ISSN: 2230-7346

Shanti Prathyusha et al. / JGTPS / 6(2)-(2015) 2643– 2649

(Review Article)

Journal of Global Trends in Pharmaceutical Sciences

Journal home page: www.jgtps.com

A REVIEW – BIOANALYTICAL METHOD DEVELOPMENT AND ITS VALIDATION BY RP HPLC

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ABSTRACT

Method validation is a process that determines whether a method will successfully meet the minimum standards recommended in the Food and Drug Administration (FDA) guidance. In today's drug environment, highly sensitive and selective methods are required to quantify drugs in biological samples such as blood, plasma, serum, or urine. Chromatographic methods (high-performance liquid chromatography [HPLC] or gas chromatography [GC] have been widely used for the bioanalysis of small molecules, with liquid chromatography coupled to mass spectrometry (LC/MS/MS) After developing a method with required attributes, the method is validated for the parameters like accuracy, precision,etc. Bioanalytical method is the process used to establish that a quantitative analytical method is suitable for biomedical applications.It includes all of the procedures that demonstration of quantitative measurement of analytes in a given biological samples, such as blood, plasma, serum, or urine. The present review article focuses on various extraction techniques like liquid-liquid extraction, solid phase extraction and protein precipitation which play important role in sample preparation and detection by RP HPLC and consistent evaluation of the key bioanalytical method validation .Validation parameters include accuracy, precision, sensitivity, selectivity, standard curve, limits of quantification,limits of detection, range, recovery stability etc.

Keywords: Bioanalytical method validation; Validation parameters; extraction techniques, bioanalysis.

INTRODUCTION

Bioanalytical method is a set of procedures that are involved in the collection, processing, storage, and analysis of biological samples for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish suitable quantitative analytical method for biochemical applications.[1] Reassurances to the quality of the method and its reliability comes from performing a minimum series of experiments of validation and obtaining satisfactory results.

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Maintenance of stability of analytes in biological samples collected during clinical studies together with that of assay reagents, including analyte stock solutions, is considered as an important component of biological assay and validation.[2] Quality control and quality assurance are the major important areas in the pharmaceutical industry which deals with the analysis of materials starting from the raw material, intermediate products, APIS as well as finished products. Now new techniques are being developed all over the world. As a result of this, classical methods have changed to instrumental methods and finally it changed to sophisticated technique. Each technique is found to be much superior than the previous technique.[3] Bioanalytical methods which are employed for the quantitative determination of drugs and their metabolites in biological matrix (plasma, urine, saliva, serum etc) play an important role in evaluation and interpretation of

bioavailability, bioequivalence and pharmacokinetic data. Both HPLC and LCMS-MS can be used for the bioanalytical method development and validation of drugs in plasma and biological samples. Each of the instruments has its own merits and demerits. HPLC coupled with UV, PDA or fluorescence detector can be used for determination and estimation of many compounds.[4] The main advantages of LCMS/MS include low detection limits, the ability to generate structural information in clear picture, minimal requirement of sample treatment and the possibility to cover a wide range of analytes which differs in their polarities. Bioanalytical method validation includes all of the procedures that determine and demonstrate that a particular method used for quantitative measurement of analytes in a given biological samples, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. The fundamental parameters for this validation to perform include selectivity, accuracy, precision, linearity and range, limit of detection, limit of quantification, recovery, robustness and stability.[5]

Estimation of Drugs in Biological Fluids

The choice of sampling media is estimated by the nature of the drug study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and saliva. A bioavailability study may require drug level data in blood or urine. Steps involved in the estimation of drugs in biological samples are collection of the sample, sample treatment and separation of the compound of interest from the matrix and analysis of respective samples.[6]

Extraction Procedures for Drugs and Metabolites from Biological Samples

Sample preparation **three** major objectives

1. The dissolution of analyte in solvent which is suitable Eg: Acetonitrile, methanol
2. Removal of interfering compound
3. Pre concentration of the analyte

Different types of extraction techniques are:

a. Liquid-Liquid Extraction

Liquid-liquid extraction is mainly useful for separating analytes from interferences by partitioning the sample between two immiscible liquids. One phase in LLE often is aqueous and second phase is an organic solvent. More hydrophilic compounds prefer the polar aqueous phase; whereas more hydrophobic compounds will prefer the organic solvents. Analyte extracted into the organic phase is easily recovered by evaporation of the solvent, whereas analytes extracted in to the aqueous phase can be injected directly into a reverse-phase column. The technique is simple, rapid and has relatively economic factor per sample when compared to other samples. The extraction containing drug sample can be evaporated till dryness and the residue reconstituted in a smaller volume of a appropriate solvent (preferably mobile phase). Nearer quantitative recoveries of about 90% of most of the drugs can be obtained through multiple continuous extractions.

b. Solid phase extraction:

Solid phase extraction is the most widely preferable technique used in sample pretreatment for HPLC. SPE occur between a solid and a liquid phase. SPE is more efficient process of separation than LLE. It is easier to obtain a high recovery of analyte sample. SPE contains a small plastic disposable column or cartridge, in which the barrel of a medical syringe packed with 0.1 to 0.5 g of sorbent.[7] The sorbent is commonly a reverse phase material (C18-silica), and a reverse phase SPE (RP-SPE) combines both LLE and reversed phase HPLC in its separation characteristics. In SPE, a liquid sample is added to the cartridge and cleaning solvent is selected so that the analyte is either strongly retained ($k>>1$) or unretained ($K=0$). When the analyte is strongly retained, interferences are eluted or washed from the column so as to minimize their presence in the final analyte concentration as well as minimizing the errors in the method. The analyte is then eluted in a small volume with strong elution solvent, and then collected, and either injected directly or evaporated to dryness and dissolution is performed in the HPLC mobile phase. In the other case, where analyte is low retained, interferences are strongly held on the cartridge and the analyte is collected for the further treatment in study.

Advantages of SPE V/s. LLE

- Analyte is completely extracted.
- High efficient separation of interferences from analyte and Low consumption of organic solvent.
- Easy to collect the total analyte fraction
- Manually convenient procedures and Removal of particulates easily
- Easily automated[8]

c. Precipitation method

Protein precipitation is the simple method of extraction when compared to the LLE and SPE. This can be carried out by selecting the suitable organic solvents with good solubility of the analyte and protein precipitating properties. Acetonitrile is the widely preferred solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant. After the protein precipitation, the supernatant obtained can be collected and injected directly in to the HPLC or it can be evaporated and reconstituted with the mobile phase and further washing of the sample can be carried out by using micro centrifuge at very high speed.[9]

Method Development

Methods for analyzing drugs by HPLC can be developed when one has knowledge about the nature of the sample, mainly its molecular weight, polarity, ionic character, pKa values and the solubility etc. Method development cannot be standardized across the board as method development is unique and specific for each drug substance. It also depends on the nature of the sample and sensitivity.[10] While there are a number of HPLC methods available to the development, but the most commonly applied method is reversed phase and reverse phase coupled with ion-pairing. The typical pharmaceutical compounds is considered to be an active pharmaceutical ingredient of less than 1,000 Daltons, either soluble in water or an organic solvent such as acetonitrile, methanol etc.[11]

General conditions to initiate HPLC Method Development

Method development mainly starts with literature survey for the molecule in which we find the nature of the molecule, its pKa, solubility, molecular weight etc. Either isocratic or gradient mode may be used to determine the initial conditions of the separation, followed by the suggested experimental conditions given in the Table 1. Depending on the number of active components to be separated, the mode of run can be determined. If the number of components is large or the pKa values of components are wide then gradient mode is preferred than isocratic mode.[12] In order to decide whether a gradient mode or isocratic mode requirement which is adequate, an initial gradient run is performed and the ratio between the total gradient time and the difference in gradient time between the first and last component are noted and calculated.[13] When the calculated ratio is <0.25, isocratic is adequate over gradient mode; when the ratio is >0.25, gradient would be beneficial for the separation of complex mixture and when there are many compounds or degradation products, a long gradient run is required. In this case, two separation modes using an isocratic method for product release and gradient method for stability assessment is done.

In general, one begins with reversed phase chromatography, when the compounds are hydrophilic in nature with more number of polar groups and are soluble in water. The organic phase concentration required for the mobile phase can be determined by gradient elution method. For aqueous sample mixtures, the best preferred way to start is with gradient reversed phase chromatography.

Validation assures that-

1. The analysis results are reliable, consistent and moreover there is reproducibility in the experiments.
2. It also provides an assurance that the method is suitable for the intended purpose.
3. It gives assurance of quality of products and formulations.
4. Achieving and enhancing of products by international agencies is possible.
5. For compulsive requirement of registration for pharmaceutical product or formulations.[14]

Types of validation:

Validation is of three types depending upon the method used. They are

- Full validation
- Partial validation
- Cross validation

Full Validation

When bioanalytical method development of an analyte is performed for the first time, full method validation is employed here. Full method validation is much mandatory for any new drug entity. It is also recommended to use when metabolites are added to current assay of already existing analyte.

Partial Validation

Modification of validated bioanalytical methods that do not necessarily require for full revalidation. Partial Validation can range from as small as one intra-assay accuracy and precision determination to a Full Validation. Partial validation can also be carried out when there is use of different species within matrix (e.g. rat plasma to mouse plasma), changes in biological matrix within a species (e.g., human plasma to human urine), change in analytical method (e.g., change in detection systems), change in sample processing procedure, change in anticoagulant in biological fluid .[15]

Cross Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same analysis or different studies. The evaluations are to be done by considering an innovative and validated bioanalytical method as the reference and the repeated bioanalytical method as the comparator and vice-versa. Cross validation with spiked matrix and subject samples should be carried out at each site of laboratory to create inter laboratory reliability when sample analysis within a single study are carried out at more than one site, or more than one laboratory. Cross-validation should also be considered when data obtained using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different experimental studies are included in a regulatory submission. The important parameters for validation include: carry over effect, dilution integrity sensitivity, stability,

matrix effect, accuracy & precision, selectivity, recovery, purity of working standard.[16]

Selectivity/Specificity

For every step of product development, the analytical method must demonstrate specificity. The method must have the ability to assess the analyte of interest perfectly while in the presence of all expected components, which may consist of degradants, excipients, and sample blank peaks. The terms selectivity and specificity are often used alternatively. The term "specific" generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of analytes that may or may not be differentiated from each other. As there are very few methods that respond to only one analyte, the term selectivity is usually appropriate in exact. Optimized sample preparation can eliminate most of the components of the matrix.[17] The absence of matrix interferences for a quantitative method should be determined by the analysis of atleast five independent sources of control matrix. The method must be able to differentiate between the analyte of interest and compounds with a similar chemical structure that may be present in the matrix.

Accuracy

Accuracy of an analytical method describes the closeness of mean experimental results obtained by the method to the true value of the analyte. This is sometimes referred as trueness. It is usually expressed as bias% or relative error%. The two most commonly used paths to determine the accuracy or method bias of an analytical method, are analyzing control samples injected with analyte and by comparison of the analytical method with a reference standard method. It is determined by repeated analysis of samples containing known amounts of the analyte. It is measured using atleast minimum of five determinations for each concentration. A minimum of atleast three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not show deviation by more than 20%. The deviation of the mean from the true value of analyte serves as the measure for accuracy.[18]

Precision

The precision of an analytical method is nothing but the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple concentrations of a single homogeneous concentration of biological matrix. Precision should be measured using a minimum of five determinations of analytes per concentration. A minimum of atleast three concentrations in the range of expected concentrations is recommended. The precision determined at each level of concentration should not exceed 15% of the coefficient of variation except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-day precision or repeatability, which assesses precision during a single analytical run, and between-run, interday precision or repeatability. Repeatability expresses the precision under the same operating conditions over a short interval of time. It is sometimes also termed within-run or within-day precision. It shows how the method is performed in one lab on one instrument, within a given day for study. Repeatability can be determined by evaluating the precision from a minimum of atleast nine analytical determinations that encompass the specified range of the method. The nine determinations may be composed of triple repetetions of determinations at each of three different concentration levels, in which one would represent the 100% test concentration. Intermediate precision reflects within variations in laboratory such as different days, different analysts, and different equipment (between batches, different assay). Intermediate precision testing mainly consist of two different analysts, each preparing a total of six sample concentrations, as per the analytical method. The analysts perform their testing on different days using separate instruments and analytical columns. It is also called as between-run, between-day, or inter-assay precision. Reproducibility, i.e., the precision between laboratories i,e., interlaboratory studies, is not required, but can be taken into account for standardization of analytical procedures.

Linearity

Linearity determines the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. ICH7 guidelines recommend analysing a minimum of five concentrations to determine linearity. The five concentration levels should cover the upper and lower concentration levels analysed during the accuracy study. If the total range cannot be determined by a single calibration curve, two calibration ranges can be validated. A calibration curve should be plotted for each analyte in the sample. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by injecting the matrix with known concentrations of the analyte. Concentrations of standards should be selected on the basis of the concentration range expected in a particular study. A calibration curve should contain

i) A blank sample (matrix sample prepared without internal standard)
ii) A zero sample (matrix sample prepared with internal standard)
iii) Six to eight non-zero samples which covers the expected range, including LLOQ.

Matrix effect

The recent 3rd bioanalytical workshop proposed determines the matrix factor as a way of determining the matrix effect. Since ionization of analytes will be affected by presence of endogenous components in biological sample of matrix, it could be either suppression or enhancement.

Matrix Factor (MF) = Peak response in presence of matrix ions/ Peak response in absence of matrix ions

MFs for an analyte and its stable isotope are usually similar. It is advised that matrix factor or IS normalized MF being determined in six different lots of matrices. The variability in matrix factors that is measured by coefficient of variation (%CV) should be less than 15%.[19]

Carry over effect

Carry over effect in the assay is used to study the rinsing cycles or wash program of auto sampler required to clean up the injection needle properly so as not to get any interference from the previous sample injection. For carry over recovery comparison, sample of 1.5 to 1.8 times of non extracted standard H containing internal standard, extracted sample containing 1.5 to 1.8 times concentration of standard H containing internal standard, LLOQ sample in replicate with internal standard from biological matrix and extracted blank samples from the same matrix lots are used.

Stability

Stability is defined as the chemical stability of an analyte in a given matrix sample under specific conditions for a given intervals of time. Stability of the analyte during the whole analytical study is a prerequisite for reliable quantification.

Long-term stability

The stability in the sample matrix should be established under storage conditions, i.e. in the same vessels, at the same temperature and at over period of time at least as long as the one expected for genuine samples.

Freeze/thaw stability

As samples are often frozen and thawed, e.g. for reanalyis, the stability of analyte during several freeze/thaw cycles should also be analysed. It requires a minimum of three cycles at two concentrations which are spiked three times.

In-process stability

The stability of analyte under the conditions of sample preparation (e.g. ambient temperature) is evaluated here. There is general agreement, that this type of stability should be performed and evaluated to find out, if any preservative have to be added to prevent degradation of analyte during sample preparation.

Processed sample stability

Instability cannot only occur in the sample matrix, but also in prepared samples. It is therefore also necessary to test the stability of an analyte in the prepared samples under conditions of analysis (e.g. auto sampler conditions for the expected maximum time of an analytical run). One should also test the stability of prepared samples under storage conditions, e.g. refrigerator, in case prepared samples must be stored prior to analysis.

ROBUSTNESS/ RUGGEDNESS

According to ICH guidelines, the robustness/ ruggedness of an analytical procedure is the measure of the capacity of method to remain unaffected by little, but deliberate variations in method parameters and provides an indication for its reliability during normal usage. Robustness can be defined as the ability to reproduce the results of method in different laboratories or under different circumstances without producing of unexpected differences in the obtained results, and a robustness test as an experimental set-up to evaluate the robustness of a method.

DETECTION LIMIT:

The limit of detection (LOD) is the least concentration of analyte in the sample that can be detected but cannot quantify under the stated experimental conditions. It is also defined as the lowest concentration that can be differentiated from the background noise with a certain degree of confidence. There is an overall agreement that the LOD should represent the smallest detectable amount of concentration of the analyte sample of interest. The detection is usually expressed in terms of concentration of the analyte in the sample, for example, percentage, parts per million (ppm), or parts per billion (ppb).[20]

LIMIT OF QUANTIFICATION

Determining LLOQ on the basis of precision and accuracy is the most practical approach and defines the LLOQ as the lowest conc. of the sample that can still be quantified with desired precision and accuracy. LLOQ based on signal and noise ratio (s/n) can only be applicable only when there is baseline noise, for example in chromatographic methods. Upper limit of quantification: ULOQ is the maximum analyte conc. of a sample that can be quantified, with desirable precision and accuracy. The ULOQ is similar with the conc. Of the highest calibration.

Table 1 General Experimental Condition for HPLC

INITIAL PARAMETERS			
Chromatographic variables	**Neutral compounds**	**Ionic-acidic compounds (carboxylic acids)**	**Ionic-basic compounds (amines)**
Column	25cm x 0.46cm	25cm x 0.46cm	25cm x 0.46cm
Dimension			
(length, ID)			
Stationary phase	C18 or C8	C18 or C8	C18 or C8
Particle size	10μm or 5μm	10μm or 5μm	10μm or 5μm
Mobile phase			
Solvents A and B	Buffer-acetonitrile	Buffer-acetonitrile	Buffer-acetonitrile
%B			
(organic)	50%	50%	50%
isocratic			
%B	20%/80%	20%/80%	20%/80%
(organic)			
Gradient	Phosphate	Phosphate	Phosphate
	50 mM	50 mM	50 mM
Buffer	3.0	3.0&7.5(gradient)	3.0&7.5(gradient)
Type			
Concentration	10mM triethylamine and	1% acetic acid	25 mM Triethylamine
Ph	1%		
	acetic acid	1.5-2.0 mL/min	1.5-2.0 mL/min
Peak modifier			
		Ambient to 35°C	Ambient to 35°C
Flow rate	1.5-2.0 mL/min		
		10μL-25μL	10μL-25μL
Temperature	Ambient to 35°C		
		< 100mcg	< 100mc
Sample size	-	-	-
Volume	10μL-25μL		
Mass	< 100mcg		

CONCLUSION

The present review work concludes the validation parameters that are necessary according; to the requirements of ICH guidelines. The method validation process and the minimum requirements that are to be included in a regulatory method are discussed. The objective of this paper is to provide steps for determining selectivity, limit of detection, lower limit of quantitation, linearity, accuracy, precision, carry over effect, stability, robustness of liquid chromatographic methods to Determine pharmacokinetic, toxicokinetic, bioanalytical, bioavailability, and bioequivalence studies. An attempt has been made in order of easy understanding of validation parameters to every analyst. The relatively new concepts and recent progress made in several areas of analysis including sample preparation, separation, how to reduce matrix effect and specific requirements for bioanalytical method validation discussed in this review.

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How to cite this article:

M. Shanti Prathyusha *, R. Shanmugam, A. Kirthi, D. Jamal Basha, D. Mounika, a Review – Bioanalytical method development and its validation by RP HPLC, 2643 – 2649 (2015)

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