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REVIEW ON ANALYTICAL METHOD DEVELOPMENT AND VALIDATION BY RP-HPLC

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Abstract:

Analytical method development, validation, and transfer are essential elements of any pharmaceutical development program. Effective method development confirms that laboratory resources are optimized, while methods meet the objectives required at each stage of drug development. High performance liquid chromatography is most accurate methods extensively used for the qualitative and quantitative analysis of drug product. Analytical method development and validation play vital role in the drug discovery, Drug development and manufacture of pharmaceuticals. It includes detection of the purity and toxicity of a drug substance. A number of chromatographic parameters have been evaluated in order to optimize the methods in the analysis of method development in HPLC. An appropriate mobile phase, column, column temperature, wavelength, and gradient are developed.

Key Words: *Analytical method validation, ICH, HPLC, Method Validation, Regulatory Requirements.*

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INTRODUCTION:

The number of drugs presented into the market is growing every year. These drugs may be either new entities or partial structural variation of the existing one. The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in succeeding this goal [1]. Analytical method development, validation, and transfer are essential elements of any pharmaceutical development program. Effective method development confirms 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 stage of the drug approval process, is defined as "Process of signifying that analytical procedures are suitable for their intended use" [2]

Method validation is the process used to confirm that the analytical procedure employed for a particular test is suitable for its proposed use. Results from method validation can be used to critic the quality, reliability and consistency of analytical results; it is an essential part of any good analytical practice [8]. High performance liquid chromatography is most accurate methods extensively used for the qualitative and quantitative analysis of drug product. Analytical method development and validation play vital role in the drug discovery, Drug development and manufacture of pharmaceuticals. It includes detection of the purity and toxicity of a drug substance. A number of chromatographic parameters have been evaluated in order to optimize the methods in the analysis of method development in HPLC. An appropriate mobile phase, column, column temperature, wavelength, and gradient are developed [9, 10].

High Performance Liquid Chromatography (HPLC) is one of the most widely used analytical techniques in industry. It is used to separate and analyse compounds through the mass-transfer of analytes between stationary phase and mobile phase. The technique of HPLC uses a liquid mobile phase to separate the components of a mixture. The components themselves are first dissolved in a solvent and then forced to flow (via the mobile phase) through a column (stationary phase) under high pressure. The mixture is resolved into its components within the column and the amount of resolution is dependent upon the interaction between the solute components and the column stationary phase and liquid phase. The interaction of the solute with the mobile and stationary phases can be manipulated through different choices of both solvent and stationary phases. HPLC can be divided into two broad categories, normal phase and reversed phase. For normal phase chromatography, a polar stationary phase (usually silica) is used to retain analytes, which are polar and mobile phase is non-polar (heptane, chloroform, hexane, cyclohexane), whilst reversed phase chromatographic separations are based upon forces between non-polar compounds and non-polar functional groups, which are bonded to the silica support and the mobile phase is polar (methanol, acetonitrile, water or buffer). The majority of applications today are based on reversed phase separations [9, 11, 12, 13].

NEED OF ANALYTICAL METHOD DEVELOPMENT AND VALIDATION:

- Available method may be too expensive, time consuming or energy intensive, or that may not be easily automated.
- Existing method may be too much error, contamination prone or they may be unreliable.
- There may be need for an alternative method to confirm, for legal or scientific reasons, analytical data originally obtained by existing methods.
- There may not be a suitable method for a particular analyte in the specific sample matrix.
- Existing method may not provide adequate sensitivity.
- For regulatory requirements it is required [10].

The need of validation of the analytical method development and validation emerged due to international competition, maintaining the standard of products in high commercial and market valve and ethical reasons. Various International Regulatory Agencies have set the standard and fixed the protocol to match the reference for granting approval, authentication and registration [3].

It is essential to employ well-characterized and fully validated analytical methods to yield reliable results in the laboratories while analyzing the registration batch and accelerated stability testing samples. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. In these instances, specific validation criteria may need to

be developed for each analyte. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site and commercial batch for people consumption, it is necessary to validate the analytical method(s) as per ICH guidelines and to provide proper validation information for different sites and different parameter and to establish inter and intra laboratory reliability [14].

BASIC DRUG SELECITION CRITERIA FOR NEW ANALYTICAL METHOD DEVELOPMENT:

- 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 method for the quantization 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 [15, 16, 17].

CURRENT BEST PRACTICE IN ANALYTICAL METHOD VALIDATION

Validation should not be implicit separately from the development of a method. Therefore entire process of analytical method development and validation can be considered in an entirety as represented in the general scheme. The method's performance characteristics should be based on the proposed use of the method. These include analyte, its expected concentration, sample matrix, possible inquisitive substances, regulatory requirement, application (qualitative/quantitative), necessity for robustness, detection and quantization limit, accuracy and precision expectation, different types of equipment and the locations where the method will be run, ability requirements for analyst, etc. Before an instrument is used to validate a method, its performance should be verified. But still after method development it needs to be validated as per requirement which gives certain level of confidence for its intended use [15].

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 [15].

ANALYTICAL METHOD DEVELOPMENT:

Analytical method development finally results in official test methods. Consequently quality control laboratories used these methods to check the efficacy, identity, purity, safety as well as performance of products of the drug. Regulatory authorities give utmost importance on analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods.

Development in scientific and concrete analytical methods has been resulted from the advancements of analytical instruments. The improvements of the analytical method development and analytical instruments have reduced the time and cost of analysis and enhanced precision and accuracy. Techniques pertaining to analysis are developed and validated for active pharmaceutical ingredients, excipients, related substances, drug products, degradation products and, residual solvents, etc. Resulting which become an integral part of the required necessities for regulatory organization.

The numerous novel drugs are being introduced and are constantly growing day by day. Therefore it is absolutely imperative to evolve novel methods and introduced them for controlling their quality. Modern pharmaceutical analysis needs the following requirements.

1. The analysis should take a minimal time and should be economical.

2. The accuracy of the analysis must accept the guidelines of Pharmacopoeia.
3. The chosen method should be precise and selective [17].

STEPS IN METHOD DEVELOPMENT [18]

- Physicochemical Properties of drug molecule
- Selection of Chromatographic conditions
- Developing the approach of analysis
- Preparation of sample
- Method Optimization
- Method validation

A) PHYSICOCHEMICAL PROPERTIES OF DRUG MOLECULE:

a) Chemical properties:

Chemical structure of the known and expected product chemical structures are good inputs for initiating the method development and it will give scientific approach for the method development. Draw the comparative difference between impurities, starting materials, by-products intermediate and degradation products with final products. Solubility study at different pH values information for all targeted molecules is best inputes for selecting the common diluent for all molecules. Slecting the polar/ non polar HPLC column the information of polarity of molecules is important.

b) pH and pKa value of compound

The nature and polarity of the compound is assumed based on pH and pKa values. The compound is half ionized, when pH is equivalent to pKa. Almost all the pH related change occurs within the ± 1.5 units of the pKa values. Outsides the range the compound is either ionized or non-ionized, and its retention does not change much with pH [19].

B) **SELECTION OF CHROMATOGRAPHIC CONDITIONS:**

During initial method development, a set of initial conditions (column, mobile phase and detectors) is selected. In most cases, these are based on reversed phase separations on a C18 column with UV detection. A decision on developing either an isocratic or a gradient method should be made at this point.

a) Selection of column:

The principle part of a HPLC system is the column. Changing a columnwill have the greast effect on the resolution of analytes during method development. An appropriately selected column can produce a good chromatographic separation and it provides accurate and reliable analysis [10].

Reversed phase separation employ a polar eluent and a nonpolar (hydrophobic) stationary phase. The hydrophobic layer (or phase) is bonded or coated onto a rigid support that can endure the high pressure commonly used in HPLC. Until recently, about 80% of all HPLC methods specified silica-based stationary phases [20].

- C18 and C8 bonded phases are the best for initial rapid method development with typical sample types
- Choosing the most sample appropriate bonded phase and using special, targeted bonded phases, such as SB-Aq for polar, difficult to retain compounds can decreases method development time.
- Rapid resolution columns are needed to reduce the method development time.
- Rapid resolution columns reduce both gradient and isocratic analysis time and permit high throughput rapid analysis [21].

b) Chromatographic conditions :

Selection of appropriate chromatographic conditions is the next step in the method development. This includes selection of temperature, selection of composition of mobile phase, pH of mobile phase and flow rate etc. A decision of developing either an isocratic or a gradient method should be made at this point. In most cases, these are based on reversed-phase separations on a C18 column with UV detection [22].

c) Optimization of mobile phase:

When samples contain ionizable compounds, the mobile phase pH can be one of the most important variables in the controls in the control of retention in a reversed-phase HPLC (RP-HPLC) separation. However, if it is not controlled properly, pH can be a source of many problems. Since most compounds analyzed by RP-HPLC contain one or more acidic or basic functional groups, most mobile phases require pH control. For this reason, buffers are widely used [23].

In RP chromatography, water and buffer are weak solvent and CAN, methanol, THF is strong solvents and this are most widely use solvents.
Other considerations when selecting solvents :

Methanol – High viscosity may limit use of smaller particle size or longer columns at elevated flow rates.
Acetonitrile – Relatively high cost.
THF – UV absorbance at low wavelengths; high viscosity [24].

d) Column temperature:
The use of temperature in HPLC method development presents a challenge because it can have unpredictable effects on selectivity.
The use of elevated temperatures will :
Reduce mobile phase viscosity and back-pressure. This can allow you to operate at higher flow rates, or to use longer columns smaller particle sizes.
Reduce elution time.
Improve method reproducibility (as opposed to operating at room temperature).

However, it is impossible to determine if the use of elevated temperatures will help or hinder a specific separation. For complex separations, improvements in one portion of the chromatogram are almost always accompanied by decreases in another part of the same chromatogram [25].

e) Selection of wavelength and detector :
All listed molecules UV/Visible and FT-1R spectrums are required to select the UV detector nm for all molecules. FTIR spectral data is the main source for understanding the functional groups activity [10].

C) SAMPLE PREPARATION:
The sample should ideally be dissolved in the initial mobile phase. If this is not possible due to stability or solubility problems, formic acid, acetic acid or salt can be added to the sample to increase solubility. These additives do not usually effect the separation so long as the volume of the sample loaded is small compared to the column volume. The only effect when large sample volumes are applied may be an extra peak or two eluting in the void volume after sample injection. Sample preparation is an essential part of HPLC analysis, intended to provide a reproducible and homogenous solution that is suitable for injection onto the column. The aim of sample preparation is a sample aliquot that, is relatively free of interferences, Will not damage the column, and Is compatible with the intended HPLC method that is, the sample solvent will dissolve in the mobile phase without affecting sample retention or resolution. Sample preparation begins at the point of collection, extends to sample injection onto the HPLC column. All of these operations form an important part of sample preparation and have a critical effect on the accuracy, precision, and convenience of the final method[26,27]

D) METHOD OPTIMIZATION:
The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type [28].

The mobile and stationary phase compositions need to be taken into account. Optimization of mobile phase parameters is always considered first as this is much easier and convenient than stationary phase optimization. To decrease the number of trial chromatograms involved, only the parameters that are likely to have a significant effect on selectivity in the optimization must be examined [10].

E) METHOD VALIDATION:
Definition: Analytical method validation is "A Documented evidence, which provides a high degree of assurance that a specific process will consistently produce, a product meeting its pre-determined specifications and quality attributes".

Parameters of analytical method validation:

1) Accuracy
2) Precision
   a) Repeatability
   b) Intermediate Precision
   c) Reproducibility
3) Specificity
4) Detection Limit
5) Quantitation limit
6) Linearity
7) Range
8) Stability
9) Robustness
10) Ruggedness
11) System Suitability

1) ACCURACY:
Accuracy of an analytical method may be defined as "The closeness of test results obtained by that method to the true value. This accuracy should be established across its range [29].
The accuracy of an analytical method may be determined by any of the following ways:

• Analysing a sample of known concentration and comparing the measured value to the 'true' value. However, a well characterized sample (e.g., reference standard) must be used.
• Spiked – placebo (product matrix) recovery method. In this method, a known amount of pure active constituent is added to formulation blank [sample that contains all other ingredients except the active(s)], the resulting mixture is assayed, and the results obtained are compared with the expected result.
• Standard addition method. In this method, a sample is assayed, a known amount of pure active constituent is added, and the sample is again assayed. The difference between the results of the two assays is compared with the expected answer. In both methods (spiked – placebo recovery and standard addition method), recovery is defined as the ratio of the observed result to the expected result expressed as a percentage.

The accuracy of a method may vary across the range of possible assay values and therefore must be determined at several different fortification levels. The accuracy should cover at least 3 concentrations (80, 100 and 120%) in the expected range.

Accuracy may also be determined by comparing test results with those obtained using another validated test method. Dosage form assays commonly provide accuracy within 3-5% of the true value. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated determination for each concentration) [30].

2) **PRECISION:**

Definition: 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 andvreproducibility [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]. Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier [34].

3) SPECIFICITY:

Definition: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

ICH divides the term specificityinto two separate categories:

Identification: to ensure the identity of an analyte.

Impurity Tests: to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e. related substances test, heavy metals, residual solvents content, etc.

Assay (content or potency): to provide an exact result this allows an accurate statement on the content or potency of the analyte in a sample. Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. It is not always possible to demonstrate that a single analytical procedure is specific for a particular analyte. Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for best selectivity. [31]

4) 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 whereas 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:

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.

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. An S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation (RSD) ≤ 10%) LOQ, an S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD) ≤ 3%) as illustrated in Figure-1

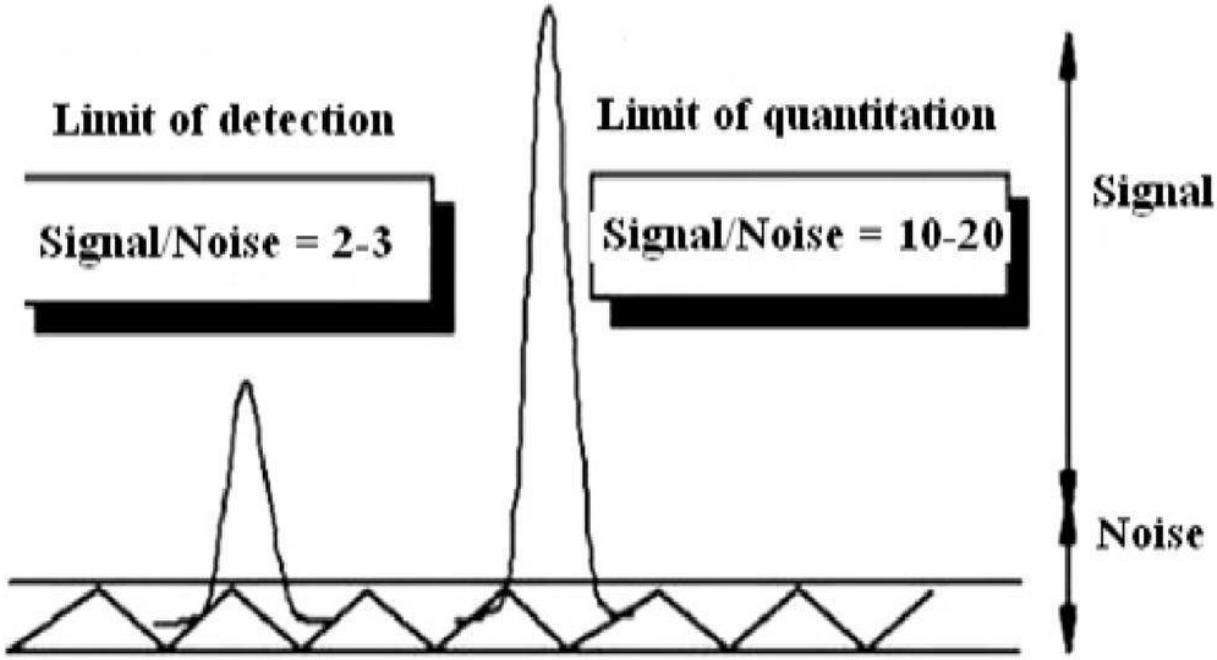

Figure-1: Limit of detection and limit of quantitation via signal to noise ratio (S/N)

The LOD and LOQ may be expressed as:

LOD = 3.3 x σ/S

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 [35].

5) LINEARITY:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. [31] Linearity is determined by a series of five to six injections of five or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method. [36].

6) RANGE:

The range of an analytical procedure is the interval between the upper and lower concentration of an analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable precision, accuracy and linearity. The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

- For Assay - 80 to 120% of test concentration
- Content uniformity - • 70 to 130% of test concentration
- Dissolution - Q-20% to 120%
- Impurities - reporting level – 120% of impurity specification limit
- Assay & Impurities - Reporting level to 120% of assay specific.

Linearity is limited to 150% of shelf life specification of impurities

- Test concentration can be used to determine impurities
- To determine drug substance (assay) the test concentration must be diluted
- The range is 0 – ~ 150% of impurity specification [1].

7) STABILITY:

Solution stability is stability of standard and extracted sample solution (ready to inject) from the sample or matrix and analyzed as per specified method, and it should be stored properly in room temperature and refrigerated condition depending upon the stability of the sample and standard solution. The stability of standard and sample solution should be established in room temperature and refrigerated, if refrigerated before analyzing it should be thawing to room temperature. A minimum two preparation of standard and sample solution should be prepared and analyzed as per specified method. The analyzed solutions stored in necessary condition and the stability can be established for two days or solution stability can be established by an hour basis depending upon the nature of the product[37]

Chemical compounds can decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes and standards. It is a measure of the bias in assay results generated during a preselected time interval[38]

Stability testing is termed as a complex process because of involvement of a variety of factors influencing the stability of a pharmaceutical product. These factors include stability of the active ingredient(s); interaction between active ingredients and excipients, manufacturing process followed, type of dosage form, container/closure system used for packaging and light, heat and moisture conditions encountered during shipment, storage and handling. In addition, degradation reactions like oxidation, reduction, hydrolysis or racemization, which can play vital role in stability of a pharmaceutical product, also depend on such conditions like concentration of reactants, pH, radiation, catalysts etc., as well as the raw materials used and the length of time between manufacture and usage of the product. A pharmaceutical product may undergo change in appearance, consistency, content uniformity, clarity (solution), moisture contents, particle size and shape, pH, package integrity thereby affecting its stability. Such physical changes may be because of impact,

vibration, abrasion, and temperature fluctuations such as freezing, thawing or shearing etc. The chemical reactions like solvolysis, oxidation, reduction, racemization etc. that occur in the pharmaceutical products may lead to the formation of degradation product, loss of potency of active pharmaceutical ingredient (API), loss of excipient activity like antimicrobial preservative action and antioxidants etc.[39]

8) 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.

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase
- Influence of variations in mobile phase composition
- Different columns (different lots and/or suppliers)
- Temperature
- Flow rate

The factors chosen for all the drugs under investigation were the flow rate, mobile phase composition, pH of a mobile phase and using different lot of LC column. The observation shall be

summarized and critical parameters shall be listed out in the validation report. System suitability parameter must be within the limit of acceptance criteria as mentioned in the method [31].

9) RUGGEDNESS:

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method. For the determination of ruggedness, the degree of reproducibility of test result is determined as function of the assay variable. This reproducibility may be compared to the precision of the assay under normal condition to obtain a measure of the ruggedness analytical method [40].

10) SYSTEM SUITABILITY:

According to the USP, system suitability tests are an integral part of chromatographic methods. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole.

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and reproducibility (%RSD retention time and area of repetitive injection) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability “sample” that is a mixture of main components and expected by-products.

Documentation of system suitability can be accomplished by using software specifically designed for the task to provide a review of the sepration and to summarize the data regarding reproducibility. The softwares are also used to troubleshoot the method. Results stored in a relational database can be compared and summarized on a peak-by-peak or system-by-system basis to provide the feedback necessary to determine system performance [41].

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