OriginalArticle

A REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY HYPHENATED TECHNIQUES

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Abstract

Bio-analysis is related to the analysis of analytes (drugs, metabolites, biomarkers) in biological matrix and it involves several steps from sample collection to analysis and data reporting. Sample collection from clinical or preclinical studies; then sending the samples to laboratory for analysis, later the sample preparation will be carried. Stable sample preparation method could be achieved. The role of sample preparation is to remove interferences from the biological matrix and to extract the analyte of interest and to improve analytical system performance. Sample preparation is often labor intensive and time consuming. Final step of the analysis is the quantification of analyte by using analytical instruments. Among them, Liquid chromatography-tandem mass spectrometry (LC–MS/MS) is method of choice in bioanalytical laboratories. This is due to high selectivity and high sensitivity of the LC–MS/MS technique. Development and validation of analytical method play an important role in the discovery, development and manufacturing of pharmaceuticals. Every year, number of drugs entered into the market; hence it is mandatory to develop newer analytical methods for such drugs. After the development, it becomes necessary to validate the new analytical method. Method development is the process which proves that the analytical method is acceptable for use. Validation of analytical method gives information about various stages and parameters like accuracy, precision, linearity, Limit of Detection, Limit of Quantification, specificity, range and robustness. Validation should be done as per ICH regulatory guidelines.

Keywords: Method development, Validation, stability studies, Bioanalytical, LCMS/MS.

INTRODUCTION

Bio-analytical method validation is a procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: to quantify the analyte with a degree of accuracy and precision. Both HPLC and LC-MS/MS can be used for the bioanalysis of drugs in plasma. The main advantages of LC-MS/MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities [1]. Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. [2] Measurement of drug concentrations in biological matrices is an important aspect of medicinal product development. Such data may be required to support applications for new actives substances and generics as well as variations to authorized drug products [3].

Bioanalytical methods involve the quantification of drugs from the biological matrices whole blood, serum, plasma, urine. The bioanalysis would determine the concentration of drugs and it is very important for drug and formulation development. These data can be used to maintain the safety and efficacy of the drug, or based on toxic kinetics, pharmacokinetics and biology, etc. Various methods are adopted for extraction of analyte from the biological matrixes. Solid Phase extraction (SPE) is a sample preparation technology that uses solid particle, chromatographic packing material, usually in a cartridge type device, to chemically separate the different components of a sample. Liquid-liquid extraction (LLE) is a common sample preparation choice in regulated bioanalysis. LLE is useful for separating analytes from interferences by partitioning the sample between two immiscible liquids or phases. One phase in LLE will usually be aqueous and the second phase an organic solvent. LLE can generate high analyte recoveries, clean extracts, and is perceived as low cost. Extraction solvents may need to be acidified, basified or low percentages of more polar solvents may be required to simultaneously achieve high recoveries for metabolites and related compounds, as well as the primary analyte[4].Protein precipitation extraction method is simplest ,cheap but very poor cleaned sample.

Bioanalytical results of the efficacy trials make key decisions. Therefore, it is necessary to fully verify and document the

applied bioanalytical method for reliable results. Various guidelines provide requirements for bioanalytical method validation and also address non-clinical requirements.

METHOD DEVELOPMENT [5, 6]:

The objective of the method development is too develop and validate highly specific, reliable and cost effective LC-MS/MS method for determination of drug in human plasma. The Scope of developing and validating the bioanalytical method is to get a suitable method which is more accurate and precise for the analyte of interest under given set of lab conditions by available resources.

Steps in Method Development (MD)

1. Literature search for drugs (Structure, Solubility, pKa etc.,)
2. Identification of analytical techniques and optimization
3. Reference standard preparation
4. Selection of Internal Standard (ISTD)
5. Sample pre-treatment (Extraction Procedure)
6. Sample Storage

During the process of developing your drug, method development processes can also determine the multitude of constituents in your formulation. It's important to use method development to work out the product's purity, composition, and potency.

Method validation assures the scientific veracity of analytical results and is a key component of total quality management. Proper validation of a method provides documented evidence of method performance and prescribes on-going measures to ensure quality monitoring for the life of the method. For quantitative analysis, high-pressure liquid chromatography, gas chromatography, LC, and GC are used in conjunction with mass spectrometric (MS) processes such as LC-MS, LC-MS-MS, GC-MS, and GC-MS-MS.

Conclusions of Method Development

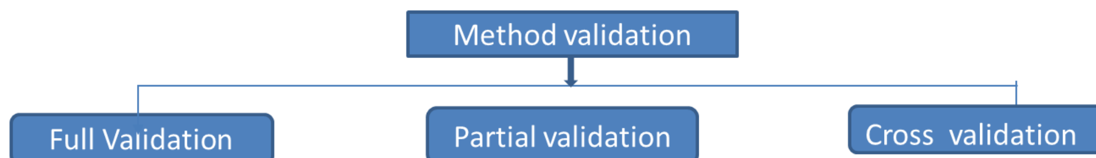
- Instrument conditions are optimized
- Sample processing procedure is finalized.
- All the required materials are finalized.
- Selectivity, Specificity, Recovery, COT, Matrix effect and P & A parameters were proved.
- Calibration range and linearity experiment is confirmed.
- LOQ response is confirmed as more than 5 times of S/N ratio to the baseline S/N ratio.
- Method validation protocol shall be prepared for performing method validation

METHOD VALIDATION [7]:

Method Validation is the process of determining the suitability of a given methodology for providing useful analytical data. Validation is required for any new method to ensure that it is capable to give reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories.

BMV include all procedure that demonstrate that a particular method used for quantitative measurement of analyte in given biological matrix are reliable and reproducible for intended purpose

Types of Method Validation



Full Method Validation

Any new bioanalytical method for the analysis of a new drug entity and its metabolite(s). Any revisions to an existing

validated method that adds metabolite or an additional analyte.

Partial Validation

Partial validation is performed in case of modifications of already validated bioanalytical methods. Partial validation can range from a single batch validation to a nearly full validation. The extent of validation to be performed shall depend on the parameters of validation that would be affected by the modification of the method.

Cross Validation

Two or more bioanalytical methods or techniques that are used to generate data within the same study or across different studies.

When sample analyses within a single study are conducted at more than one site or more than one laboratory.

- **Method Validation Parameters**

✓	System suitability	✓	Stabilities
✓	Selectivity/Specificity	✓	Standard stock solution
✓	Matrix selectivity	✓	Auto sampler stability
✓	Analyte Selectivity	✓	Bench Top stability
✓	Sensitivity	✓	Freeze and Thaw stability
✓	Ruggedness	✓	Re –injection stability
✓	Matrix effect (for LC-MS/MS only)	✓	Wet extract stability
✓	Recovery	✓	Whole blood stability
✓	Carry –over test	✓	Long Term stability
✓	Accuracy and precision	✓	Stock solution stability
✓	Run size Evaluation		
✓	Dilution integrity		

A. ACCURACY:

The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added.

Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added both above and below the normal levels expected in the samples. Dosage form assays commonly provide accuracy within 3-5% of the true value. It has 3 concentration levels, covering the specified range (i.e., three concentrations and three replicated of each concentration).

B. PRECISION

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions. ICH has defined precision to contain three components: repeatability, intermediate precision and reproducibility.

a. Repeatability

Sensitivity is the ability to detect small changes in the concentration of the analyte in the sample. Sensitivity can be partially controlled by monitoring the specification for injection reproducibility (system suitability testing).

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. One of the most common statistical terms employed is the standard deviation of a population of observation. Standard deviation is the square root of the sum of squares of deviations of individual results for the mean, divided by one less than the number of results in the set. The standard deviation S, is given by

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2},$$

The square of standard deviation is called variance (S2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

Recommendations

As part of methods validation, a minimum of 10 injections with an RSD of 2% is recommended. With the methods for release and stability studies, an RSD of 2% for precision of the system suitability tests for at least five injections (n=5) for the active drug either in drug substance or drug product is desirable. For low-level impurities, higher variations may be acceptable.

b. Intermediate Precision

Intermediate precision was previously known as part of ruggedness. The attribute evaluates the reliability of the method in a different environment other than that used during development of the method.

C. SPECIFICITY:

The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selectivity refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other .If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them.

D. LIMIT OF DETECTION

The limit of detection is the parameter of limit tests. It is the lowest level of analytes that can be detected, but not necessarily determined n a quantitative fashion, using a specific method under the required experimental conditions.

The determination of the limit of detection of instrumental procedures is carried out by determining the signal -to-noise ratio by comparing test results from the samples with known concentration of analytes with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted.

The detection limit (DL) may be expressed as

$$DL = \frac{3.3\,\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. Estimation of S may be carried out in a variety of ways, for example

E. LIMIT OF QUANTIFICATION

Limit of quantification is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantification is the lowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied. It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable.

The quantitation limit (QL) may be expressed as

$$QL = \frac{10\,\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

F. LINEARITY

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well-defined mathematical transformation) proportional to the analyte concentration in samples within a given range. The linear range of detectability that obeys beers law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression.

Data is processed by linear least square regression co-efficient and intercept of the linear equation y=ax +b together with the correlation co-efficient (R).For the method to be linear the R value should be close to 1.

G. SYSTEM SUITABILITY

Six replicates of standard stock solution for each drug were injected sample was given to evaluate equipment, electronics and analytical operations and samples suitability. Parameters calculated for system suitability were percentage of relative standard deviation of retention time, number of theoretical plates and tailing factor.

H. ROBUSTNESS

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated. During robustness testing each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0 ± 0.2ml/min).

ICH defines robustness as a measure of the method capability to remain unaffected by small, but deliberate variations in method parameters. Robustness can be partly assured by good system suitability specifications. In the case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature and flow rate.

I. RUGGEDNESS

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay.

STABILITY STUDY [8]:

Stock solution stability

The stock solution stability of drug sample and internal standard should be evaluated at room temperature for minimum of 6 h. The stock solutions are kept at frozen or refrigerated over the period. Then these stock solutions are evaluated by comparing with the response of freshly prepared stock solutions. The stock solution stability should be performed at least for one concentration in duplicate.

1.Post preparative (extracted samples or autosampler tray) stability

This stability is determined for extracted samples. To cover expected run time for the analytical batch and to allow delayed injection due to some instrument malfunctioning or long term storage of the samples, this stability is determined for ~48 to 96 h. This stability is determined on QC samples which are kept for measurable time at the autosampler tray. These samples are analyzed with fresh standards.

2. Benchtop stability

The QC samples at minimum of two concentration levels are kept at room temperature for 4–24 h. It covers the time to extract the samples. The concentrations are analyzed by comparing with their nominal values. The samples are analyzed in replicates generally in triplicate.

3. Freeze-thaw stability

This stability is determined at a minimum of two concentration levels. The samples are frozen overnight for −20 or −70°C.

Then it is removed and thawed at room temperature. After that, the samples are frozen again at the same temperature for 12–24 h and again thawed. This freeze thaw cycle is repeated for two more times. After completion of third cycle the samples are analyzed. If more degradation is observed than normal values then the first and second freeze-thaw cycle is repeated and the step in which instability occurred is determined. The freeze-thaw cycle can be extended as per requirement.

4. Freezer storage stability

The freezer storage stability should be carried out at nominal freezer storage temperature during the validation process.

5. Post validation long-term stability

This stability is performed after validation. The two QC samples in the matrix low and high concentration level are analyzed by keeping at long-term storage temperature and analyzed in triplicate. The post validation long-term stability should be performed in regular intervals starting from 1, 3, 6, 9 and 12 months in accordance with the length of stability required. The long-term stability of incurred samples at storage data should be assessed with stored in vivo samples.

6. Matrix stability

At lower temperature, there may be denaturation of matrix proteins. Therefore matrix stability should be validated. For that purpose, additional stability should be carried out at lower temperatures for sample matrix .

7. Whole blood stability evaluation

The immediate spinning down of aliquot of whole blood containing the drug taken immediately following preparation (time zero) followed by the spinning down of another aliquot following the stability period. The whole blood stability should be performed during method validation. However, there are various approaches for determination of whole blood stability.

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