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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY RP HPLC: A COMPREHENSIVE REVIEW

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ABSTRACT

The bioanalytical techniques plays an essential role in quantitation of drugs and metabolite concentration in biological fluids such blood, plasma, serum during drug discovery and development. Appropriate sample preparation, as well as method development and validation, are essential to provide accurate results. The bioanalyses are important in pharmacokinetic studies, and also contribute to the study of animal bioavailability and bioequivalence. These studies can be used to make critical decisions about the safety and efficacy of a drug. High performance liquid chromatography (HPLC) is a critical method for the detection and quantitation of drugs and metabolites in biological matrices at low concentrations. Accordingly establishing HPLC based bioanalytical methods are handy for low dose drugs. It covers novel methods of sample preparation, including liquid-liquid extraction solid-phase extraction and the protein precipitation as well as the validation parameter such as accuracy, precision, sensitivity, selectivity linearity limits of quantitation and range recovery in addition to stability in chromatographic bioanalysis.

Keywords: RP-HPLC, Bioanalytical Method Development, Validation, Validation parameter
INTRODUCTION:

Bioanalysis is the measurement of drugs and their metabolites in the biological matrices like blood, plasma, serum, urine and saliva. The procedure entails collection, preparing,

storing, and analyzing a drug's in biological matrix [1]. Bioanalytical Method Validation is a laboratory-based testing that supports the measurement of drug levels in biological

matrices to determine concentration, and its way into pharmacokinetic and toxicology studies. The overall and the results support preclinical and clinical studies [2, 7, 22].

Biological matrices are complex substances which contain different endogenous substances and hence separation of the drug in its pure form is essential using suitable method. HPLC is used extensively for identification and quantification of drugs in biological fluids i.e. plasma, serum and urine for successful HPLC analysis suitable detection system, stationary phase, eluent and separation schedule are pre-requisite. The best suitable detector is UV/VIS since they supply rapid quantitative analysis. This article focuses on the development and validation of bio-analytical method using HPLC, sample preparation and validation [4, 5, 16, 30, 43].

METHOD DEVELOPMENT:

Analytical method development is the procedure in which compound was detected and quantified in matrix. It has an important significance for the drug research and development based on accurate and reliable detection. The sample preparation, analyte separation and analyte detection are three vital steps to developing a method [11, 20, 25, 40].

Sample collection and preparation:

The biological media is usually blood, plasma, urine and serum which includes the

analyte. Blood samples (5-7 mL) are taken from humans, rats or mice. Natural and exogenous proteins can interfere with the analyte in biological fluids, the goal of sample preparation is to isolate the analyte from these unwanted components without significant loss. After collecting venous blood in tubes then added anticoagulants like Heparin or EDTA, plasma is separated by centrifugation at 4000 rpm for 15 minutes [11, 19, 34].

Cleaning and isolating the sample before the sample analysis is the goal of sample preparation. Proteins, salts, endogenous substances found in biological samples that can block the analysis.

General procedures for sample preparation like: Three methods have been used to extract analytes from biological matrices: liquid-liquid extraction (LLE), solid-phase extraction (SPE), and precipitation of plasma proteins (PP) [1, 31].

Liquid-liquid extraction (LLE):

In bioanalytical studies the liquid-liquid extraction (LLE) has found wide applicability as a sample preparation technique to separate analytes from complex biological specimens like plasma, serum, blood and urine. It depends on the distribution of analyte between two non-mixing liquid phases, usually an aqueous biological sample and a water insoluble organic solvent in proportion to the solubility and partition coefficient of an

analyte. LLE is a method appreciated for its simplicity and effectiveness. It gives fairly clean extracts, which would be suitable for chromatographic analysis, mainly the HPLC and GC techniques. Furthermore, LLE increases sensitivity and enables focussing of the analyte and improves precision and accuracy in bioanalytical assays [6, 37].

Solid phase extraction (SPE):

Solid phase extraction is a sample preparation methodology commonly employed in bioanalysis to remove analytes (drugs, metabolites: biomarkers) from biological matrices such as plasma, serum, urine or tissue homogenates prior to instrumental analysis (LC-MS/MS, HPLC and GC) [7, 8, 46].

Solid-phase extraction (SPE) involves four main steps

Conditioning: Activates the column using an organic solvent to wet the sorbent and solvate its functional groups, followed by water or buffer to prepare it for proper adsorption.

Sample loading: After pH adjustment the sample is allowed to pass through column by gravity or pumping or vacuum.

Washing: During column washing the matrix is washed out and separated from analyte, which remains on the sorbent.

Elution: An appropriate solvent breaks the analyte-sorbent interactions and removes the analyte with minimal interference.

Protein Precipitation (PP)

Protein precipitation is one of the easiest and simplest sample preparation processes in bioanalysis to remove proteins from biological matrices (like plasma, serum) prior analysis. It comprises denaturation and precipitation of proteins by an addition of organic solvent or acid, followed by HPLC analysis. Here, protein precipitation is an easy and fast method of sample preparation requiring low-cost amounts of organic solvents. However, it has limitations in that protein residues may be present and it is non-selective for clean-up. This may permit native compounds or other drugs to interact with the RP-HPLC system. The organic solvents used are mostly methanol which gives a clear supernatant for direct HPLC analysis [9, 27].

Bioanalytical Method Validation (BMV):

Bioanalytical Method Validation is the process used to establish that an analytical method employed for test in a particular biological matrix (such as plasma, serum, urine) is reliable and reproducible for the quantitative analysis of drugs or metabolites in body fluids. It maintains the reliability and quality of data from pharmacokinetics, bioavailability, bioequivalence and toxicokinetic studies [28, 29, 35, 48].

Selectivity, specificity, accuracy, precision (intra - and inter-day variability), linearity range and sensitivity ([LOD] Lower Limit of Detection and [LLOQ] Lower Limit Of Quantification), recovery, matrix effect as

well as stability on different conditions are among the key validation parameters. Validation generally takes place according to guidelines of for example ICH regulatory authorities. Appropriate validation of a bioanalytical method is necessary for the acceptance of medical products in compliance with regulations, as well as to guarantee confidence in interpretation of drug concentration data during drug development and clinical trials [10, 12, 13, 21, 42].

Need Of Bioanalytical Method Validation [3, 27, 38, 39]

- I. For accurate and precise quantitation of drugs, metabolites, and biomarkers in biological samples.
- II. To confirm the analytical results are reliable and repeatable.
- III. To prove the specificity and selectivity of the method for analyte in presence of biological matrix components.
- IV. To ensure data integrity for pharmacokinetic, bioavailability, bioequivalence, and toxicokinetic studies.
- V. To comply with regulatory requirements (FDA, ICH guidelines).
- VI. To verify the stability of the analytes in samples from collection, storage and analysis.
- VII. To support dose optimization and safety evaluation during drug development.
- VIII. To ensure regulatory acceptance of bioanalytical study results

Common parameters for validation of an analytical method are selectivity, accuracy, precision, linearity range, LOD/LOQ (Limit of detection and limit of quantification), recovery, robustness and stability. It is well recognized that it is necessary to validate an analytical method to confirm that the method is accurate, reproducible and intended use appropriate [18, 26, 44].

1. Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy shall be performed at least at three concentration levels of the expected range with six determinations in each level. This should be within $\pm 15\%$ of the true value [17, 33].

2. Precision:

Precision is the closeness of individual measurements obtained from a sample of homogeneous material under prescribed conditions using an appropriate analytical method. The precision of the method should be determined at not less than three concentration levels throughout the range and not fewer than 5 concentrations being tested at each level.

The variation does not exceed 15% at each concentration [11, 41].

3. Repeatability: Repeatability reflects the variation between repeated measurements of a method under controlled conditions in a short period of time. It represents the in-assay (intra-assay) reproducibility, referring to how accurate of the method is within a single laboratory, on one apparatus and in one day. Repeatability thus reflects precision under optimal condition, i.e. short time interval and analyzed by the same analyst.

4. Reproducibility:

Reproducibility describes the closeness of agreement between results obtained under changed conditions including but not limited to, different laboratories, analysts, instruments and days. It is generally not necessary during the course of regulatory submission to perform interlaboratory reproducibility studies, which are essential for ensuring that laboratory procedures are standardized. Reproducibility refers to the ability of a method to return equivalent concentration values for the same sample analysed on different occasions and even under different circumstances [1].

5. Linearity: Linearity is the correlation between the response of an instrument and sample concentrations of an analyte.

It measures a methods capability to respond in a linear relationship with the analyte concentration of an assayed sample [27, 36].

6. Selectivity and Specificity:

Selectivity and specificity are the ability to measure an analyte in the presence of other components such as metabolites, impurities, degradation products, matrix components (including those induced by sample preparation), etc. that might be present together with the intended constituent from the sample matrix. Specificity is the selectivity of a method that only measures the analyte as an unequivocal result, e.g. by ensuring that a chromatographic peak truly represents a single chemical species. The importance of both selectivity and specificity cannot be overstated, as it constitutes the cornerstone for robust analytical techniques [12, 24].

7. Limit of detection (LOD):

The detection limit is the minimum quantity of an analyte that can be detected, but not necessarily quantified. It can be calculated differently, depending on whether laboratories calculate it against reference solutions or from the biological samples [38].

8. Limit of Quantitation:

LOQ, also known as minimum quantifiable limits (MQL), is the minimum concentration of an analyte in

a sample that can be determined and reported with acceptable precision under experimental conditions [15, 23].

9. Recovery:

Recovery is defined as the percentage of known amounts of an analyte that must be successfully achieved during the sample workup utilized in the analytical method; it does not have to be 100%. They should both be accurate, consistent, precise, and reproducible [5].

10. Standard curve (calibration curve):

A (calibration) standard curve that dependence of an instrument's response (e.g., peak A height or absorption) on known concentrations of analyte. It must be monotonic (ever increasing, or ever decreasing) to give reliable and accurate measurements [11].

11. Stability:

Analyte stability is defined as the ability of a chemical (analyte) to be preserved in time, under predetermined conditions and in a matrix. Stability testing aims to determine any degradation the analyte may undergo from the moment of sampling through to analysis, following storage and preparation methods. The stability requirements are analyte-, biological matrix- and expected storage time-dependent [5].

a) Short-term stability: The short term stability demonstrate whether the analyte intact at ambient (room

temperature) in the biological matrix. Samples are stored at room temperature for a minimum of 24 hours and then assayed [12].

b) Long – term stability: Long-term stability studies are performed to justify if an analyte is stable in a biological matrix over certain periods of time. Such studies guarantee that the analyte remains stable during storage from the moment it is collected to the time for analysis [5].

c) Freeze and Thaw Stability: Freeze–thaw stability in a bioanalytical context investigates whether an analyte (i.e., drug, metabolite or biomarker) is chemically and quantitatively stable in biological matrix (e.g. plasma, serum, urine) when exposed to repeated cycles of freezing and thawing during sample processing and storage. Freeze–thaw evaluation must be carried out in a way that mimics the realistic sample handling during analysis. Stability samples (time point and concentration) shall be subjected to three freeze–thaw cycles with adequacy of the analyte stability across a minimum three cycles [11].

d) Bench-Top Stability: A bench-top stability examination assesses the

stability of samples stored at room temperature in normal laboratory use situations. It is carried out to confirm the stability of the analyte when preparing, processing and analysing the sample [32].

- e) **Stock solution stability:** Stock solution Stability test is determined the stability of drug stocks solution during Attest time. Stability data should be generated when the stock solution is prepared in a different solvent or buffer system than that of the certified reference standard. This will justify the proper storage condition as well as its stability period of storage for stock solution.

12. Range: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

13. Robustness: Robustness is an important validation parameter defined by ICH guidelines. It refers to the ability of an analytical method to remain unaffected by small, variations in method parameters, such as changes in pH, temperature, flow rate, or mobile phase composition. A robustness study is carried out to evaluate whether the method can consistently produce

accurate and precise results under varied experimental conditions [45].

14. Ruggedness: Robustness is defined as the ability of an analytical method to provide acceptance results for identification, content and assay in this case, under normal variation in factors like sample preparation and analyst. These differences can be attributed to different venues for testing, analyzers, operators, columns and possibly sources of reagents, chemicals and solvents. Ruggedness is tested by analyzing the same sample under deliberately modified experimental conditions (ie, independent operators performing the testing in the same laboratory) to demonstrate that a method provides consistent results [10].

Specific Recommendation for Bioanalytical Method Validation:

- I.** In the case of method validation, Accuracy and Precision are to be calculated from at least five determinations for each Analyte and concentration level. The center of the mean value should not deviate by more than $\pm 15\%$ from the theoretical value.
- II.** Precision and accuracy using known concentrations of Analyte in biological matrix shall be established. This can be achieved by analyzing duplicate sets of (known

concentration) QC samples prepared in the same biological matrix.

- III. The stability of the analyte in biological matrix at experimental storage temperature must be determined.
- IV. The analyte/matrix mixture should be stable at ambient temperature for similar time period as the sample preparation, sample handling and analytical run time.
- V. The reproducibility of reinjection should be tested in order to establish whether it would be possible to reanalyze an analytical batch if an instrument failed.
- VI. The Specificity of the methods under test is to be demonstrated with a minimum of six individual sources of same matrix [1, 12, 14, 47].

CONCLUSION:

Drug metabolism data is required in both pharmaceutical research and drug development processes. The bioanalysis method has been developed for researching the pharmacokinetic (PK) and metabolic profile detection of adverse effects to enhance or adjust original drugs. New methods of sample preparation, instrumental analysis and elimination of matrix effects together with bioanalytical method validation have recently raised the reliability of analytical data. The results successively confirm that RP-HPLC is a

preferred medium in small molecules bioanalysis. The novel ideas and concepts presented in this paper will aid the development of RP-HPLC methods for decreased matrix effects caused by endogenous interfering substances met in bio-related samples.

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