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Development and Validation of Analytical Method for Quantitative Determination of Drugs by HPLC - A Review

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ABSTRACT
Development and validation of analytical method play an important role in the discovery, development and manufacturing of drugs. The number of drugs entered into the market every year; hence it is essential to develop an analytical methods for those drugs. Once it is developed, it is mandatory to validate the developed 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 system suitability, specificity, accuracy, precision, linearity & range, limit of deduction, limit of quantitation and robustness. Validation should be done as per regulatory guidelines, ICH guidelines. This article was prepared with an aim to review development and validation of analytical methods for quantitative determination of drugs.
Keywords: Analytical method development, Validation, Chromatography, HPLC.

I. INTRODUCTION

In every year, the number of drugs introduced into the market is increasing. These drugs may be either new entities or partial structural modification of the existing ones. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. There is a scope, therefore, to develop newer analytical methods for such drugs[1].

ANALYTIC METHODS

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs that we use. Methods are developed to support drug testing against specifications during manufacturing and quality release operations, as well as during long-term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance.

Analytical methods are often classified as classical / traditional and instrumental methods. In classical / traditional method, the analyses are carried out by separating the components of interest (the analytes) in a sample by precipitation, extraction or distillation. The separated components are then treated with different types of reagents for qualitative analysis based on the nature of analytes that yielded products which could be identified by their colors, odours, boiling and melting points, solubility study, optical activities or refractive indices. Gravimetric and titrimetric measurements are used to quantify the analytes present in the supplied sample.

In instrumental methods, scientific instruments like spectrometers, chromatographs, electrochemical instruments etc are used to investigate analytes which are available in the pharmacopoeias[2]. Modern analytical chemistry is dominated by sophisticated instrumentation. These methods are extremely sensitive and they require only small amount of sample material to provide precise and detailed information. Because of these reasons analytical methods are in widespread use, particularly in quantitation of drugs and medicines, product development and stability studies [3].

The various analytical techniques[4-6] have differing degrees of sensitivity, selectivity, cost of analysis, sophistication etc. For a given determination, analyst has to select the best procedure considering all these parameters.

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For proper selection of analytical method following criteria must be considered.

- The type of analysis required - molecular or elemental, occasional or routine.
- The nature of substance to be analyzed - Hygroscopic substances, radioactive substances, corrosive substances etc.
- The concentration range, which needs to be investigated.
- The accuracy required.
- The analytical instruments and other facilities available.
- The time required for complete analysis.

ANALYTICAL METHOD DEVELOPMENT

It is the process of selecting an accurate assay procedure to determine the composition of a drug formulation. It is the process of proving that an analytical method is acceptable for use in laboratory to measure the concentration of subsequent samples. The prerequisites for method development are qualified and calibrated instruments, documented methods, reliable reference standards, qualified analysts, sample selection and integrity and change control [7].

The **basic criteria for new method development** of drug analysis are as follows:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve some extraction and separation procedures and these may not be reliable.

METHOD VALIDATION

Analytical method development and validation are continuous and interconnected activities conducted throughout the drug development process. Method validation is defined as the "process of demonstrating that analytical procedures are suitable for their intended use".

The validation of an analytic method demonstrates the scientific soundness of the measurement or characterization. It is required to varying extents throughout the regulatory submission process. The validation practice demonstrates that an analytic method measures the correct substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to understand the behavior of the method and to establish the performance limits of the method.

In order to perform method validation, the laboratory should be following a written standard operating procedure (SOP) that describes the process of conducting method validation. The laboratory should be using qualified and calibrated instrumentation with a corresponding operating SOP. There should be a well-developed and documented test method in place and an approved protocol should be in place prior to the execution of any validation experiments.

The protocol is a plan that describes which method performance parameters will be tested, how the parameters will be assessed, and the acceptance criteria that will be applied. Finally, samples of active pharmaceutical ingredient (API) or drug product, placebos, and reference standards are needed to perform the validation experiments.

The advantages of the analytical method validation are as follows:

i. It builds a degree of confidence, not only for the developer but also to the user.
ii. Although the validation exercise may appear costly and time consuming, it results inexpensiveness, eliminates frustrating repetitions and leads to better time management in the end.
iii. Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

REGULATORY ASPECT OF METHOD DEVELOPMENT

International Conference on Harmonisation (ICH) guidelines recommend that stability indicating methods need to be used in determination of the assay of drug product in stability test sample. Stability-indicating methods, according to United States-Food and Drug Administration (US-FDA) stability guideline of 1998 are defined as validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other

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components of interest can be accurately measured without interference [8-10].

A lot of requirements that are currently prescribed by regulatory authorities deals with the approach to assure that the process at every stage must not impact the quality. The strategy for method development must be adequately specific and sensitive to check the known and unknown impurities. Also there is need to write the format in a systematic way so that the method can be reproduced by other analyst with other parameter such as robustness and ruggedness over a timeframe. In other words the method must be validated [11].

CHROMATOGRAPHY

Chromatography is one of the analytical methods used wildly in drug analysis. The importance of chromatography is increasing rapidly in pharmaceutical analysis. The exact differentiation, selective identification and quantitative determination of structurally closely related compounds are possible with chromatography. Another important field of application of chromatographic methods is the purity testing of final products and intermediates (detection of decomposition products and by-products).

Chromatography is used to separate the mixtures of substances into their components on the basis of their molecular structure and molecular composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. This difference in rates causes the separation of various components. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography). Among the various chromatographic techniques, Reversed Phase High Performance Liquid Chromatography (RP-HPLC) is an advanced technique which can give both qualitative and quantitative data of an analyte.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography[12] (HPLC) is basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres. That makes it much faster. All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts occurs because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

INSTRUMENTATION OF HPLC

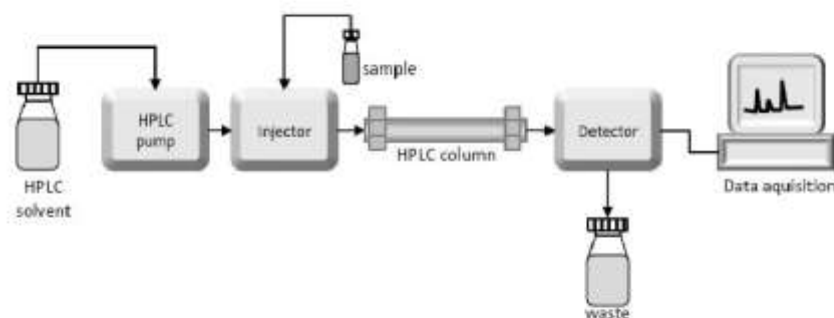

Figure 1.1 : A block diagram of HPLC

As shown in the schematic diagram in Fig.1.1, HPLC instrumentation[12] includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

Solvent Reservoir

Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

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Pump

A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

Sample Injector

The injector can be a single injection or an automated injection system. An injector for the HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

Detector

The HPLC detector, located at the end of the column detects the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

TYPES OF HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

Normal Phase HPLC:

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

Reversed Phase HPLC:

The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions and hence the more nonpolar the material is, the longer it will be retained[12-15].

Size-exclusion HPLC:

The column is filled with material having precisely controlled pore sizes, and the particles are separated according to their molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the pores of the packing particles and elute later.

Ion-Exchange HPLC:

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionisable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

VALIDATION PARAMETERS

There are various parameters[16-21] for validating an analytic method which confirm that the method is intended for its use for the determination of the analyte. The method performance parameters that are applicable to most methods are given below:

- System suitability
- Specificity
- Linearity and Range
- Accuracy
- Precision
- Robustness
- Limit of Detection (LOD)
- Limit of Quantification (LOQ)

System Suitability: System suitability testing is usually performed to decide whether a chromatographic system that is being utilized day to day in a routine manner in pharmaceutical laboratories is suitable for a definite analysis with quality. The parameters used in the system suitability test report are as follows:

Number of theoretical plates or Efficiency (N): This is a measure of the sharpness of the chromatogram peaks and therefore the efficiency of the column. This

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can be calculated in various ways, for example the United States Pharmacopeia (USP) uses the peak width at the base and the British Pharmacopeia (BP) at half the height.

Capacity factor (K): Capacity factor is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase.

Separation or Relative retention (α): Selectivity or separation factor is the measure of relative retention of a two component in a mixture. Selectivity is the ratio of the capacity factor of both the peaks and the ratio of the adjusted retention times. This describes the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

Resolution (Rs): Resolution is the parameter describing the separation power of the complete chromatographic system relative to the particular component of the mixture. It is defined as the ratio of the distance between two peak maxima. This is not only a measure of the separation between two peaks, but also the efficiency of the column.

Tailing factor (T): The tailing factor is a measure of peak tailing. It is defined as the distance from the front slope of the peak to the back slope divided by twice the distance from the center line of the peak to the front slope, with all measurements made at 5% of the maximum peak height. The acceptance criteria for System suitability parameters are shown in Table 1.1.

System suitability parameters	Acceptance criteria
Number of theoretical plates or Efficiency (N)	> 2000
Capacity factor (K)	< 1
Separation or Relative retention (α)	> 1.0
Resolution (Rs)	> 1.5
Tailing factor (T)	< 2
Relative Standard Deviation (RSD)	< 2

Table 1.1 Acceptance criteria for system suitability parameters

Specificity

One of the significant features of HPLC is its ability to generate signals free from interference. Specificity refers to the ability of the analytical method to differentiate and quantify the analyte in complex mixtures. Specificity can be assessed by measurement of the active pharmaceutical ingredient (API) in samples that are spiked with impurities or degradants, if available. If API related compounds are not available, drug can be stressed or force-degraded in order to produce degradation products. In chromatographic separations, apparent separation of degradants may be confirmed by peak purity determinations by photodiode array, mass purity determinations by mass spectroscopy (MS), or by confirming separation efficiency using alternate column chemistry. During forced degradation experiments, degradation is targeted at 5 to 20% degradation of the API, in order to avoid concerns about secondary degradation.

Linearity and range

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least-squares regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy: Comparison to a reference standard, Recovery of the analyte spiked into blank matrix and Standard addition of the analyte.

Precision

The precision of an analytical procedure represents the nearness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the similar analytical conditions. Samples may be analyzed on different days, by different analysts, on different instruments, or in different laboratories.

There are three levels of precision validation evaluations.

- **Repeatability**: Repeatability is a measure of precision under the same conditions, same analyst over a short period of time.
- **Intermediate precision**: Intermediate precision is a measure of precision within the same laboratory by different operators, using different instruments, and making measurements on different days.
- **Reproducibility**: Reproducibility assesses precision between two or more laboratories (inter-laboratory studies).

Robustness

Robustness is typically assessed by the effect of small deliberate changes to chromatographic methods on system suitability parameters such as peak retention, resolution, and efficiency. Experimental factors that are typically varied during method robustness evaluations include age of standards & sample preparations, sample extraction time, variations to pH of mobile phase, variation in mobile phase composition, analysis temperature, flow rate, column lot and/or manufacturer, type and use of filter against Centrifugation.

The limit of detection

The limit of detection (LOD) is determined by the analysis of samples with known concentration of analyte and by establishing a minimum level at which the analyte can reliably be detected, under the stated experimental conditions. The detection limit is generally expressed in the concentration of analyte (ppm) in the sample.

The acceptable approaches are visual evaluation, signal-to-noise ratio, standard deviation of the response, standard deviation of the slope of linearity plot.

The formula for calculating LOD is given in equation 1.1

$LOD = 3.3\ \delta / S$ --------------- 1.1;

Where, δ = standard deviation of intercepts of calibration curves.

S = the slope of linearity plot.

Limit of Quantification

Limit of quantification (LOQ) is the least concentration of drug in a sample which is estimated with appropriate precision and accuracy under the affirmed experimental conditions.

The formula for calculating LOQ is given in equation 1.2

$LOQ = 10\ \delta / S$ --------------- 1.2

Where, δ = standard deviation of response.

S = Mean of slopes of the calibration curves.

II. CONCLUSION

Analytical method development is the activity of selecting an accurate assay procedure to find out the composition of a formulation. Method development and validation are continuous and interrelated processes that measure a parameter as intended and establish the performance limits of the measurement. The primary objectives of development of analytical methods are for identification, purification and eventually to qualification any necessary drug etc. The development of analytical methods helps in understanding the critical process parameters and to reduce their effects on precision and accuracy. Validation is a necessary technique in the Pharmaceutical sector and that used to ensure that quality work is done in the process which supports the development of drugs and their formulations.

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