EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 5
280-286
Year: 2018

A COMPARISON AND DISCUSSION OF INTERNATIONAL GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION

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Article Received on 08/03/2018 **Article Revised on 29/03/2018** **Article Accepted on 19/04/2018**

ABSTRACT
US FDA guideline comes into effect in 2001 for Bioanalytical Method Validation that Provide Guidance for Industry. This guidance is adopted universally by all over Pharma Industries and contract research industries as standard approach for conducting validation and for implementing bio-analytical methods that are used to obtain data to support medical applications. ANVISA and EMA get developed on the basis of US FDA. General agreement is there between this guideline to evaluate the different validation parameters based on which methodology is employed. This review compares regulatory guidelines by US FDA, ANVISA and EMA and summarizes it for bioanalytical method validation. Present review include discussion on evaluation of several validation parameters those are specificity, calibration curve/linearity, sensitivity, accuracy, precision, recovery various stability analysis.

KEYWORDS: Bioanalysis, Validation, US FDA, ANVISA, EMA, Regulatory guidance.

INTRODUCTION[11-13]

Bio-Analytical Method: The methods employed for detection and measurement of drug concentrations in biological fluids are referred to as Bio-Analytical Methods. Bio-analytical methods are widely used to quantitate drugs and their metabolites in physiological matrices. **Need of Bio-Analytical Method:** To investigate the pharmacokinetic of new drug candidates. To compare pharmacokinetic profiles of different formulations, To monitor drug levels to establish the appropriate dose or frequency of administration .For fast and reliable measurement of the compounds in biological matrices.[11]

Bio-Analytical Method Validation

Validation may be defined as documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes. Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Bioanalytical method validation is vital not only in terms of regulatory submissions but also for ensuring generation of high quality data during drug discovery and development. Bio- analytical method validation assures that the quantification of analyte(s) in biological fluids is reproducible, reliable and suitable for the application. The general approach in bioanalysis use a calibration curve with or without internal standard (IS) and the concentration of unknown samples is calculated based on response of other samples known as calibration curve standards (CC).

Objective of Validation: To yield reliable result, to minimize the error**,** to define the requirements for establishing & implementing an effective method**,** to ensure that a particular method for quantitative measurement of an analyte in a biological matrix is reliable and reproducible.

Different Types and Levels of Validation: There types are there 1. Full Validation, 2. Partial Validation**,** 3. Cross Validation.

Comparision of Various International Guidline For Bioanalytical Method And Its Validation Parameters: [1-10]

1. Specificity

Table No 1: Comparison of Available Guidelines for Validation Parameter 'specificity' [1-10]

Specificity	EMA	US FDA	ANVISA
Definition	• Ability of the bioanalytical method to measure and differentiate the analyte in the presence of components which may be expected to be present	• Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample	• Ability of the bioanalytical method to measure and distinguish the analyte from components that may be present in the sample, such as metabolites, impurities, degradation compounds or components of the matrix
Method	• Prove selectivity by using at least 6 sources of blank matrix and evaluate for interference • Evaluate interference caused by metabolites, degradation products, co-administered medications • Evaluate the possibility of back conversion of metabolite into parent drug by spiking metabolite of interest in blank matrix	• Analyze blank samples of appropriate biological matrix obtained from at least six different sources and test for interference • Ensure selectivity at LLOQ (lower limit of quantitation)	• Analyze blank samples of biological matrix obtained from six individuals (four normal samples, one haemolyzed and one lipemic) • Ensure specificity by comparing response obtained with aqueous solution of analyte spiked at LLOQ and IS at working concentration • Consider OTC medications, metabolites of drugs during specificity evaluation
Acceptance criteria	• Absence of interfering component is accepted if response in blank samples is >20% of LLOQ for analysis	• Not mentioned	• The response of interfering peaks at retention time of analyte and IS must be >20 & 5%, respectively of response in aqueous solution

2. Calibration Curve/ Linearity

Table No 2: Comparison of Available Guidelines for Validation Parameter 'Calibration Curve/Linearity'[1-10]

Calibration Curve/ Linearity	EMA	US FDA	ANVISA
Definition	• Calibration curve is a relationship which can simply and adequately describe the response of the instrument with regard to the analyte	• Calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte	• The calibration curve represents the ratio between the response of the instrument and the known analyte concentration
Method	• Prepare calibration standards in the same matrix as the matrix of the intended study samples by spiking the blank matrix with known concentrations of the analyte (and IS) • The range should cover LLOQ to upper limit of quantitation (ULOQ) based on scientific justification • Use minimum of six calibration levels excluding a blank and zero sample Calculate slope, intercept, back • calculated concentrations	• Prepare sufficient number of standards in the calibration curve by spiking in matrix with known concentration of analyte • Select the concentration range based on concentrations expected in study • Include one blank sample (matrix without analyte and IS) one zero sample (matrix with IS) 6–8 non-zero • samples covering whole range including • LLOQ • Use appropriate weighting and statistical tests for	• Build calibration curve for each analyte or run using same biological matrix proposed in the study • Calibration curve must include one blank sample, one zero sample and at least six samples containing drug and IS from LLOQ to 120% of highest concentration expected • Calculate coefficient of linear correlation, angular coefficient and intercept using • method of squares minimums

		goodness of fit	
Acceptance criteria	• The back calculated concentration should be within ± 15% of nominal value, except for LLOQ for which it should be within ± 20% • At least 75% calibration standards six must fulfill this criteria	• LLOQ: response should be at least 5 times as compared with blank. Response should be accurate (80–120%) and precise (20%) and at other points: up to 15% deviation from nominal value • At least 4 out of 6 should meet above criteria including LLOQ and highest concentration	• LLOQ: deviation B20% with respect to nominal concentration and B15% at all other points of curve • At least 4 out of 6 concentrations should meet above criteria including LLOQ and largest concentration of the curve • Coefficient of linear correlation must be more than 0.98

3. Sensitivity

Table No 3. Comparison of Available Guidelines for Validation Parameter 'Sensitivity'[1-10]

Sensitivity	EMA	US FDA	ANVISA
Definition	• LLOQ: lowest amount of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision	• LLOQ: is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision	• LLQ: lowest amount of an analyte in a sample that can be quantified with acceptable precision and accuracy • Detection limit (DL): lowest concentration of an analyte that the bioanalytical procedure can distinguish reliably from the background
Method	• LLOQ analyte signal must be five times of blank signal • The LLOQ adapted to aim of study and accepted concentration	• Five sample analysis of LLOQ should be done independent of standard and determine confidence interval or % cv	• At LLQ minimum 5 determination should be carried out • By analyzing solutions of known and decreasing concentrations of the drug up to detectable level DL is established
Acceptance criteria	• at LLOQ accuracy should be within 80–120% with precision 20%	• LLOQ: response as compared with blank it should be at least 5. With precision of 20% and accuracy of 80–120% analyte peak should be identifiable	• Ratio of 5:1 between signal to noise of baseline should be obtained for LLQ. • LLOQ response should be at least 5 times greater than interference in blank sample at retention time of analyte. • With precision of 20% and accuracy of 80–120% peak should be identifiable. • At least 2–3 times higher than noise of baseline for DL.

4. Recovery

Table No 4: Comparison of Available Guidelines for Validation Parameter 'Recovery' [1-10]

Recovery	EMA	US FDA	ANVISA
Definition	• Not mentioned	• The extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method	• Measures the efficiency of the extraction procedure of an analytical method within a variation limit
Method	• Not Mentioned	• Establish recovery By comparing analytical results for extracted samples at three levels (high, medium and low) with un-extracted samples we can establish recovery, • That represent 100% recovery	• By comparing analytical results of samples extracted from three concentrations levels (high, medium and low) covering whole range with the results obtained with non-extracted standard solutions presenting 100% recovery. • Calculation should be based on the ratio of area of non-extracted and extracted standard for both IS and analyte respectively.
Acceptance criteria	• Not mentioned	• Recovery of analyte and IS recovery should not to be 100%, • Consistent, precise and reproducible that should be	• Recoveries are accurate and precise if it is near 100% are desirable but never less than that values are accepted provided

5. Accuracy

Table No 5: Comparison of Available Guidelines for Validation Parameter 'Accuracy'[1-10]

Accuracy	EMA	US FDA	ANVISA
Definition	• Describes the closeness of the determined value obtained by the method to the true concentration of the analyte (expressed in %)	• The closeness of mean test results obtained by the method to the true value (concentration) of the analyte	• Represents the degree of match between the individual results found and a value accepted as reference
Method	• Determine accuracy by using quality control samples at 4 different levels such as within three times of LLOQ, LQC , near 50% of range MQC and about 75% of upper range HQC • Obtained accuracy by using minimum replicate analysis of five results for each one quality control level • Accuracy is determine within a run (within run accuracy) and inter run or in different runs (between run accuracy) • Also asses accuracy of QC samples for at least one of the runs which is equivalent to prospective analytical run	• By analysis of replicate concentrations accuracy is determined • At minimum three concentration levels use minimum five determinations for covering whole range	• By using at least five determinations at 3 levels of concentrations (high, medium and low) covering whole range accuracy is determined • Accuracy is determine as within analytical run (intra-day) and between analytical runs (inter-day)
Acceptance criteria	• In calculation of accuracy outliers are included • Mean accuracy should be between 15% of nominal value, except at LLOQ where it should be within 20% of nominal value	• Mean value should be between 15% of actual concentration except for LLOQ, where it should not deviate more than 20%	• Deviation from nominal value must not exceed 15% except at quantification limit, where it can be up to 20%

6. Precision

Table No 6: Comparison of Three Guidelines for Validation Parameter 'Precision'[1-10]

Precision	EMA	US FDA	ANVISA
Definition	• The closeness of repeated individual measures of analyte	• 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	• Represents the degree of repeatability between the results of individual analyses, when the procedure is applied various times to the same homogeneous sample, in identical assay conditions
Method	• Precision is determine for LLOQ, high, medium and low of QC samples • Within single run (within run precision) and between different runs (between run precision that should be demonstrated	• By using minimum five determinations at minimum three concentration levels covering whole range precision is determined • Within-run, intra-batch precision or repeatability (variation with in an analytical run), between-run, inter batch precision or repeatability (variation with time and may involve different equipments, analysts, reagents and laboratories) is also determined	• Check precision using at least 3 concentrations high, medium and low) covering whole range using at least 5 determinations per concentration • Also demonstrate precision within same run (intra-run) and in different runs (inter-run)
Acceptance criteria	• %CV should not exceed 20 at LLOQ and 15 at other points for both within run and between run precisions.	• %CV should not exceed 20% at LLOQ, and should not exceed 15% at other points	• RSD should not exceed 20% at LLQ, and should not exceed 15% at other points

7. Stability

- FT stability
- Short term (bench top stability)
- Post preparative stability
- Long term stability

Table No 7: Comparison of Available Guidelines for Validation Parameter 'Stability'[1-10]

Stability	EMA	US FDA	ANVISA
Definition	• The chemical stability of analyte in a given matrix under specific conditions for given time intervals	• The chemical stability of analyte in a given matrix under specific conditions for given time intervals	• Parameter aim at determining if an analyte remaining chemically unchanged in a given matrix in a specific conditions, at certain time interval
Freeze Thaw (FT) stability			
Method	• Determine FT stability by using at least triplicates of LQC and HQC, stored at intended temperature and thereafter thawed at room temperature • At least for 12 h Samples should be frozen between each and every cycle • Based on study samples number of cycle is determine • Against freshly prepared calibration standards quantification of sample is	• We can Demonstrate FT stability after conducting minimum of three FT cycles using at least three aliquots of LQC and HQC, which should be stored at intended temperature for at least 24 h • Samples are thawed unassisted at room temperature and re-freeze for 12–24 h. Repeat same procedure and analyze after third cycle • Stored samples result are	• By using minimum of three samples of LQC and HQC we can determine FT stability after 3 freeze– thaw cycles • The samples are frizzed at intended storage for at least 24 h and then defrost them at room temperature. Freeze the samples again for at least 12–24 h and repeat until three cycles • Samples are quantify after three cycles and compare them with recently prepared samples

	done	compare with freshly prepared samples	
Short term (bench top stability)			
Method	• By using at least triplicates of LQC and HQC evaluation of bench top stability is done • Quantification of samples is done against freshly prepared calibration standards	• By thawing three sets of LQC and HQC and storing them at room temperature over 4–24 h (based on duration for which study samples will be stored on bench) Establishment of short term stability is done • After required time samples are analysed and compare against freshly prepared samples	• By using at least three sets of LQC and HQC. Store them at room temperature over 4–24 h (based on time for which study samples are kept at room temperature) determination of short term stability is done • After required time samples are analysed and compare against freshly prepared samples
Post preparative stability			
Method	• Post preparative stability is divided into three categories such as stability of reconstituted sample at room temperature, dry extract stability, and stability of sample in autosampler maintained at autosampler temperature • Post preparative stability is evaluated by using at least triplicates of LQC and HQC • Samples are quantified against freshly prepared calibration standards	• Post preparative stability is define as combination of processed sample stability at room temperature and autosampler stability • Assessment of this stability for drug and IS over anticipated duration of batch analysis is done by determining concentrations against original calibration standards	• It is autosampler stability and recommends to evaluate drug and IS stability at temperature at which analysis is carried out and over a duration of analytical batch analysis • It must be obtained by using three sets of LQC and HQC and compare against freshly prepared samples
Long term stability			
Method	• At least the time required for study sample analysis that should be covered • Evaluation of long term stability is done by using at least triplicates of LQC and HQC • Samples are qualified against freshly prepared calibration standards	• Duration between long term stability should exceed time between first sample collection and last sample analysis • Establishment of long term stability is done by analyzing at least three sets of LQC and HQC and compare back calculated concentrations against results obtained on first day of analysis	• Storage period for long term stability should exceed duration between from first sample collection till last sample analysis • Comparison of back calculated concentrations with average values obtained on first day of test is carry out using at least three sets of LQC and HQC
Acceptance criteria for all stability			
Acceptance criteria	• For study sample analysis it should cover at least required time	• Not properly specified	• Considered Samples as stable when there is no deviation higher than 15% of value obtained from recently prepared sample and at LLQ, it should not be more than 20%

SUMMARY AND CONCLUSION

During preclinical and clinical phases of drug, bioanalytical method validation is essential for generation of quality data, its acceptance by regulatory agencies. Bioanalytical method validation assures the suitability of method for desired purpose, and the method performance under defined conditions for analysis of study samples. In present time there is

mainly three referred bioanalytical method validation guidelines are 2001 **US FDA:** United states food and Drug Administration (guidance for Industry), 2003 **ANVISA:** Agencia Nacional de Vigilancia Sanitaria (Guideline for validation of analytical and Bioanalytical method), 2009 **EMA**: - European Medicines Agency (Guideline on Bioanalytical method validation). For the evaluation of validation parameters, guidelines are same but the significant difference of addressing few validation parameters are there. So the differences are identified and that have been explained in these paper. So it will be helpful to the bioanalysts and researcher to easily understand these widely refereed guidelines.

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