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DEVELOPMENT AND VALIDATION OF METHOD BY HPLC TECHNIQUES

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Abstract:
The primary focus of the review article is on general approaches and considerations toward development of chromatographic methods for separation, identification, and quantification of compounds, which may be applied within the various functions in the drug development continuum. This article also discusses the issues and parameters that must be considered in the validation of analytical methods. At the end of the review, a scope of the present research study is covered.
Keywords: *Introduction, Method development, Steps for HPLC method development, Method validation, Advantages of method validation and Parameters for validation.*

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INTRODUCTION:

Analytical method development and validation play a vital role in the discovery, development and manufacture of pharmaceuticals. Those pharmaceutical products that can be formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. Various analytical methodologies are employed for the determination of related components in pharmaceuticals. There is a great need for development of new analytical methods for quality evaluation of new emerging drugs.

Analytic method development and validation are key elements for any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This technical will focus on development and validation activities as applied to drug products.

Basic criteria for new method development of drug analysis:

- The drug or combination of drug 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 cumbersome extraction and separation procedures and these may not be reliable.

METHOD DEVELOPMENT:

Effective method development ensures that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. Method validation, required by regulatory agencies at certain stages of the drug approval process, is defined as the "process of demonstrating that analytical procedures are suitable for their intended use" [1-2].

Understanding of the physical and chemical characteristics of drug allows one to select the most appropriate high performance liquid chromatography method development from the available vast literature. Information concerning the sample, for example, molecular mass, structure and functionality, pKa values and UV spectra, solubility of compound should be compiled. The requirement of removal of insoluble impurities by filtration, centrifugation, dilution or concentration to control the concentration, extraction (liquid or solid phase), derivatization for detection etc. should be checked. For pure compound, the sample solubility should be identified whether it is organic solvent soluble or water soluble, as this helps to select the best mobile phase and column to be used in HPLC method development.

Method development in HPLC can be laborious and time consuming. Chromatographers may spend many hours trying to optimize a separation on a column to accomplish the goals. Even among reversed phase columns, there is astonishing diversity, owing to differences in both base silica and bonded phase characteristics. Many of these show unique selectivity. What is needed is a more informed decision making process for column selection that may be used before the chromatographer enters the laboratory. The method of column selection presented here involves a minimal investment in time initially, with the potential of saving many hours in the laboratory.

Analytic 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 that support safety and characterization studies or evaluations of drug performance are also to be evaluated. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate the potential degradation of the API in the presence of formulation excipients [3].

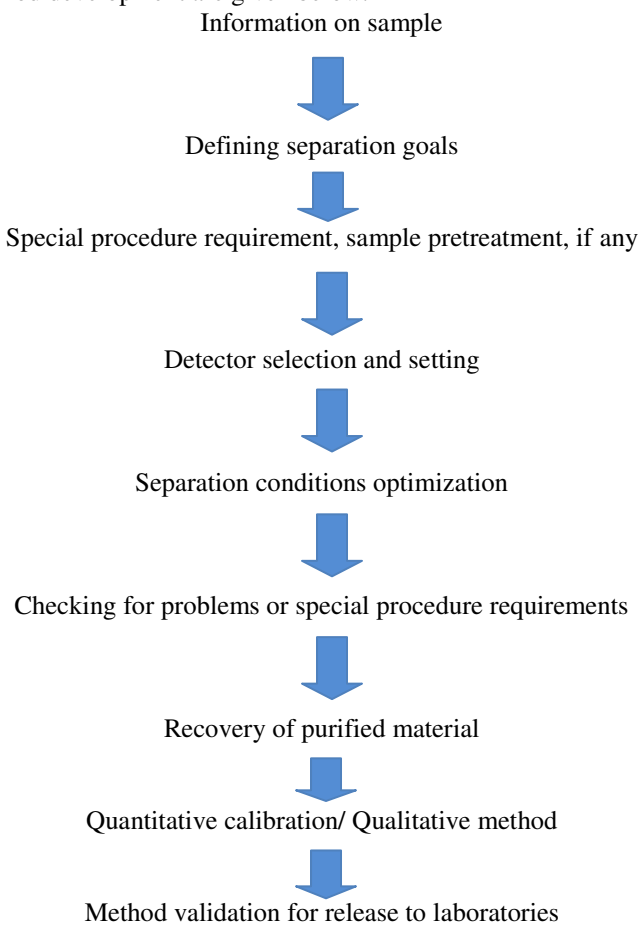
The three critical components for a HPLC method are: sample preparation (% organic, pH, shaking/sonication, sample size, sample age) analysis conditions (%organic, pH, flow rate, temperature, wavelength, and column age), and standardization (integration, wavelength, standard concentration, and response factor correction). During the preliminary

method development stage, all individual components should be investigated before the final method optimization. This gives the scientist a chance to critically evaluate the method performance in each component and streamline the final method optimization [4]. The percentage of time spent on each stage is proposed to ensure the scientist will allocate sufficient time to different steps. In this approach, the three critical components for a HPLC method (sample preparation, HPLC analysis and standardization) will first be investigated individually [5-7].

The degraded drug samples obtained are subjected to preliminary chromatographic separation to study the number and types of degradation products formed under various conditions [8]. Scouting experiments are run and then conditions are chosen for further optimization [9]. Resolving power, specificity, and speed are key chromatographic method attributes to keep in mind during method development [10]. Selectivity can be manipulated by combination of different factors like solvent composition, type of stationary phase, mobile phase, buffers and pH. Changing solvents and stationary phases are the most comfortable approaches to achieve the separation. The proper range of pH is an important tool for separation of ionizable compounds. Acidic compounds are retained at low pH while basic compounds are more retained at higher pH. The neutral compounds remain unaffected. The pH range 4-8 is not generally employed because slight change in pH in this range would result in a dramatic shift in retention time. However, by operating at pH extremes (2-4 or 8-10), not only is there a 10-30 fold difference in retention time that can be exploited in method development but also the method can be made more robust which is a desirable outcome with validation in minutes [11,12].

STEPS FOR HPLC METHOD DEVELOPMENT

Various steps for HPLC method development are given below.



Method development should be based on several considerations. It is preferable to have maximum sample information to make development fast and desired for intended analytical method application, physical and chemical properties are most preferable as primary information. Moreover, separation goal needs to define at beginning so; appropriate method can be developed for the purpose. An LC method development is very huge area for even pharmaceuticals with regulatory requirement of international standards. So, prior to method validation and usage at quality control many aspects need to focus as per ICH guidelines.

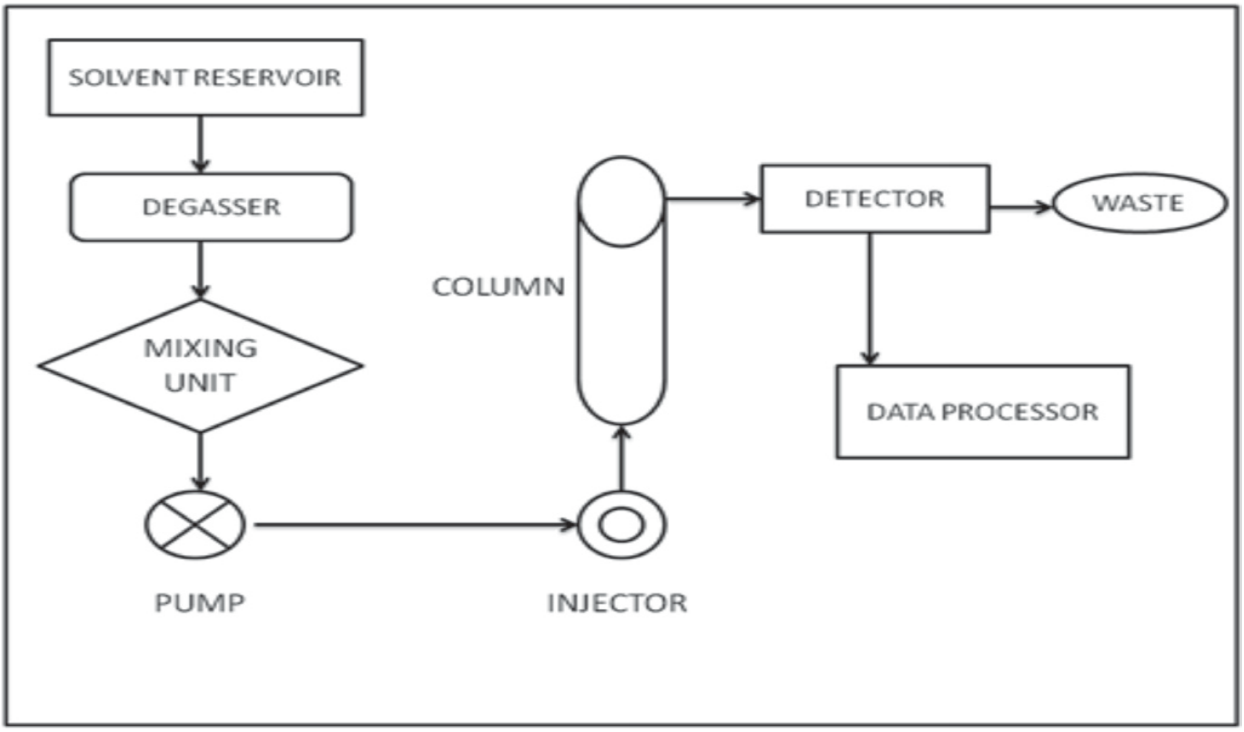
Sample information

- Number of compounds present
- Chemical structure of compounds
- Chemical nature
- Molecular weight of compounds
- pKa Value(s) of compounds
- Sample solubility
- Sample stability and storage
- Concentration range of compounds in sample
- UV spectra of compounds or properties for detection of compounds

METHOD VALIDATION

The need to validate an analytical or bioanalytical method is encountered by analysis in the pharmaceutical industry on an almost daily basis, because adequately validated methods are a necessity for approvable regulatory filings. What constitutes a validated method, however, is subject to analyst interpretation because there is no universally accepted industry practice for assay validation.

The validation of an analytical method demonstrates the scientific soundness of the measurement or characterization. It is required to provide validation data throughout the regulatory submission process. The validation practice demonstrates that an analytical 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 [13,14]. The goal is to identify the critical parameters and to establish acceptance criteria for method system suitability.



ADVANTAGES OF ANALYTICAL METHOD VALIDATION

The advantages of the analytical method validation are as follow:

- The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user.
- Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating repetitions and leads to better time management in the end.
- 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.

Validation is defined by the International Organization for Standardization (ISO) as "verification, where the specified requirements are adequate for an intended use", where the term verification is defined as "provision of objective evidence that a given item fulfills specified requirements" [15]. The applicability and scope of an analytical method should be defined before starting the validation process. It includes defining the analytes, concentration range, description of equipment and procedures, validation level and criteria required. The validation range is **d**efined by IUPAC as "the interval of analyte concentration within which the method can be regarded as validated" [16,17]. This range is not the highest and lowest possible levels of the analyte that can be determined by the method. Instead, it is defined on the basis of the intended purpose of the method [18,19]. The method can be validated for use as a screening (qualitative), semi-quantitative (5-10ppm) or quantitative method. It can also be validated for use on single equipment, different equipments in the laboratory, different laboratories or even for international use at different climatic and environmental conditions.

PARAMETERS FOR VALIDATION

The criteria of each type of validation will ofcourse be different with the validation level required [20]. The various validation parameters include linearity, accuracy, precision, ruggedness, robustness, LOD, LOQ and selectivity or specificity.

1. Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration of the analyte in the sample [21,22]. It is essential to determine the useful range at which the instrumental response is proportional to the analyte concentration. Generally, a value of correlation coefficient (r) > 0.998 is considered as the evidence of an acceptable ft of the data to the regression line [23]. Significance of deviation of intercept of calibration line from the origin can be evaluated statistically by determining confidence limits for the intercept, generally at 95% level [24,25].

Linearity is determined by a series of three to six injections of five or more standards. Peak areas (or heights) of the calibration standards are usually plotted on the Y-axis against the nominal standard concentration, and the linearity of the plotted curve is evaluated through the value of the correlation coefficient (r). Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used to evaluate the linearity of the plot. The first one is to plot deviations from regression line versus concentration or versus logarithm of concentration. For linear ranges, the deviations should be equally distributed between positive and negative values. Another approach is to divide signal data by their respective concentrations yielding the relative responses. A graph is plotted with the relative responses on Y-axis and the corresponding concentrations on X-axis on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn in the graph corresponding to, for example, 95 % and 105 % of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 % line [26,27].

2. Accuracy

Accuracy is defined by ISO as "closeness of agreement between a measured quantity value and a true quantity value". It is a qualitative characteristic that cannot be expressed as a numerical value. It has an inverse relation to both random and systematic errors, where higher accuracy means lower errors [28]. Accuracy is evaluated by analyzing test drug at different concentration levels. Typically, known amounts of related substances and the drug substance in placebo are spiked to prepare an accuracy sample of known concentration of related substance. Samples are prepared in triplicate. ICH recommends accuracy

evaluation using a minimum of nine determinations over a minimum of three concentration levels covering the range specified. It is determined by comparing the found concentration with the added concentration. The methods of determining accuracy include analysis of analysis of known purity (reference material), comparison of results of the proposed analytical procedure with those of a second well characterized procedure and standard addition method. The accuracy may also be inferred once precision, linearity and specificity have been established [29,30].

3. Precision

It expresses closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility [31]. Repeatability is also referred to as intra-assay precision. It is a measure of precision of analysis in one laboratory by one operator using one piece of equipment over a relatively short time-span. It is degree of agreement of results when experimental conditions are maintained as constant as possible, and expressed as RSD of replicate values. ICH recommends a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/three replicates as in the accuracy experiment), or a minimum of six determinations at 100% of the test concentration for evaluation of repeatability which should be reported as standard deviation, relative standard deviation (coefficient of variation) or confidence interval. ICH defines intermediate precision as long-term variability of the measurement process and is determined by comparing the results of a method run within a single laboratory over a number of weeks. It is also called as intraday precision [32]. It reflects discrepancies in results obtained by different operators, from different instruments, with standards and reagents from different suppliers, with columns from different batches or a combination of these but in the same laboratory. The objective of intermediate precision validation is to verify that the method will provide same results in the same laboratory once the development phase is over. Reproducibility expresses precision of analysis of the same sample by different analysts in different laboratories using operational and environmental conditions that may differ but are still within the specified parameters of the method [33]. The objective is to verify that the method will provide the same results despite differences in room temperature and humidity, variedly experienced operators, different characteristics of equipments (e.g., delay Volume of an HPLC system), variations in material and instrument conditions (e.g. in HPLC, mobile phase composition, pH, flow rate of mobile phase), equipments and consumables of different ages, columns from different suppliers or different batches and solvents, reagents and other material with different quality [34].

4. Selectivity and Specificity

Selectivity and specificity are sometimes used interchangeably to describe the same concept in method validation. Selectivity of an analytical method is defined by the ISO as "property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measured such that the values of each measured are independent of other measured or other quantities in the phenomenon, body, or substance being investigated". Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. The specificity of a test method is determined by comparing test results from an analysis of samples containing impurities, degradation products, or placebo ingredients with those obtained from an analysis of samples without impurities, degradation products, or placebo ingredients. Specificity can best be demonstrated by resolution between the analyte peak and the other closely eluting peak [35].

5. Detection limit (LOD) and Quantitation limit (LOQ)

LOD of an analytical procedure is the lowest concentration of an analyte in a sample which can be detected but not necessarily quantitated as an exact value where as LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. ICH guidelines describe three methods for determining LOD and LOQ that include:

a. Visual evaluation:

It may be used for both non instrumental and instrumental methods. The LOD and LOQ is determined by analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected or quantified with acceptable accuracy and precision respectively.

b. Signal to noise ratio approach:

This method can only be applied to analytical procedures which exhibit baseline noise. It is determined by comparing measured signals from samples of known low concentrations of analyte with those of blank samples and establishing minimum

concentration at which the analyte can be reliably detected. A S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation (RSD) ≤10%) LOQ, a S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD) ≤ 3%) as illustrated in Figure3.12.
The LOD and LOQ may be expressed as: LOD = 3.3 x σ/S and LOQ = 10 x σ/S
Where σ = the standard deviation of the response
S = the slope of the calibration curve of analyte

The slope S may be estimated from the calibration curve of the analyte. The value of σ may be taken from as standard deviation of analytical background responses of an appropriate number of blank samples. Alternatively, it can be taken as residual standard deviation of a regression line or standard deviation of y-intercepts if regression lines are obtained for samples containing an analyte in the range of LOD and LOQ.

6. Range

The range of an analytical procedure is the interval between the upper and lower concentrations of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity [36]. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range of an analytical method varies with its intended purpose. It is generally 80-120% of the test concentration for the assay of a drug substance or a finished (drug) product, 70-130% of the test concentration for content uniformity, ±20% over the specified range for dissolution testing, reporting level of an impurity to 120% of the specification for the determination of an impurity. It should commensurate with LOD or LOQ (the control level of impurities), for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

7. **Robustness**

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of method robustness, a number of chromatographic parameters, for example, flow rate, column temperature, injection Volume, detection wavelength and mobile phase composition are varied within a realistic range and quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range. Obtaining data on these effects will allow to judge whether a method needs to be revalidated when one or more of parameters are changed, for example to compensate for column performance over time [37,38]. Variation in method conditions for robustness should be small and reflect typical day-to-day variation. Critical parameters are identified during the method development process. Only these critical method parameters should be investigated for robustness. Common critical method parameters can be divided into two categories. The HPLC conditions include HPLC column (lot, age, and brand), Mobile-phase composition (pH ± 0.05 unit, organic content ± 2%) and HPLC instrument (dwell Volume, detection wavelength ± 2 nm, column temperature ± 5°C and flow rate). The sample preparation variations include sample solvent (pH ± 0.05 unit, organic content ± 2%), sample preparation procedure (shaking time, different membrane filters) and HPLC solution stability. The variations in chromatographic parameters for robustness study are given in Table-1.

Table-1: Variations in chromatographic parameters for robustness study

S. No.	Robustness	Change
1.	Detection wavelength	±5 nm
2.	Flow rate	±0.05ml/min
3.	Buffer pH	±0.1 unit
4.	Mobile phase	±2 ml
5.	Column	Different brand or batch number

CONCLUSION:

Pharmaceutical analytical chemistry is an important part in monitoring the quality of pharmaceutical products for safety and efficacy. With the advancement in synthetic organic chemistry and other branches of chemistry including bioanalytical sciences and biotechnology, the scope of analytical chemistry has enhanced to much higher levels. The emphasis in current use of analytical methods particularly involving advance analytical technology has made it possible not only to evaluate the potency of active ingredients in dosage forms and APIs but

also to characterize, elucidate, identify and quantify

important constituents like active moiety, impurities, metabolites, isomers, chiral components and prediction of the degradations likely impurities being generated. Pharmacopoeias rely more on instrumental techniques rather than the classical wet chemistry method. A modest attempt has been made to develop validated analytical methods for the determination of single or combined dosage form. Estimation of degradants generated during formulation and storage of finished products using a UPLC technique.

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