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## Review Article

# An Overview of the Development and Validation of Bioanalytical Methods using HPLC

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### ABSTRACT

A key component of successful drug development is the creation of sensitive, dependable, and selective bioanalytical techniques for the quantitative assessment of medications and their metabolites in biological matrices. The pharmacokinetic and toxicokinetic analyses of investigational new drug applications (INDs), new drug applications (NDAs), and abbreviated new drug applications (ANDAs) all require the data gathered from these techniques. Critical judgments supporting a drug's safety and efficacy are based on the findings of human clinical trials, including bioavailability and bioequivalence studies that require pharmacokinetic evaluation, as well as animal toxicokinetic research. Therefore, in order to acquire accurate results, it is crucial that the proposed bioanalytical procedures be well-designed, sufficiently validated, and documented to a satisfactory quality for application in drug analysis. A flexible analytical method for identifying and quantitatively estimating low concentrations of medications and metabolites in biological matrices is high pressure liquid chromatography. Therefore, developing and validating a bioanalytical HPLC approach for low-dose medicines is beneficial. This article examines recent developments in the creation of bioanalytical methods based on HPLC and the validation of various medications. Thus far, bioanalytic analysis has been performed on medications such as Omeprazole, Clofarabine, Palonosetron HCl, and antimalarials.

### INTRODUCTION

The technique used to ascertain the levels of medications, their metabolites, and/or endogenous chemicals in the biological matrices, including urine, saliva, serum, blood, plasma, and

cerebrospinal fluid. The procedure entails gathering, processing, storing, and analyzing a drug's biological matrix. Validating bioanalytical methods entails recording established and confirmed specifics inside a particular biological

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matrix. All factors that determine the quality of data, including selectivity, sensitivity, precision, calibration stability, model, accuracy, lower limit of quantification (LLOQ), recovery, linearity, limit laboratory investigations for quantitative measurement of a drug substance of detection, reproducibility, and ruggedness, are included in the basic parameters of validation.<sup>1-5</sup> Quality control labs use the results of established bioanalytical techniques to verify the identification, potency, quality, purity, and bioavailability of pharmaceutical goods. In order to ensure inter-laboratory dependability, it is crucial to test the bioanalytical method or methods at each lab and provide appropriate validation information for various labs when a study calls for sample analysis in numerous labs. Current bioanalytical techniques and procedures are frequently appropriately adjusted to meet the needs of an analytical process. Therefore, in order to obtain accurate data that can be sufficiently inferred, it is crucial to establish well-defined and validated bioanalytical procedures.<sup>6-8</sup> Matrix of biological concentrations of frequently have low drug concentrations and a variety of high endogenous chemicals. Occasionally, the endogenous substances share structural similarities with the medicine that needs to be assessed. A proper extraction process must be used to isolate the medication in a pure state for analysis. Modern analytical tools and extraction methods led to the development and validation of sophisticated bioanalytical techniques. Drugs and their metabolites can be identified and quantitatively determined in biological fluids, especially plasma, serum, or urine, using high performance liquid chromatography (HPLC) analysis. Good detector selection, a suitable stationary phase, eluents, and a suitable protocol during separation are all necessary for HPLC. The most adaptable detector in HPLC is the UV/VIS detector.

## METHOD DEVELOPMENT

When developing new drugs, a methodical approach is crucial. Method development includes of three crucial, interconnected elements: sample preparation, analyte separation, and analyte detection. Sample Pretreatment: If the analyte is protein-bound, serum and plasma samples do not need to be pretreated. In these situations, one of the following techniques is able to be followed. To get the sample's pH down to 3 or up to 9, use 0.1M or more of acids or bases. The resultant supernatant should be separated and used as the extraction sample. Using a polar solvent like acetonitrile, methanol, or acetone in a 1:2 ratio, precipitate the proteins from the biological fluid by centrifugation. Then, use the supernatant for extraction. Proteins can be precipitated by treating the biological fluid with acids or inorganic salts such zinc sulfate, ammonium sulfate, sodium sulfate, trichloroacetic acid, perchloric acid, or formic acid. Centrifuge the resultant supernatant, dilute it with water or buffer, sonicate it for 15 minutes, adjust its pH, and use the supernatant used in the extraction process.<sup>9</sup> An essential first step in the study of medications and their metabolites is sample preparation. bioanalytic research. Proteins and other endogenous and exogenous materials may interfere with the analyte in biological samples. The goal of sample preparation is to remove any potential undesirable components from the analyte of interest without causing a sizable loss of analyte.<sup>10</sup>

### **Analyte extraction techniques for drug and metabolite separation from biological samples:**

Traditional methods for removing analytes from biological matrices include (a) liquid-liquid extraction (LLE), (b) solid-phase extraction (SPE), and (c) plasma protein precipitation.

**a) Liquid-Liquid Extraction (LLE):-** LLE is a technique that uses water and an immiscible organic solvent to separate components in a combination. Analyte separation happens



according to its partition coefficient between two immiscible liquids, and extraction can be carried out with the use of an appropriate solvent. When compared to alternative approaches, the LLE process is straightforward, quick, and reasonably priced. The majority of medications may be recovered up to 90% of the time using several continuous extraction techniques. Add an immiscible after dissolving the component mixture in an appropriate solvent. solvent with the first solvent. After properly mixing the ingredients, put aside to allow the two insoluble solvents to separate into layers.<sup>20</sup> The partition coefficients of the two immiscible solvents will determine how the mixture's constituents are divided between them. After separating the two layers of immiscible solvents, move and separate the component from each solvent. Hydrophilic molecules enter the aqueous phase upon extraction, while hydrophobic compounds are present in the organic solvents. By allowing the solvent to evaporate and then reconstituting the residue with a small volume of a suitable solvent, ideally mobile phase, non-polar analytes that have been extracted into the organic phase can be readily recovered. Direct injection of polar analytes that have been extracted into the aqueous phase into a column in reverse phase (RP). Occasionally, the extraction process calls for pH control of the materials. Because the evaporation process uses a high temperature, the approach is not appropriate for thermolabile chemicals.<sup>10-20</sup>

**b) Solid Phase Extraction (SPE):-** Analyte isolation and concentration in trace levels in a range of sample matrices can be accomplished with SPE, a popular and efficient approach. With To increase analyte sensitivity, SPE can limit the final sample amount and lower the level of interferences. A tiny, disposable plastic column or cartridge containing 0.1 to 0.5 g of sorbent—typically RP material (C18 or C8)—can yield a higher analyte recovery. The relevant components

might either stay in the liquid phase or be preferentially adsorbed on the solid. Washing with the right solvent can selectively desorb the desired analyte if it has been adsorbed on the solid phase. The component of interest can be recovered by evaporation or concentration if it is still in a liquid phase. or the process of recrystallization. High density materials are challenging to extract with SPE, and the process takes longer.<sup>20</sup>

**c) Protein precipitation (PP):-** Protein precipitation is a fairly basic method for removing the analyte from plasma or blood. The principal prerequisite for According to this method, the analyte must dissolve easily in the reconstituting solvent. This method uses salts (ammonium sulphate) or acids (trichloroacetic acid and perchloric acid) in combination with organic solvents (methanol, ethanol, acetone, and acetonitrile) to prepare the sample via protein precipitation. Following precipitation, the sample is centrifuged, and the analyte is separated into the supernatant. Since it yields a clear supernatant that is appropriate for direct injection, methanol is the solvent of choice. PP can be used to extract both hydrophilic and hydrophobic materials. PP has the potential to jam the column.<sup>21-22</sup>

#### **Detection of analyte**

#### **HPLC Instrumentation:**

**HPLC Equipment:-** Biochemistry and analysis employ high-performance liquid chromatography (HPLC). To distinguish, recognize, and measure the active ingredients. A pump, injector, column, detector, integrator, and display system make up HPLC equipment. The column where separation takes place is the central component of the system. Since the stationary phase is made up of porous particles that are micron in size, a high-pressure pump is needed to transfer the mobile phase through the column. A tiny volume of the sample to be examined is added to the mobile phase stream. The detector displays the compounds' retention periods. The retention time is the



moment at which a particular analyte elutes or emerges from the end of the column.<sup>23-24</sup>

**Sample Injection:-** When the mobile phase is flowing or halted, septum injectors can be used to inject the sample solution. Results can be replicated with a new, sophisticated rotary valve and loop injector.<sup>24</sup>

### **Bioanalytical Method Validation:**

Regulatory bodies require validation. Showing the dependability of a specific technique created for the quantitative measurement of an analyte in a given biological matrix is the primary goal of method validation. When creating and implementing a bioanalytical method for the first time, when developing and implementing an analytical method for the analysis of a new drug entity, or when modifying an existing assay method-for example, by adding metabolites to an existing assay for drug quantification complete validation is required.<sup>6</sup>

### **Validation Parameters**

The fundamental factors for validating a chemical test include all standards for assessing the quality of the data, including selectivity, Precision, accuracy, recovery, linearity, calibration model, lower limit of quantification (LLOQ), limit of detection (LOD), stability, reproducibility, and ruggedness.<sup>25-27</sup>

**1. Selectivity:** - The capacity of an analytical technique to distinguish and measure the analyte in the presence of other components in the example. A biological matrix may contain endogenous matrix constituents, metabolites, breakdown products, and, in the case of the study, concurrent medications and xenobiotics as interfering chemicals. Analyzing blank samples of the relevant biological matrix from a minimum of six sources is necessary for selectivity. Selectivity should be guaranteed at LLOQ, and each blank should be examined for interference from other drugs.<sup>3-4</sup>

**2. Accuracy:** - An analytical method's accuracy is defined by how closely the mean test results produced by the technique to determine the analyte's actual value (concentration). Using at least six determinations per concentration, accuracy should be assessed for a minimum of three concentrations within the anticipated concentration range. With the exception of LLOQ, where it shouldn't vary by more than 20%, the mean should be within 15% of the actual value. The accuracy is determined by this mean deviation from the actual value.<sup>4</sup>

**3. Precision:** - An analytical method's precision refers to how closely each analyte measure is measured. when several aliquots of a single homogeneous volume of biological matrix are subjected to the process repeatedly. Precision during a single analytical run is known as intraday precision. Time-based interday precision measurements may use various analysts, tools, chemicals, and labs. A minimum of three concentrations within the anticipated range of concentrations, with five determinations per concentration, should be used to quantify precision. With the exception of the LLOQ, which should not exceed 20% of the CV, the precision calculated at each concentration level should not be greater than 15% of the CV.<sup>4</sup>

**4. Recovery:** - In an experiment, recovery of an analyte is the detector response derived from a quantity of the detector response for the actual concentration of the pure authentic standard, in contrast to the analyte that was introduced to and removed from the biological matrix. Three concentrations (low, medium, and high) should be used in recovery tests, and un-extracted standards representing 100% recovery should be used. Analyte recovery does not have to be 100%, but it should be consistent, accurate, and repeatable in terms of both analyte and internal standard recovery.<sup>4</sup>



**5. Linearity:-** This concept is the connection between the instrument's reaction to known analyte concentrations. The ability of the procedure to produce test findings that are exactly proportionate to the analyte concentration in the sample is measured by linearity.

**6. Calibration curve:-** It is the connection between known analyte concentrations and experimental response values. A calibration curve ought to be created by adding known analyte concentrations to the same biological matrix that will be used for the planned investigation. The response from both matrices should be compared if there is insufficient blank sample available, such as in the case of cerebrospinal fluid, where 0.9% NaCl can be used as the calibration matrix. The concentration range anticipated in a given investigation should be the foundation for selecting the standards' concentration. A blank sample (matrix sample processed without an internal standard) should be included in a calibration curve. 5-8 non-zero samples that span the anticipated range of the analyte, including LLOQ, and a zero sample (matrix sample processed with internal standard). Using appropriate weighting and statistical tests for goodness-of-fit, the simplest model that adequately captures the concentration response relationship should be used to build the calibration curve. When creating a calibration curve, the following requirements must be fulfilled: 15% departure of standards other than LLOQ from actual concentrations and 20% variance of LLOQ from actual concentrations. The LLOQ and the calibration standard at the maximum concentration shall be among the minimum of four of the six non-zero standards that satisfy the aforementioned requirements.<sup>4</sup>

**7. Detection Limit (LOD):-** It is the minimum detectable amount of analyte in a sample, while it is not always measured in the specified experimental setup.<sup>4</sup>

**8. Quantification's Lower Limit (LLOQ):-** It is the smallest concentration of analyte in a sample that can be identified but must be quantified with sufficient precision and accuracy under the specified experimental circumstances. If the following conditions are satisfied, the analyte response must be at least five times that of the blank response in order for the lowest standard on the calibration curve to be accepted as LLOQ. The analyte peak should be distinct, discrete, and repeatable with an accuracy of 80–120% and a precision of no more than 20%.<sup>4</sup>

**9. Reproducibility:-** This quality conveys the accuracy of laboratory-to-laboratory investigations, which are typically used to standardize methods. Only when a procedure is intended to be utilized in several laboratories does reproducibility need to be examined.<sup>28</sup>

**10. Ruggedness:-** (Robustness) Ruggedness is a metric that quantifies a method's vulnerability to potential minor modifications. because of temperature, mobile phase composition, pH, and other factors during normal analysis. If a procedure is meant to be transferred to another lab, its robustness should be examined. Although robustness is not required for complete validation, it would be beneficial for method creation since potential issues during validation are frequently identified beforehand.<sup>5</sup>

**Sample quality control:-** QC samples at three concentration levels in duplicate (one close to the three-x LLOQ, Each test run should include one in the mid-range and one in the high end. At least 5% of the total samples in a run or six QCs in total, whichever is higher, should be the minimum number of QCs.<sup>4</sup>

**Criteria for acceptance:** The QC sample results serve as the foundation for approving or disapproving a run. At least Four of every six ought to fall within 15% of their corresponding nominal values. Though not both at the same concentration, two of the six might be below 15%.<sup>4</sup>

**Repeat Analysis:-** It is necessary to specify the rules for repeat analysis. The explanation for the recurrence Analyte separation and resolution issues, equipment malfunctions, and sample processing problems should all be properly documented.<sup>4</sup>

## CONCLUSION

In recent years, adjustments to current bioanalytical With the advent of new biological matrix purification techniques and contemporary instrumentation, method development and validation have become commonplace. Existing bioanalytical techniques can be improved with the use of data produced by a well-developed, verified, and documented bioanalytical method. Improvements in bioanalytical techniques employing HPLC and validation help with pharmaceutical quality control and the faster acquisition of pharmacokinetic and toxicokinetic data. The development of bioanalytical methods aids in the identification, potency, purity, and bioavailability of pharmaceuticals. Consequently, the development and validation of bioanalytical HPLC methods is crucial for monitoring and controlling drug contaminants as well as for determining a drug concentration in pharmaceutical dosage forms and bulk.

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