. As per MHLW, the accuracy of back-calculated concentrations of calibration standards at each level should be within ± 20% deviation of the theoretical concentration at the LLOQ or ±15% deviation at all other levels. At least 75% of the calibration standards with a minimum of 6 levels should meet the above criteria. In case the calibration standard at LLOQ and ULOQ does not meet the criteria in study sample analysis, the next lowest/highest level calibration standard may be used as the LLOQ and ULOQ, but the modified calibration range should still cover at least 3 different QC sample levels [6]. On the other hand, USFDA guidelines recommend the inclusion of QC samples, calibration standards and the processed unknown samples in a single analytical run. All the processed samples should be analyzed as a single batch or a batch comprised of processed unknown samples of one or more volunteers in a study, and the CC should cover the expected unknown sample concentration range in addition to a calibrator sample at LLOQ. USFDA does not recommend estimating the concentration in the unknown sample by extrapolation of standard curves below LLOQ or above the highest standard; instead, the standard curve should be redefined. When the bioanalytical method necessitates separation of the overall analytical run into distinct processing batches, new USFDA draft guidance recommends processing, at least, duplicate QCs at all QC levels in each distinct processing batch along with study samples [1].

2.2.9.2. Accuracy and precision. The accuracy values of the QC samples should be within ±15% of the nominal values, and at least 67% of the QC samples and at least 50% at each concentration level should comply with this criterion, and if the criterion is not fulfilled then the analytical run should be rejected, and the study samples should be re-extracted and analyzed. In case the overall mean accuracy and precision exceed 15%, this should lead to additional investigations justifying this deviation [2,6]. As per the USFDA guidance, a number of QC samples separately prepared

should be analyzed with processed test samples at intervals based on the total number of samples and the QC samples in duplicate at three concentrations should be incorporated in each run. At least four out of every six QC samples should be within 15% of the respective nominal value; two of the six may be outside the 15%, but not both at the same concentration [1,3].

2.3. Reanalysis of study samples

All the guidelines suggest performing a reanalysis of study samples in cases where the rejection is due to failure of the run to fulfil the criteria of accuracy, improper sample injection, poor chromatography, etc. EMA and MHLW also consider reanalysis due to pharmacokinetic reasons, and if the reanalysis is due to positive pre-dose samples, the reanalysed samples should be identified, and the initial value, the reason of reanalysis, the values obtained in the reanalysis, the finally accepted value and justification for the acceptance should be provided. Reinjection of sample can be made only in the case of instrument failure and if reinjection reproducibility and on-injector stability have been demonstrated during validation [1–4,6].

2.4. Chromatogram integration

All the guidelines recommend the procedure for chromatogram integration and re-integration to be predefined in the protocol or SOP. The reasons for re-integration should be recorded, and the chromatograms obtained both before and after the re-integration should be kept for future reference [1–4,6].

2.5. System suitability

It is advised to confirm the system suitability prior to each run to ensure optimum performance of the instrument used for bioanalysis. MHLW do not mandate the system suitability as the validity of analysis is routinely checked by evaluation of calibration curves and QC sample in each analytical run, while USFDA and EMA lack to provide any guidance regarding system suitability. The recent USFDA draft guideline includes system suitability but did not make any compulsion and suggests to follow a specific SOP while the apparatus conditions should be determined using spiked samples independent of the study calibrators, QCs, and study samples. According to ANVISA guidelines, the number of QC samples (in multiple of three) incorporated in each analytical run should not be lower than 5% of the number of unknown samples. For analytical runs consisting of up to 120 samples, at least 6 QC samples (duplicate of each concentration) should be used. The acceptance criteria were similar to that of USFDA guidance [1–4,6].

2.6. Incurred sample reanalysis

Incurred sample reanalysis (ISR) has become an integral part of the bioanalytical process to access the quality of bioanalytical assay which has been widely accepted within the pharmaceutical and bioanalytical community. ISR has a significant contribution regarding building confidence in well planned and executed validation studies as it reaffirms the reproducibility and reliability of a validated bioanalytical method. It is also required as the use of calibration standards and QC samples during validation may not fully mimic the actual study samples. It is recommended to evaluate the accuracy of incurred samples by reanalysis of study samples in separate runs on different days as the differences for instance in protein binding, back-conversion of metabolites during storage, sample inhomogeneity or co-medication may affect the accurate quantification. The major principle behind ISR is to demonstrate the validity and reproducibility of the method during study sample analysis and in the case of the failed ISR, the underlying reasons should be investigated, and remedial measures for the analytical method should be taken. The reasons for the failure of ISR may be attributed to pipetting errors, sample inhomogeneity due to improper vortexing, inadequate thawing, contamination, other operational errors, interference of biological components unique to the study samples or of unknown metabolites and wrong labeling. EMA and MHLW highlight the reasons mentioned above for evaluating the accuracy of incurred samples by reanalysis of study samples in separate runs at different days and recommends 10% of the samples to be reanalysed in the case when the samples are less than 1000 and 5% of the number of samples in case the samples are exceeding 1000. USFDA draft guidelines suggest that the total number of ISR samples should be 7% of the study sample size. EMA and MHLW also advises to take the sample around C_{max} and in the elimination phase and should be done at least in: toxicokinetic studies once per species; for all pivotal bioequivalence trials; first clinical trial in subjects; first patient trial; and the first trials in patients with impaired hepatic and/or renal function. MHLW additionally suggests that the ISR should be performed with samples from as many subjects or animals as possible. In the case of animal studies, the ISR can be done in early phase studies, and the samples should not be pooled, as pooling may limit anomalous findings. In addition, MHLW also suggests the ISR for non-clinical studies with samples obtained in an independent non-GLP study, if the study is similar to the relevant toxicokinetic study. The results of ISR are evaluated as assay variability. Assay variability can be calculated as the difference between the concentration obtained by ISR and that in the original analysis divided by their mean and multiplied by 100.

$$\%\text{difference or Assay Variability} = \frac{(\text{Repeat value - Initial value})}{\text{Mean value}} \times 100$$

As per EMA and MHLW the % difference during repeat analysis should not be greater than 20% of their mean for at least 67% of the repeats, while USDA draft guidelines state that two-third (67%) of the repeated sample results should be within 20% for small molecules and 30% for large molecules. If the ISR data fails to comply with the above criteria, the cause should be investigated, and necessary measures should be taken by considering the potential impact on study sample analysis [1–4,6,10,11,22,24].

3. Ligand binding assays

These are the immunoassays that use a specific antigen or antibody capable of binding to the analyte to identify and quantify substances and are mainly used for macromolecules such as peptides and proteins [25]. USFDA and EMA provide a single guideline for both small and large drug molecules while MHLW has provided a separate guidance for the LBA validation in 2014 [1,13,26]. Regulatory guidelines are issued for the validation of LBA as the analytical methods for the measurement of drugs in biological samples obtained in toxicokinetic studies and clinical trials, as well as applicable to the analyses of study samples using such methods. These guidelines are generally applicable to the quantification of peptides and proteins as well as low molecular weight drugs that are analyzed by LBAs. The validation principles and the considerations about the analysis of study samples are similar to those of the small molecules. These assays are often 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. These assays do not measure the

macromolecule directly but indirectly measure the binding reaction with reagents employed in the assay.

3.1. Reference standard

The reference standards should be well characterized and documented as their potency and immunoreactivity may vary due to their heterogeneous nature. As per the MHLW guidance, the reference standard should accompany the certificate of analysis which provides the information regarding the standard, and the material should be procured from an authenticated source. EMA recommends using the same batch of reference standard used for the dosing in the non-clinical subjects, for the preparation of calibration standards and QC samples. If there is a change in the batch then the analytical characterisation and bioanalytical evaluation should be carried out prior to its use to ensure that the performance characteristics of the method are not altered [2,26]. As per the guidelines, the minimum required dilution for full validation should be defined prior to diluting samples with buffer solution. In the case when the plate-based assay is used, the analysis should be performed in at least 2 wells per sample and the sample concentration should then be determined either by calculating a mean of responses from the wells or by averaging the concentration calculated from each response [26].

3.2. Ligand binding assay method validation

3.2.1. Specificity

In the case of LBAs, it is important that the binding reagents should specifically bind to the target analyte, but do not cross-react with the coexisting related substances. If the presence of the related substance is anticipated in the biological samples, then the extent of their impact should be evaluated. Specificity of any method is its ability to detect and differentiate the analyte of interest in the presence of other substances, including its related substances. Earlier, USFDA guidance has not mentioned LBA and later in the new draft guidance LBA method validation was included, still specificity is not included. The comparison in methodology and acceptance criteria between different guidances is detailed in Table 6.

3.2.2. Selectivity

It is the ability of the method to measure the analyte of interest in the presence of unrelated compounds in the matrix. There is no extraction due to the inherent characteristic of macromolecules, but the unrelated compounds present in the matrix may interfere with the analyte of interest in the LBA. The USFDA draft guidelines have subdivided selectivity into interference from substances physicochemically similar to analyte and matrix effect. As per this guidance, the evaluation of interference from substances physicochemically similar to analyte should be done by individually evaluating cross-reactivity of metabolites, concomitant medication, and their significant metabolites or endogenous compounds. The CC in biological matrices should be compared with calibration standards in the buffer to detect matrix effect using blank matrix from at least ten sources and the non-specific binding should also be determined. EMA and MHLW have provided a common methodology and acceptance criteria for selectivity which is discussed in Table 7.

Specificity and selectivity in the case of LBA are dependent on the ligand reagent and the species biology. If the macromolecular drug is a monoclonal antibody against any targeted protein, then the presence of a soluble form of the protein may cause interference with LBA, especially when the target protein is used as a ligand in the binding reaction. It is a challenge to the analyst to recognize the particular reaction between analyte and reagent along with the type of experiment to be employed for proving specificity and selectivity of LBA method [27].

3.2.3. Accuracy, precision and recovery

As per EMA, for the estimation of precision and accuracy QC samples should not be freshly prepared, but should be frozen and treated the same way as for the analysis of study sample. Recovery has not been considered in the EMA and MHLW guidelines but USFDA draft guidelines have provided recovery studies for the LBAs, which employed sample extraction. Comparison of methodology and acceptance criteria has been included in Table 8.

3.2.4. Calibration curve

Most LBA calibration curves are generally nonlinear and often sigmoidal. In general, more concentration points may be recommended to define the fit over the standard curve range than the chromatographic assays. The response-error relationship for immunoassay standard curves is a variable function of the mean response, due to which the standard curve should consist of a minimum of six non-zero calibrator concentrations in duplicate covering the entire range including LLOQ and excluding blanks. A 4–5-parameter logistic model is generally preferred for the regression equation of a calibration curve, the validation report should include the regression equation and weighting conditions used [1,3]. As per EMA and MHLW guidance, a minimum of 6 concentration levels of calibration standards including LLOQ and ULOQ, and a blank sample should be evaluated during the validation and should be used for the generation of CC which is to be reported in a Table to establish the overall robustness of the regression model of the CC. Along with the calibrators, anchor points lying outside the calibration range can be used to facilitate better curve fitting. The accuracy of back-calculated concentration of each calibration standard should be within 20% (25% at LLOQ and ULOQ) of nominal concentration for at least 75% of calibration standards excluding anchor points, and minimum of 6 levels of calibration standards, including the LLOQ and ULOQ, should meet this criterion [2,26]. On the other hand, USFDA draft guidelines suggest that the standard curve should consist of a minimum of six non-zero calibrator concentrations in duplicate covering the entire range including LLOQ and excluding blanks. LLOQ is established using, at least five samples and the analyte peak should have the back-calculated concentration whose precision should not exceed 25% CV and accuracy within 25% of the nominal concentration and within 20% of the nominal concentration at all other concentrations and 75% of non-zero standards should meet the above criteria, including LLOQ and the total error should not exceed 30%. Along with the acceptance criteria and discussed methodology, the draft guidelines also recommend to incorporate three concentrations in duplicate in each run i.e. LLOQ, MQC and HQC and the results of the QC samples provide the basis for acceptance and rejection; also at least 67% or two third of the QC concentration results should be within 20% of their respective nominal values and at least 50% of QC at each level should be within 20% of their nominal concentrations. Also, the minimum number of QCs should be at least 5% of the number of unknown samples or six total QC, whichever is greater [1].

3.2.5. Stability

Validation studies should determine the analyte stability after the freeze-thaw cycles, short-term and long-term storage. The stability of the analyte should be evaluated in the stock and working solutions using solutions at or near the highest and lowest concentration levels under the actual solution storage conditions. EMA, MHLW and USFDA draft guidelines recommend evaluating the stability of at least 3 replicates per QC concentration level before and after the stability storage. EMA and MHLW acceptance criteria suggest that the mean accuracy of the measurements at each level

Table 6
Comparison of "Specificity" for Ligand Binding Assay validation[1–5,26].

Specificity	USFDA draft guidelines for BMV (2013)	MHLW draft guideline for BMV(LBA) (2014)	EMA guidelines for BMV (2011)
Definition	Not specified	The ability of analytical method to detect and differentiate the analyte from other substances, including its related substances	Specificity of the binding reagent refers to its ability to bind solely to the analyte of interest
Method	Not specified	Evaluated by using blanks samples, blank samples spiked with the related substance at concentrations anticipated in the study samples and QC samples with the analyte concentration near to LLOQ and ULOQ	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 the macromolecule of interest at both LLOQ and ULOQ
Acceptance criteria	Not specified	Assay results for the blank samples and blank samples spiked with the related substance should be below LLOQ. Accuracy in the measurements of the QC samples spiked with related substance should demonstrate an accuracy of within ±20% of the theoretical concentration or within ±25% of the theoretical concentration at the LLOQ and ULOQ	Assay acceptance criteria of the QC samples should be within 25% of the nominal values

Table 7
Comparison of Selectivity for Ligand Binding Assay validation[1–5,26].

Selectivity	USFDA draft guidelines for BMV (2013)	MHLW draft guideline for BMV(LBA) (2014)	EMA guidelines for BMV (2011)
Definition	Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample	Ability of an analytical method to detect and differentiate the analyte in presence of other components in the sample.	Ability to measure the analyte of interest in the presence of unrelated compounds in the matrix.
Method	By individually evaluating cross-reactivity of metabolites, concomitant medication and their significant metabolites or endogenous compounds and when possible, the LBA should be compared with the validated reference method using incurred samples.	Evaluated by using blank samples obtained from 10 individual sources and at or near LLOQ prepared from individual blank samples	Tested by spiking at least 10 sources including lipemic and haemolysed sample matrix at or near LLOQ
	Matrix Effect should be evaluated by comparing CC in biological fluids with calibrators in buffer using at least ten sources of blank matrix and parallelism of diluted study samples should be evaluated with diluted standards to detect matrix effect	Less than 10 sources are acceptable when matrix is limitedly available	When interference is concentration dependent, it is essential to determine the minimum concentration where interference occurs
Acceptance criteria	Not defined	At least 80% of the blank samples should be below LLOQ and at least 80% of the near-LLOQ QC samples should demonstrate an accuracy of within ±20% of the theoretical concentration or within ±25% at the LLOQ	Accuracy should be within 20% (25% at the LLOQ) of the nominal spiked concentration in at least 80% of the matrices evaluated

should be within ±20% deviation of the theoretical concentrations [2,26] while USFDA draft guidelines suggest that the stability study sample results should be within ±15% of the nominal concentrations [1].

3.2.6. Analysis of study samples

An analytical run in case of LBA comprises several individual plates, but each plate should contain an individual set of calibration standards and QC samples to compensate for the difference in plate performance. As per EMA, it is recommended to assay in replicates (use at least 2 wells instead of 1). Each plate should contain at least 3 levels of QC samples at least in duplicate during and within study validation; the QCs should mimic the analysis of the study sample concerning the number of wells used per study sample. USFDA draft guidelines suggest that the runs should be rejected if the calibration standards or QCs fall outside the acceptance criteria and the minimum number of QCs to ensure proper control of the assay should be at least 5% of the number of unknown samples or a total of six QC, whichever is greater. USFDA draft guidelines and EMA do not provide a detailed methodology and acceptance criteria for the study sample analysis while MHLW has provided guidelines for each and every parameter. The accuracy of back-calculated concentrations of calibration standards at each level should be within ±25% deviation of the theoretical concentration at the LLOQ and ULOQ, and ±20% deviation at all other levels and at least 75% of the calibration standards excluding anchor points, with a minimum of 6 levels should meet the criteria. In case the calibration standard at the LLOQ and ULOQ does not meet the criteria, the next lowest/highest-level calibration standard may be used as the LLOQ or ULOQ of the calibration curve, even though, the modified calibration curve should still cover at least 3 different QC levels. MHLW guidelines state that QC samples with a minimum of 3 different concentration levels within the

Table 8
Comparison of "Accuracy, Precision and Recovery" for Ligand Binding Assay validation[1–5,26].

	USFDA draft guidelines for BMV (2013)	MHLW draft guideline for BMV(LBA) (2014)	EMA guidelines for BMV (2011)
Method	Accuracy and Precision is determined by replicate analysis of samples containing known amount of analyte using minimum of 5 determinations per concentration and a minimum of 3 concentrations in range of expected study sample concentration.	Accuracy and Precision is assessed by QC samples with a minimum of 5 different concentrations (LLOQ, low-mid- high QCs, ULOQ) within the calibration range.	At least five QC samples and measurement should be made across at least 6 independent assay runs over several days
	Recovery is applicable for the LBA that employ sample extraction, and it is the measured concentration relative to the known amount added to the matrix at three concentrations	Accuracy and precision should be evaluated by repeating the analysis in at least 6 analytical runs	The mean concentration should be within 20% of the nominal value at each concentration level for within-run and between-run accuracy
Acceptance criteria	For accuracy and precision, the mean value should be within 20% of the actual value except at LLOQ, where it should not deviate by more than 25%.	Mean within-run and between-run accuracy at each concentration level should be within 20% deviation of the theoretical concentration, except at the LLOQ and ULOQ, where it should be below 25%	With-in run and between-run precision should not exceed 20% (25% at LLOQ and ULOQ)
	For recovery, no specific criteria are provided but generally 100% recovery is required	A total error at each level should not exceed 30%, except at the LLOQ and ULOQ, where it should not exceed 40%	The total error should not exceed 30% (40% at LLOQ and ULOQ)

calibration range should be analyzed, and the analysis requires 2 QC sample at each QC level or at least 5% of the total number of study samples in the analytical run, whichever is greater and the accuracy should be within ±20% deviation of the theoretical concentrations and, at least, two-third of the QC samples and at least 50% at each concentration level should meet the criteria.

3.2.7. Incurred sample reanalysis

As described earlier, the incurred sample reanalysis refers to a reanalysis of incurred samples in separate analytical runs on different days to check the reproducibility of the originality of the analytical results. As per MHLW guidelines, approximately 10% of the samples should be reanalysed in cases where the total number of study samples is less than 1000 and approximately 5% of the number of samples exceeding 1000. USFDA draft guidelines and EMA consider the same methodology as mentioned for the chromatographic assays. As per EMA and MHLW, the concentration obtained from the initial analysis and the concentration obtained by reanalysis should be within 30% of their mean for at least 67% of the repeats. Additionally, MHLW suggests that if the ISR data failed to meet the above criteria, root cause investigation should be conducted for the analytical method, and necessary measures should be taken by considering the potential impact on the study sample analysis.

4. Conclusion

In spite of several modifications and improvements in newer guidelines regarding bioanalytical method validation, in the present authors' opinion we still lack an efficient, scientifically sound and harmonized bioanalytical method validation guidance which expedites the generation of quality and reliable data during preclinical and clinical phases of drug development for smooth registration of the product worldwide and acceptance by all regulatory agencies. To date USFDA, ANVISA, EMA and MHLW guidelines are referred for the bioanalytical method validation, recently the new draft guidelines of USFDA have also been launched which are under evaluation and may be finalized for their enactment soon. Even though the scientific basis for evaluation of parameters is same across these guidelines, but still there are differences in the acceptance criteria and methodology for few parameters. Till date USFDA (2001) and EMA are the most widely referred guidelines for the bioanalytical method validation, USFDA guidelines were lacking in some parameters which have been added in the draft guidelines issued in September 2013. But still, USFDA draft guidance lacks in providing acceptance criteria for various parameters, making the guidelines nonbinding and allowing other approaches. The draft guidance provides freedom and responsibility to the skilled and experienced bioanalyst to design and conduct appropriate protocols for all aspects of method validation. In comparison to EMA guidelines and USFDA 2001 guideline, USFDA draft guideline provides guidance on additional issues such as endogenous compounds, biomarkers, diagnostic kits and also encourages the development and use of newer bioanalytical technologies. EMA lacks in discussing important parameters such as recovery in its guidelines. These divergences among the guidelines have lead to many debates, conferences on these topics and unlimited discussion between the industrial personnel and FDA but still did not lead to a common conclusion. Hence, it can be considered to maintain a common In-House SOP for these topics as suggested by various industrial professionals in several meetings, conferences, and seminars worldwide. Also, 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 of bioanalytical method validation. As none of the guidelines appear to be restricting to their particular parameters which make it open that additional parameters which are not present in specific guidelines can be performed. Also, the regulatory agencies should consider for implementing a common guidance for the bioanalytical method validation which will lead to harmonization of the method development and validation of bioanalytical validation worldwide.

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