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AN OVERVIEW OF STANDARD GUIDELINES CONCERNING BIOANALYTICAL METHOD DEVELOPMENT

Gaurav Kumar
Student, JSPM'S Rajarshi Shahu College of Pharmacy and Research, Tathawade

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ABSTRACT
Bioanalysis plays a key role in the development of a new drug. In the present time, it is an integral part in the toxicological assessment and also in pharmacodynamics and pharmacokinetic studies in the process of developing a novel drug. It is the analysis of the desired analyte in the respected biological fluids performed by considering numerous parameters by following certain guidelines.

INTRODUCTION

This Bioanalytical method development is a procedure that is basically used to incorporate quantitative analysis useful in biomedical applications. Quantification of concentrations of drugs in biological matrices comprising serum, urine, plasma, saliva and blood are a relatively critical facet of development of a medicinal product; correspondingly these statistics might be a requisite for novel active substances and generics along with deviations to authorized drug products. The findings and repercussions of clinical trials and such animal toxicokinetic studies are utilized to make pivotal decisions assisting the potency and safety of a medicinal drug product. It is thereby crucial that the implemented Bioanalytical methods employed are considerably characterized, documented and completely validated to an adequate standard for the purpose of yielding trustworthy results. Bioanalytical method validation is used for the figuring out quantitative analysis of drugs and their further metabolites in biological fluid exerts substantial purpose in the elucidation and assessment of bioequivalence along with the bioavailability of the drug as well as the pharmacokinetic and toxicokinetic evidence of the study. It is paramount not only in terms of the regulatory submission but also for guaranteeing procreation of high standard data in the course of drug discovery and development. The caliber of these research studies is bluntly proportional to the quality of the fundamental data of bioanalysis. Thereby it's quite pivotal that steering principles for the validation of these methods of analysis be accustomed and distributed to the pharmaceutical society**.** As per the guidelines issued by globally recognized regulatory body like *European Medicines Agency (EMA)* and *The United States Food and Drug Administration (USFDA)* it is evident that methods like *high-performance liquid chromatography (HPLC)* or *gas chromatography (GC)* are predominantly employed for validation.

IMPORTANCE OF BIOANALYTICAL METHOD VALIDATION

1. It is paramount to utilize completely verified and validated methods of Bioanalysis for showcasing dependable results which can be interpreted tolerably.
2. Such methods of bioanalysis and their sets of techniques are regularly altered and developed.
3. It is vital to highlight that every single technique of Bioanalysis has unique peculiarities that may change depending on the type of analyte, there has to be development of a particular criteria for assessment of every other analyte.
4. On top of that, the suitability of the technique can also change with respect to the aim of the study that needs to be done. For e.g., during analysis of a specific sample for defined research is carried out at multiple sites, it is essential to assess the method of Bioanalysis at every site and present relevant assessment data for various sites to set up inter-laboratory stability.

METHOD VALIDATION

It comprises of 3 types of validation methods, namely:

1) FULL BIOANALYTICAL METHOD VALIDATION PARAMETERS OF FULLBIOANALYTICAL METHOD VALIDATION

- Selectivity or specificity
- Carry-over
- Lower limit of quantification (LLOQ)
- Accuracy
- Precision
- Calibration curve

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- Dilution integrity
- Stability

2) PARTIAL VALIDATION
3) CROSS VALIDATION

FULL BIOANALYTICAL METHOD VALIDATION

It is a thumb rule that even if the method of analysis is new-fangled or is based upon some sort of a literature review, a complete method validation must be performed. The primary intent of method validation is to illustrate the dependability of a peculiar method for the assessment of the concentration of an analyte in a particular biological matrix which includes saliva, blood, plasma, urine or serum. On top of that it is of utmost importance that if a specific decoagulant is being used for the study samples that same decoagulant must be used for the complete validation process. Usually, a full validation should and must be performed for every matrix and species involved. It has been observed that in a handful of cases, it is a plight in validation related purposes to procure an analogous matrix in contrast to the matrix of the study samples. Whereas a befitting substitute of matrix can also be used if it can be justified, for e.g., a synthetically produced cerebrospinal fluid (CSF).Generally, a single analyte or maybe drug is assessed, while it is also possible to determine more than one analyte. This might include use of two different drugs, however can also involve same parent drug with its enantiomers or metabolites or isomers of a drug. In such types of cases standards of analysis and validation imply to all analytes of interest.

CHROMATOGRAPHIC METHODS

Reference standards: The process is as follows:

1) Firstly, a blank biological matrix will be spiked with the analyte using solutions of reference standard (RS) to formulate calibration standards, quality control (QC) samples and stability samples.
2) Addition of favorable internal standards (IS) while processing the sample in chromatographic methods.
3) The quality of the RS and IS should be taken under consideration, as the quality the purity might most probably affect the overall outcome of the analysis and thereby the outcome of the data received through studies.

Note: It is quite significant that the reference standards used while performing the study sample analysis and validation must be acquired from fairly genuine and traceable sources to guarantee the purity of the reference standard.

PARAMETERS OF FULL BIOANALYTICAL METHOD VALIDATION

- **Selectivity or specificity**

Definition: Selectivity and specificity are often misjudged as same terms, in broader perspective they are, but there are slight distinct meanings of either of them when we observe minutely.

Difference between Selectivity & Specificity:

Selectivity	Specificity
It shows the capability of a particular method to distinguish the analyte from other analytes or impurities that are in there in the samples.	It shows the capability of a particular Bioanalytical method to distinguish the analyte but not for the other intruding components

IMPORTANCE

1) Selectivity must be verified by using no less than 6 individual sources of the appropriate blank matrix, which are analyzed, done by one and assessed for interference.
2) Utilization of a smaller number of sources is tolerable in event of rare matrices.
3) Lack of interfering compounds is sometimes approved where the response is not more than
   - 20% of the total lower limit of analyte quantification.
   - 5% for the IS.
4) It is also necessary to take under consideration the interference that metabolites of the drugs might have caused, the interference by degradation compounds created during the production of sample and also the interference from probable medications that might have co-administered.
5) Some co-medications that are ordinarily used in the subject population studied which might interfere potentially should be taken into account at the time of method validation or on a study specific and compound specific base.

- **Carry-over**

In the process of method validation, carry-over is one such element which should be used as less as possible.

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Process

- Carry-over is determined by inducing samples pertaining blank reading after a high concentration sample or calibration standard at the upper limit of quantification.
- This blank sample following the sample having higher concentration must have a carry-over of not more than
- 20% of the lower limit of quantification (LLOQ) and
- Should be 5 percent for the internal standard (IS).

Note

- If it is observed that the carry-over is unavoidable, the study samples used must not be randomized.
- Some peculiar sort of measures must be taken under consideration and tested during the validation and applied amid the analysis of the study samples just because it does not affect the accuracy and precision.
- This could probably also inculcate the injection of blank samples after samples with an expected higher concentration, before the analysis of the next study sample.

- **Lower limit of quantification (LLOQ)**

Definition: LLOQ is the lowest concentration of an analyte in a sample which can be quantified precisely, with justifiable accuracy and precision.

- The LLOQ is regarded being the lowest calibration standard.
- Note that the analyte signal of the LLOQ sample must be at least **5x** the signal given by a blank sample.
- The LLOQ must be adapted to expected concentrations and to aim of the study done.

For an example, while doing the bioequivalence studies of the LLOQ, it should not be more than 5% of the C_{max}(maximum concentration), whereas such a lower LLOQ might not be necessary for exploration of pharmacokinetic studies.

- **Accuracy**

Process:

- Accuracy ought to be measured employing a minimum of five repetitions of determinations per concentration.
- A minimum of 3 concentrations in the range of expected study sample concentrations is recommended.
- The mean value should be within 15% of the nominal value (except at LLOQ).
- This mean value should not deviate by more than 20%.
- The deviation of the mean from the nominal value serves as the measure of accuracy.
- The more the deviation, the lesser the accuracy.

Note:

- The accuracy of a method of bioanalysis elucidates the ratio of nearness of the calculated value obtained and the method to the analyte's nominal concentration.
- Accuracy should be assessed on samples spiked with known amounts of the analyte; the quality control samples (QC samples).

Method

1. Firstly, the QC samples should be spiked independently from the calibration standards, using separately prepared stock solutions, unless the nominal concentration(s) of the stock solutions have been established.
2. The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value.
3. The accuracy should be reported as percentage of the nominal value.
4. Accuracy should be evaluated for the values of the QC samples obtained within a single run (the within run accuracy) and in different runs (the between-run accuracy).

Basically, accuracy may be divided in two segments:

1) Within-run accuracy
2) Between-run accuracy

Within-run accuracy

It should be identified by analyzing in a single run for at least 5 samples per level at a minimum of 4 concentration levels which are covering the calibration curve range as described.

	Reading of 5 samples	**Mean Reading**
LLOQ (Lowest)	Not more than 5% of C_{max}	20% of the nominal value
LLOQ (Low QC)	Not more than 3x	15% of the nominal value
Medium QC	30-50%of CC Range	15% of the nominal value
High QC	At least 75% of CC Range	15% of the nominal value

Note:

CC – Calibration curve
QC – Quality control

Fig 1: Tabular comparison of readings

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Between-run Accuracy

- For the validation of the between-run accuracy, it is basically done in 3 repetitions where the LLOQ, low, medium and high QC samples are analyzed on at least two days should be assessed.
- The mean concentration should not be more than 15% of the nominal values for the QC samples, except for the LLOQ which should be under 20% of the nominal value.

Note: The data procured from validation should be reported and the assessment of accuracy should comprehend all the results acquired except those cases where inaccuracies are evident and recorded.

- **Precision**

The precision of a method of bioanalysis is a measure of the random error and is defined as the nearness of agreement between a series of measurements obtained from multiple sampling of the same analogous sample under the ordained conditions.

It is typically measured as coefficient of variation (%CV) or relative standard deviation (R.S.D.) of the replicate measurements.

Mainly, precision can be segmented into:

1) Within-run precision
2) Between-run precision

Within-run precision

In the assessment of the within-run precision, there should be at least 5 samples per concentration level at

- LLOQ (Lowest)
- Low QC
- Medium QC
- High QC samples in one run.

The within-run CV value for:
LLOQ – should not be more than 20%.
QC samples – should not be more than 15%.

Between-run Precision

In the assessment of the between-run precision, LLOQ, low, medium and high QC samples from at least 3repetitionsassessed on at least 2 different days must be determined.

The between-run CV value should not be more than 15% for the QC samples, and for the LLOQ it should not surpass 20%.

Calibration curve

The feedback of the instrument in contrast with the concentration of analyte must be unanimous, and should be evaluated through a stated range of concentration

Procedure

Preparation of calibration standards should be done in the matrix of the aimed study samples by the technique of spiking a blank matrix with an analyte whose concentrations are recognized.

Note

1) Single calibration curve for every analyte should be studied in the method development and for individual run of analysis.
2) Generally, prior to executing the validation of a bioanalytical method it must be acknowledged that what sort of range of concentration is anticipated.
3) The calibration range must be included by this particular range, where the LLOQ is the lowest standard of calibration and the upper limit of quantification (ULOQ) is the highest standard of calibration.
4) The range should be accustomed to permit appropriate explanation of the pharmacokinetics of the analyte.

Procedure

- Take a blank sample with treated matrix which has no analyte and no internal standard, a zero sample with treated matrix and internal standard and at least 6 calibration concentration levels must be utilized.
- Every single calibration standard should be examined in replicate.
- Ignore the blank and zero samples reading to reckon parameters of calibration curve.
- These calculated parameters should be stated.
- Additionally, the back concentrations reported must also be presented with the mean values of the accuracy.

Range

- For back concentration – It should be within ±15% of the nominal value.
- For LLOQ - It should be within ±20.
- For replicates – There should be fulfillment of the criteria for at least 50% of the standards of calibration examined per level of concentration.

Dilution integrity

1) In the first place, the dilution integrity must not influence the precision and accuracy. However, if it does, then it should be displayed by
- Spiking the matrix with an analyte concentration above the ULOQ&
- Diluting this sample with blank matrix with not more than five determinations per dilution factor.
2) Precision and accuracy should be within the set criteria, i.e., within ±15%.
3) Dilution integrity should cover the dilution applied to the study samples.
4) Evaluation of dilution integrity may be covered by partial validation.
5) Use of another matrix may be acceptable, as long as it has been demonstrated that this does not affect precision and accuracy.

Stability

Aim: The test for stability is for the identification of any sort of deterioration of the analytes amid the complete

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duration of collecting samples, processing, storing, preparing, and analysis.

Stability is broadly dependent of certain conditions; they are as follows:

1) Nature of the analyte
2) Biological matrix
3) Time period of storage

Note: During the preparation and analysis of samples, at each individual stage the stability must be reaffirmed, and additionally the conditions utilized for prolonged storage.

Process

1) An analyte's stability is assessed with the help of samples having low and high QC.
2) In this process, a blank matrix is usually spiked with an analyte at a concentration of a maximum of 3 times the lower limit of quantification and close to the upper limit of quantification that are evaluated directly after preparation and after the storage conditions that are to be assessed.
3) The samples of QC are evaluated upon a calibration curve which is collected from promptly spiked calibration standards and the acquired concentrations are matched to the nominal concentrations.
4) The mean concentration at every level must be under ±15% of the nominal concentration.

Types of Stability tests that should be evaluated are:

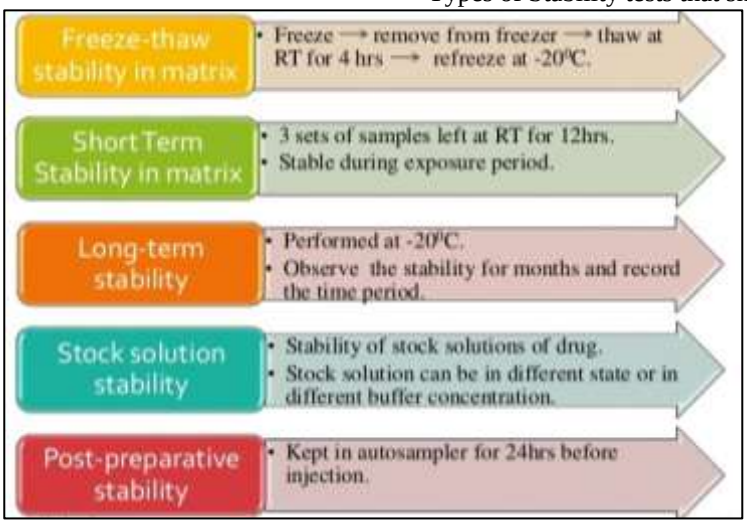

Fig 2: Types of stability tests

Long-term stability

Note

- The analyte in the sample matrix must have stability either same or more than the time amid the date of collection of first sample and the date of analysis of last sample.
- The QC samples must be kept in the freezer under the identical storage conditions and at least for the equal time period as the samples of study.
- For relatively tiny molecules, it is passable that if the stability at -70°C and -20°C has been established, it isn't mandatory to examine the stability of the temperature in its bracket.
- For relatively big molecules, stability must be studied at every temperature at which the samples for study are kept.

Freeze and Thaw Stability

- Amid freeze and thaw stability assessments, the freezing and thawing of stability samples must be identical to the conditions of sample handling to be used during the analysis of sample. Stability should be evaluated for at least 3 freeze-thaw cycles.
- After full thawing, samples are frozen again by application of equivalent conditions.
- At every cycle, samples should be frozen for at least 12 hours before they are thawed.

Bench-Top stability

Bench top stability experiments must be planned and carried out to cover the laboratory handling conditions that are expected for study samples.

Stock solution stability

The stability of stock solutions of drug should be evaluated.

When the stock solution exists in a different state (solutions vs. solid) or in a different buffer composition (generally the case for macromolecules) from the certified reference standard, the stability data on this stock solution should be generated to justify the duration of stock solution storage stability.

Processed Sample Stability:

The stability of processed samples, including the time until completion of analysis, should be determined.

FORCED DEGRADATION STUDIES (FDS)

AIM: The primary objective of forced degradation studies is to expose the finished composition and the active pharmaceutical ingredient to external factors like:

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1) base,
2) heat,
3) light conditions,
4) acid,
5) and peroxide,

To the point a sufficient amount of degradation has reached.

PROCESS

- A satisfactory degradation can range from 10–30% but is subject to change based on the API getting degraded.
- It has been observed that sometimes on account to excess degradation of the active, there has been formation of secondary form of deteriorating agents, hence it is advisable to avoid surplus degradation.

EVALUATION PROCESS BY PEAK PURITY ANALYSIS

- Assessment of these products and also assay and its related substances after forced degradations is usually done through analysis of its peak purity with the help of a special photodiode array detector or mass spectral evaluation which shall affirm that the active peak in no way co-elute over any of the degradation products formed as a consequence of this process.

CONCLUSION

1) Every single stage in the methodology of development of a composition, the method of analysis should exhibit selectivity or specificity; the same method must necessarily possess the capability to explicitly assess the analyte of in the vicinity of all other components, which might include deteriorating agents, reagents, filters and sample matrix.
2) During the tests for identifying analytes, selectivity must showcase positive results for the sample that contains analyte and negative for the one that doesn't.
3) Also, it should be capable of separating out the primary analyte which is important and the one which similar properties that might prevail.

PARTIAL VALIDATION

In many cases there is need for only small changes to method of analysis which has been validated already, so there is no space for a full validation, relying on the nature of the changes, and that's why partial validation is performed.
Partial validation includes the following factors:
1) Transfer of method of bioanalysis to another laboratory
2) Limited volume of sample
3) Procedure of sample processing
4) Change in equipment
5) Calibration concentration range
6) Another matrix or species
7) Storage conditions

CROSS VALIDATION

Where data are obtained from different methods within and across studies or when data are obtained within a study from different laboratories, applying the same method, comparison of those data is needed and a cross validation of the applied analytical methods should be carried out.

Cross validation should be performed in advance of study samples being analyzed if possible. For the cross validation, the same set of QC samples or study samples should be analyzed by both analytical methods.

For study samples, the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats.

The outcome of the cross validation is critical in determining whether the obtained data are reliable and whether they can be compared and used.

It is a comparison of validation parameters when two or more Bioanalytical methods are used to generate data within the same study or across different studies.

1. An example of cross-validation would be a situation where an original validated Bioanalytical method serves as the reference and the revised Bioanalytical method is the comparator. The comparisons should be done both ways.

a. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability.
b. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MSMS vs. ELISA) in different studies are included in a regulatory submission.

APPLICATION

The following recommendations should be noted in applying a Bioanalytical method to routine drug analysis:

1. A matrix-based standard curve should consist of a minimum of six standard points, excluding blanks (either single or replicate), covering the entire range.

2. Response Function: Typically, the same curve fitting, weighting, and goodness of fit determined during pre-study validation should be used for the standard curve within the study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation. Changes in the response function relationship between pre-study validation and routine run validation indicate potential problems.
3. The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
4. System suitability: Based on the analyte and technique, a specific SOP (or sample) should be identified to ensure optimum operation of the system used.
5. Any required sample dilutions should use like matrix (e.g., human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.

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6. Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis. Reasons for repeat analyses could include repeat analysis of clinical or preclinical samples for regulatory purposes, inconsistent replicate analysis, samples outside of the assay range, sample processing errors, equipment failure, poor chromatography, and inconsistent pharmacokinetic data.
7. Sample Data Reintegration: An SOP or guideline for sample data reintegration should be established. This SOP or guideline should explain the reasons for reintegration and how the reintegration is to be performed.

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