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Original Article

A Review on Bioanalytical Method Development and Validation by LC-MS/MS

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Abstract

The development of bioanalytical method is of paramount importance during the pre-clinical and clinical stage of drug development. Analytical method development where the appropriate bioanalytical method with its various parameters is developed and the assay is defined and application of the bioanalytical to actual analysis of sample from bioavailability, bioequivalence and pharmacokinetic studies. Bioanalytical methods are used for the quantitative analysis of drugs and their metabolites in the biological matrices like saliva, plasma, blood, serum, urine. In method development sample preparation and sample analysis are two important part. For sample analysis generally chromatographic technique are used like (HPLC, LC-MS/MS, GC, UPLC). After method development validation of that bioanalytical method is important. Method validation is a process that demonstrates that a method will successfully meet or exceed the minimum standard recommended in the USFDA or other guideline for accuracy, precision, selectivity, sensitivity, reproducibility and stability.

Keywords: Bioanalytical Method Development; LC-MS/MS; Bioanalytical Method Validation.

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Introduction

Method validation is a process that explain that a method will successfully meet or exceed the minimum standards recommend in the U.S.FDA , EMA or other regulatory guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. This article discusses the validation of bioanalytical methods for small molecules with emphasis on chromatographic techniques. Bioanalytical methods are used for the quantitation of drugs and their metabolites in biological matrices like plasma, serum, saliva, urine. this type of bioanalytical methods are develop for analyte and some time for endogenous substance like levothyroxine sodium. for the purpose of analysis or quantification of analyte there is different type of analytical methods are available Hyphenated techniques in which LC-DAD (liquid chromatography-diode array detection), CE-MS(capillary electrophoresis- mass spectrometry), LC/MS (liquid chromatography-mass spectrometry), LC/MS/MS (tandem mass spectrometry), GC/MS (gas chromatography-mass spectrometry). Chromatographic methods HPLC(high performance liquid chromatography), GC(gas chromatography), UPLC(ultra performance liquid chromatography) Supercritical fluid chromatography. Ligand binding assays Dual polarization interferometry, ELISA (Enzyme-linked immune sorbent assay), MIA (magnetic immunoassay), RIA (radioimmunoassay) are used for method development and validation .In today's drug development environment, highly sensitive and selective methods are required such as high-performance liquid chromatography (HPLC) or

gas chromatography (GC) have been widely used for the bioanalysis with liquid chromatography coupled to triple quadrupole mass spectrometry (LC/MS/MS) is most commonly used technology [1]. Typically the combined increases in selectivity and sensitivity of LC-MS/MS based methods provide more than 10-fold improvement in limit of quantification (LOQ) relative to conventional methods using UV or fluorescence detection. Particular method used for quantitative measurement of analytes is reliable and reproducible for the intended use 1. Validation involves documenting, through the use of specific laboratory investigations, that the performance of characteristics of the method are suitable and reliable for the intended analytical applications. as increased number of therapeutics has promote the pharmaceutical industry to review and redefine aspects of development and validation of bioanalytical methods for the quantification of of this therapeutics in biological matrices in support of preclinical and clinical studies. A bioanalytical method is a set of procedures involved in the sample collection, sample processing, storage, and analysis of a biological matrix for a chemical compound.

Instrumentation

Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (*m/z*) ratios. Two key components in this process are the 1) ion source, which generates the ions, and 2) mass analyzer, which sorts the ions.

Ion Sources

Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermo spray) or if did so before ionization (particle beam). The analytes were then ionized under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds. In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electro-statically separated from neutral molecules. Common atmospheric pressure ionization techniques are:

a. Electro spray ionization (ESI)
b. Atmospheric pressure chemical ionization (APCI)
c. Atmospheric pressure photo ionization (APPI)

a. Electrospray ionization

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas.

The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases.

Eventually, the repulsive force between ions with like charges exceeds the cohesive forces; ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer.

Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates.

Mass Analyzer

Four types of mass analyzer used for LC/MS:

i. Quadrupole
ii. Time-of-flight
iii. Ion trap
iv. Fourier transform-ion cyclotron resonance (FT-ICR or FT-MS)

Quadrupole

A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time.

Quadrupole Mass Analyzers can Operate in Two Modes

- Scanning (scan) mode
- Selected ion monitoring (SIM) mode

In scan mode, the mass analyzer monitors a range of mass-to-charge ratios. In SIM mode, the mass analyzer monitors only a few mass to charge ratios. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance. SIM mode is used for quantitation and monitoring of target compounds [2].

Method Development

Analytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations, such as: chemical properties of the analyte, concentrations levels, sample matrix, cost of the analysis, speed of the analysis, quantitative or qualitative measurement, precision required and necessary equipment.the analytical process of method development includes sample collection, sample prepration, separation, detection and evalution of the results.

Sample Collection

According to specific time period sample/matrix is collected from human subject by vein puncture with a hypodermic syring (volume of sample is depending on the assay sensitivity and the total number of samples taken for study being performed).withdrawn sample is fill in the tube according to intended use (if sample is used as a plasma than blood is withdrawn in tube with anticoagulant EDTA. if serum is required than anticoagulant free tube used) Plasma is obtained by centrifugation at 4000 rpm for 15 min. serum is obtain by store sample at room temperature coagulation for 25-30 min. after the blood is coagulated centrifugation at 4000 rpm for 10 min. The purpose of sample preparation is to clean up the sample before analysis and to concentrate the sample [3].

Sample Preparation

Sample preparation technique is used to clean up a sample by removing endogenous material as well as to concentrate a sample before analysis to exclude errors in its detection, also Minimize interferences, Prevent clogging of column, Minimize matrix effect, Improve reproducibility and improve specificity .various methods used for sample preparation

Solid Phase Extraction

SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed suitable sorbent. The first strategy is usually chosen when the desired sample component is present in high concentration. When components of interest are present at low levels, or multiple components of widely differing polarities need to be isolated, the second strategy is generally employed. The second strategy may also be used for trace enrichment of extremely low level compounds and concentration of dilute sample. A complex matrix may be treated by both elution strategies to isolate different target analyte. Solid phase consists of four steps; conditioning, sample loading, washing and elution. There two SPE methods

Off-Line Solid Phase Extraction (SPE)

Selective sample clean-up, Various SPE formats – phases, Sample pre concentration, Automated systems, Trend to 96-well micro SPE (avoids drying steps) these type of advantages for off-line SPE.

On-Line Solid Phase Extraction (SPE)

Disposable SPE cartridges, Minimal pre-treatment steps, Sample preparation at high pressure and controlled flow-rates, all samples are analyzed, sensitive assays, Systematic, automated method development, Fully automated and integrated process these are advantages of On-line SPE.

Liquid-Liquid Extraction (LLE)

Liquid-liquid extraction is based on distribution of solutes between an aqueous phase and a water immiscible organic phase. Distribution of different solutes depends on their degree of solubility in different solvents. Analyte extracted into the organic phase can be evaporated to dryness and the residue reconstituted in a smaller volume of an appropriate solvent (preferably mobile phase), while analyte extracted in to the aqueous phase can often be injected directly on to a reversed-phase column. The technique is simple and rapid. Good quantitative recoveries are obtained through multiple continuous extractions. Solvents normally used are Ethyl Acetate, TerButyl methyl ether, Diethyl ether, n-Hexane and sometimes mixtures of the two or more solvent for efficient extraction.

Advantages: Low LODs are possible, Cost effective as compare to the On-line and Off line SPE techniques, Clean sample obtain as compare to protein precipitation technique.

Protein Precipitation: Protein precipitation is the simple method of extraction as compared to the LLE and SPE. It is utilized when high throughput of plasma and serum samples is Desired. Protein precipitation is very useful method in field of clinical toxicology, the drug discovery and the therapeutic drug monitoring in which high throughput is

required. Proteins are denaturated with acid, base, salts or organic solvents. (e. g. TFA, TCA, NaOH, ZnSO4, Acetonitrile, Methanol). Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents. After protein precipitation centrifugation is carried out and the supernatant obtained is used for analysis. The supernatant can be injected directly into the chromatographic column or it can be evaporated and reconstituted with the mobile phase. Simple, generic, easy to perform, just mix solvents centrifuge, No Method Development time, Fast sample preparation (96-well titer plate automation),Very low volume require for sample processing, Very few step to get final drug concentrate ready for inject into LC-MS/MS [4-6].

Bioanalytical 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. method validation To ensure that a particular method for quantitative measurement of an analyte in a biological matrix is reliable and reproducible. the possibility of a bioanalytical method not being used on a regular basis will require adequate revalidation data when needed to be used, in order to document and demonstrate that a method is still valid prior to analyses of samples in a study. there are different levels and types of method validations, including “Full Validation, Partial Validation, and Cross Validation”. These different types of bioanalytical method validations are defined and characterized as follows:

Full Validation

Full validation is important when developing and implementing a bioanalytical method for the first time. Full validation is important for a new drug entity. A full validation of the revised assay is important if metabolites are added to an existing assay for quantification. Generally, a full validation should be performed for each species and matrix (mainly plasma, serum, whole blood, or urine) to be analyzed.

Partial Validation

Partial validations are modifications of already validated bio-analytical Methods. Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. A set of parameters to be evaluated in a partial validation are determined according to the extent and nature of the changes made to the method.

Cross Validation

Cross-validation is a comparison of validation parameters when two or more bio-analytical methods 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, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.

Need of Bioanalytical Method Validation

It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactory interpreted, It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology, It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria ma need to be developed for each analyte [7-8].

Validation Parameters

Parameters to validate method are include;

Specificity or Selectivity

Selectivity is ability of an analytical method to differentiate and quantify analyte in the presence of other components in sample. For selectivity, analysis of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics.

Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by method to the true value (concentration) of an analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15 % of actual value except at LLOQ, where it should not deviate by more than 20 %. The deviation of mean from true value serves as measure of accuracy.

Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in range of expected concentrations is recommended.

Recovery

Recovery is a measure of efficiency at which an analytical method recovers analyte through sample comparing analytical results for extracted samples at three concentrations (low, medium, and high) and three replicates that represent 100 % recovery. Recovery of analyte need not be 100 %, but extent of recovery of an analyte and of internal standard should be consistent, precise, and reproducible.

$$\%\text{Mean recovery} = \frac{\text{Mean extracted peak area}}{\text{Mean un-extracted peak area}} \times 100$$

Matrix Factor

The ratio of analyte response in presence of matrix ions to response in absence of matrix ions. matrix factor is determined by comparing analyte response in presence of matrix with that in absence of matrix. Matrix factor may be normalized using an internal standard.

$$\text{Matrix factor} = \frac{\text{Peak are in presence of matrix ion}}{\text{mean aqueous peak area}}$$

$$\text{ISTD Normalized factor} = \frac{\text{Matrix factor of drug}}{\text{matrix factor of ISTD}}$$

Linearity

Linearity is determined by using mean of two calibration curve standards and it includes STD BL, standard zero and at least six calibration standards. r2 value should be more than ≥ 0.98.

Stability

Short Term Stock Solution Stability (STSS)

Stability of drug and ISTD stock solution should be evaluated for at least 06 hours. For STSS stock solutions are stored in refrigerator for minimum of 06 hours and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Long Term Stock Solution Stability (LTSS)

Stability of drug and ISTD stock solution should be evaluated for relevant time period. For LTSS stock solutions are stored in refrigerator for 20 days and after stability period retrieve it and make ULOQ vial from drug stock and ISTD dilution vial from ISTD stock solution. Inject aqueous ULOQ and ISTD dilution vial compare it with freshly prepared aqueous ULOQ and ISTD dilution.

Bench Top Stability (BT) (Short Term Stability of Analyte in Matrix)

It should be performed at higher quality control and lower quality control level for six replicates. Prepare spiked sample of HQC and LQC and stored at room temperature for a specific time period. Generally, time period is about time required from spiking of sample to transfer in to vials. Use freshly spiked calibration curve and quality control standard for determination of stability samples. For BT kept spiked HQC and LQC for 06 hours at room temperature.

Stability of Dry Extract (DE)

evaporation step. DE stability was conducted by using previously processed and dried stability samples. Freshly spiked replicates of each LQC and HQC samples were prepared and processed as per sample preparation procedure. After drying, dry extract stability samples were stored at -20 ± 5 ºC for a period of at least 24 hours or as per requirement.DE stability samples were analyzed along with freshly spiked CCs and freshly prepared QCs 6 replicates of each LQC and HQC samples as per procedure.

Freeze and Thaw Stability

QC samples (at high and low level) are stored and frozen in freezer at intended temperature and thereafter thawed at room or processing temperature. After complete thawing, samples are refrozen again applying same conditions. At each cycle, samples should be frozen for at least 12 hours before they are thawed. Number of cycles in freeze-thaw stability should equal or exceed that of freeze/thaw cycles of study samples. It is perform to demonstrate that accuracy and precision is not change upon freezing and thawing cycle. Freeze and thaw stability experiment is performing by processing n ≥ 5 sample (at high and low level) of freeze thaw stability along with freshly spiked calibration curve and quality control sample. Storage temperature: -20 ± 5 °C and -78 ± 8 °C.

Long Term Stability of Analyte in Matrix

It should be performed at higher quality control and lower quality control level which are analysed immediately after preparation (first day stability assessment) and after applied storage conditions that are to be evaluated. Prepare spiked sample of 2 set of HQC and LQC and stored at-70 °C and -20°C for a specific time period. Generally, time period is about time required from sample collection to last sample analysis. Use freshly spiked calibration curve and quality control standard for determination of stability samples. Stability is evaluated by 3 replicates of each quality control level Concentration [9-11].

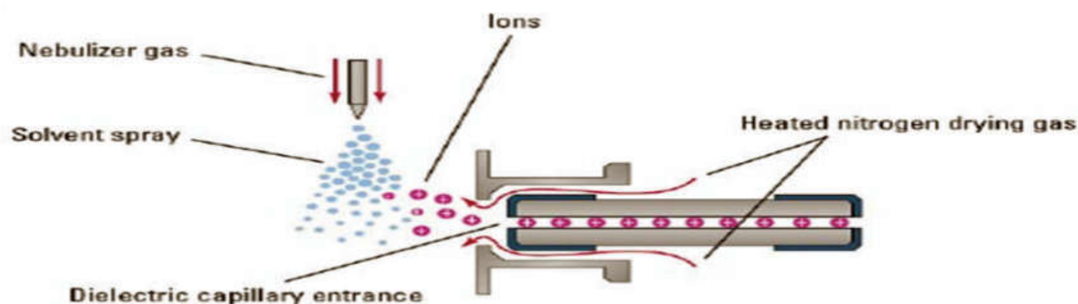

Fig. 1: Electrospray ionization

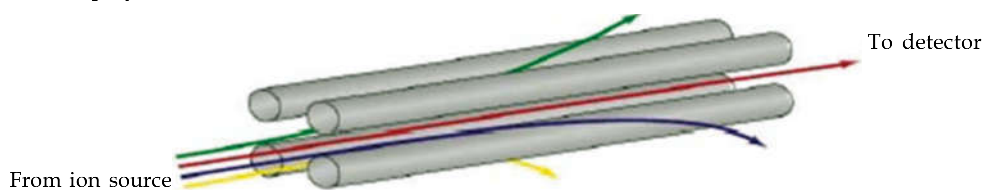

Fig. 2: Quadrupole

Fig. 3: off-line solide phase extraction (SPE)

Fig. 4: on-line solid phase extraction (SPE)

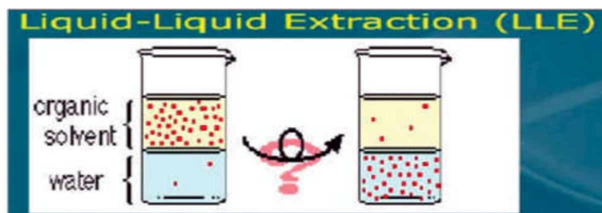

Fig. 5: Liquid-Liquid extraction (LLE)

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