

A review on analytical method development

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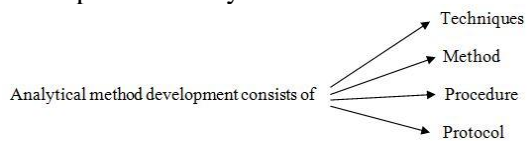
ABSTRACT

Pharmaceutical analysis plays a very prominent role in quality assurance as well as quality control of bulk drugs and pharmaceutical formulations. Rapid increase in pharmaceutical industries and production of drug in various parts of the world has brought a rise in demand for new analytical techniques in the pharmaceutical industries. As a consequence, analytical method development has become the basic activity of analysis. Recent development in analytical methods has been resulted from the advancement of analytical instruments. The improvement of the analytical method development and analytical instruments have reduced the time of analysis, increased precision and accuracy and reduced costs of analysis. As a consequence, most of pharmaceutical organizations are investing huge amount of money for the establishment of advanced analytical laboratories. Analytical techniques are developed and validated for active pharmaceutical ingredients (API), excipients, drug products, degradation products and related substances, residual solvents, etc. As a result, it has become an integral part of the requirements of the regulatory organization. Analytical method development finally results in official test methods. These methods are used in quality control laboratories to ensure the identity, purity, safety, efficacy and performance of drug products. Regulatory authorities are placing greater emphasis 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.

Key words: Analytical method development, validation, Quality control.

INTRODUCTION

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. 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. Thus it becomes necessary, to develop newer analytical methods for such drugs.



The method development provides the following requirements to the analyst so as to enable him to estimate the drug.

- The required data for a given analytical problem.
- The required sensitivity.
- The required accuracy.
- The required range of analysis.
- The required precision.

The method validation / evaluation imply the process of documenting or providing that: analytical method provides analytical data for the intended use.

Validation analytical method require the following

- Assuring quality
- Achieving acceptance of products by the international agencies.
- Mandatory requirement purposes for accreditation as per ISO 17025 guidelines.
- Mandatory requirement for registration of any pharmaceutical product or pesticide formulation.
- Validation methods are only acceptable for under taking proficiency testing.
- Validated/Evaluated method undergoes quality control procedures for further evaluation.

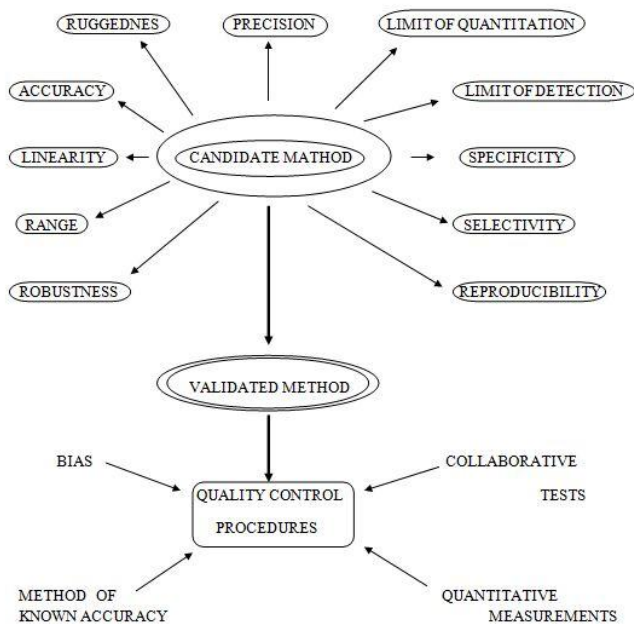


Figure.1. Flow chart showing different steps in analytical method development

Criteria for the Development of New Analytical Method: Drug analysis is the basis for the determination of the product. Very often, there is a time lag from the date of introduction of a drug in to the market to the date of its inclusion in pharmacopias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, report of new toxicities and development of patient resistance and introduction of better drugs by the competitors (Connors, 1994).

Under these conditions, standard and analytical procedures for these drugs may not be available in pharmacopias. Therefore, it becomes necessary to develop new analytical methods for such drugs. In brief the reasons for the development of newer methods of drugs analysis are (Mendham, 2001).

The new drug or drug combination may not be official in any pharmacopias. 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 formulation excipients. Analytical methods for a drug in combination with other drugs may not be available. Analytical methods for the quantitation of the drug in biological fluids may not be available. The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedure and these may not be reliable.

Steps involved in method development:

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database.

1. Analyte standard characterization

- All known information about the analyte and its structure is collected i.e., physical and chemical properties.
- The standard analyte (100 % purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators and freezer).
- When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
- Only those methods (spectroscopic, MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

2. Method requirements: The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature search and prior methodology: The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical

methods, books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, are reviewed. Chemical abstracts service (CAS) automated computerized literature searches are convenient.

4. Choosing a method: Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.

a) If there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for each analytical method already exist that is similar to the analyte of interest.

5. Instrumental setup and initial studies: The required instrumentation is to be setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified. Always new consumables (e.g. solvents, filters and gases) are used. For example, method development is never started on a HPLC column that has been used earlier. The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

6. Optimization: During optimization one parameter is changed at a time and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan, and every step is documented (in a lab notebook) in case of dead ends.

7. Documentation of analytical figures of merit: The originally determined analytical figures of merit are limit of quantitation (LOQ), limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

8. Evaluation of method development with actual samples: The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

9. Determination of percent recovery of actual sample and demonstration of quantitative sample analysis: Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average + / - standard deviation) from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100 % recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

HPLC method development: High Performance Liquid Chromatography (HPLC) is one of the most widely used analytical techniques. More than 85% of general pharmaceuticals are analyzed by HPLC. The technique of chromatography was originally developed by Russian Botanist M.S. Tswett in 1903 but after that, plenty of revolutions and amendments were done and it is still going on. HPLC is the separation module which contain mainly stationary phase and mobile phase having opposite polarity equipped with high pressure pumps and the separation is achieved by the interaction of stationary phase and the mobile phase. A proper choice of stationary phase and mobile phase is essential to reach desired separation. Ph of mobile phase, different types of buffer, column temperature, sample diluents, detection wavelength and many more are the variables which play a major role in method development.

During the preliminary method development stage, all individual components should be investigated before the final method optimization. This gives us a chance to critically evaluate the method performance in each component and to streamline the final method optimization. A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

Separation goals: The goals of HPLC separation need to be specified clearly, are represented in Table 1.

Table.1. Separation goals in brief

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5
Separation time	< 5-10 min is desirable for routine procedures
Quantization	$\leq 2\%$ for assays $\leq 5\%$ for less-demanding analyses $\leq 15\%$ for trace analyses
Pressure	< 150 bar is desirable < 200 bar is usually essential (for a new column)
Peak height	Narrow peaks are desirable for large signal/noise ratios
Solvent consumption	Minimum mobile phase use per run is desirable

Choice of the Column: Column is the heart of HPLC system. Good silica and bonding process will provide the reproducible and symmetrical peak necessary for accurate qualification. Commonly used RP columns include C_{18} (USP L1), C_8 (USPL8), Phenyl (USP L11) and Cyno (USP L18). There is no good or bad column. They are chemically different bonded phases and demonstrate significant changes in selectivity

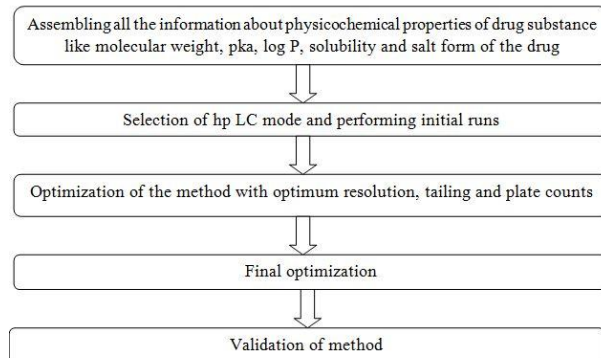
using same mobile phase. Column vary from manufacturer to manufacturer relative to their pore volumes, pore size, surface area, particle size, carbon load and whether they are end capped or not. Column length also plays a vital role in the separation resolution. Various types of columns and their applications are shown in Table 2.

Table.2. Various types of columns and their applications

Column	Phase	Solvents	Application
C_{18}	Octadecyl	ACN, MeOH, H_2O	General, nonpolar
C_8	Octyl	ACN, MeOH, H_2O	General, nonpolar
Phenyl	Styrl	ACN, MeOH, H_2O	Fatty acids, double bond
Cyano	Cyanopropyl	ACN, MeOH, H_2O , THF	Ketones, aldehydes
Amino	Aminopropyl	ACN, MeOH, H_2O , THF, $CHCl_3$, CH_2Cl_2	Sugars, anions
Diol	Dihydroxy hexyl	ACN, MeOH, H_2O , THF	Proteins
SAX	Aromatic quaternary amine	SALT Buffers, ACN, MeOH, H_2O	Anions
SCX	Aromatic sulfonic acid	SALT Buffers, ACN, MeOH	Cations
DEAE	Alkyl ether, ethyl 2° amine	SALT Buffers, ACN, MeOH, H_2O	Protein cations

There is no absolute end to the method development process. The question is which is the “acceptable method performance”? The acceptable method performance is determined by the objectives set in this

step. This is one of the most important considerations often overlooked by scientists. In this section, the different end points (i.e., expectations) will be discussed in descending order of significance.

**Figure.2. Flow chart of method development**

Analytes: For a related substance method, determining the “significant and relevant” related substances is very critical. With limited experience with the drug product, a good way to determine the significant related substances is to look at the degradation products observed during stress testing. Significant degradation products observed during stress testing should be investigated in the method development. Based on the current ICH guidelines on specifications, the related substances method to active pharmaceutical ingredients (API) should focus on both the API degradation products and synthetic impurities, while the same method for drug products should focus only on the degradation products. In general practice, unless there is any special toxicology concerns, related substances below the limit of quantification (LOQ) should not be reported and therefore should not be investigated.

Resolution (Rs): A stability indicating method must resolve all significant degradation products from each other. Typically the minimum requirement for baseline resolution is 1.5. This limit is valid only for 2 Gaussian-shape peaks of equal size. In actual method development, $R_s = 2.0$ should be used as a minimum to account for day-to-day variability, non-ideal peak shapes and differences in peak sizes.

Limit of Quantitation (LOQ): The desired method LOQ is related to the ICH reporting limits. If the corresponding ICH reporting limit is 0.1%, the method LOQ should be 0.05% or less to ensure the results are accurate up to one decimal place. However, it is of little value to develop a method with an LOQ much below this level in standard practice because when the method is too sensitive, method precision and accuracy are compromised.

Precision, Accuracy: Expectations for precision and accuracy should be determined on a case-by-case basis. For a typical related substance method, the RSD of 6 replicates should be less than 10%. Accuracy should be within 70 % to 130% of theory at the LOQ level.

Analysis time: A run time of about 5-10 minutes per injection is sufficient in most routine related substance analyses. Unless the method is intended to support a high-volume assay, shortening the run time further is not recommended as it may compromise the method performance in other aspects (e.g., specificity, precision and accuracy.)

Adaptability for Automation: For methods that are likely to be used in a high sample volume application, it is very important for the method to be

“automatable”. The manual sample preparation procedure should be easy to perform. This will ensure the sample preparation can be automated in common sample preparation workstations.

Understand the Chemistry: Similar to any other research project, a comprehensive literature search of the chemical and physical properties of the analytes (and other structurally related compounds) is essential to ensure the success of the project.

Chemical Properties: Most sample preparations involve the use of organic-aqueous and acid-base extraction techniques. Therefore it is very helpful to understand the solubility and pKa of the analytes. Solubility in different organic or aqueous solvents determines the best composition of the sample solvent. pKa determines the pH in which the analyte will exist as a neutral or ionic species. This information will facilitate an efficient sample extraction scheme and determine the optimum pH in mobile phase to achieve good separations.

Potential Degradation Products: Subjecting the API or drug product to common stress conditions provides insight into the stability of the analytes under different conditions. The common stress conditions include acidic pH, basic pH, neutral pH, different temperature and humidity conditions, oxidation, reduction and photo-degradation. These studies help to determine the significant related substances to be used in method development, and to determine the sample solvent that gives the best sample solution stability. In addition, the structures of the analytes will indicate the potential active sites for degradation. Knowledge from basic organic chemistry will help to predict the reactivity of the functional groups. For example, some excipients are known to contain trace level of peroxide impurities. If the analyte is susceptible to oxidation, these peroxide impurities could possibly produce significant degradation products.

Sample Matrix: Physical (e.g., solubility) and chemical (e.g., UV activity, stability, pH effect) properties of the sample matrix will help to design an appropriate sample preparation scheme. For example, Hydroxypropyl Methylcellulose (HPMC) is known to absorb water to form a very viscous solution; therefore it is essential to use mostly organic solvents in sample preparation.

Initial method conditions: The objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments. Initial HPLC conditions are represented in Table 3.

Table.3. Initial HPLC conditions

Stationary phase	C ₈ or C ₁₈		
Column length and internal diameter	250 mm x 4.6mm		
Particle size	10 or 5 μm		
Mobile phase	Buffer : Acetonitrile		
% Buffer isocratic	50%		
% Buffer gradient	20-80%		
Buffer concentration	Phosphate 50 mM		
pH of mobile phase	3 for neutral compounds	3 and 7.5 for ionic acidic	3 and 7.5 for ionic basic
Modifier	10 mM TEA and 1% HSA	1% HAS	10 mM TEA
Flow rate	1.5 - 2mL/minutes		
Column temperature	Ambient to 35°C		
Injection volume	10 – 25 μl		

Preliminary HPLC Conditions: In order to develop preliminary HPLC conditions in a timely fashion, we should use artificial mixtures of active pharmaceutical ingredients and related substances at relatively high concentrations (e.g., 1-2% of related substance relative to API) to develop the preliminary HPLC conditions. The concentration ratio between API and the related substances should be maintained to ensure the chromatography represents that of a real sample. Alternatively, a highly stressed sample (e.g., 5% degradation) can also be used at this stage. With the known composition and high levels of degradation products in the sample, one can evaluate the chromatography to determine whether there are adequate separations for all analytes. The high concentrations of related substances are used to ensure all peaks will be detected. Computer assisted method development can be very helpful in developing the preliminary HPLC conditions quickly. Since the objective at this stage is to quickly develop HPLC conditions for subsequent method development experiments, scientists should focus on the separation of the significant related substances instead of trying to achieve good resolution for all related substances. These significant related substances should be baseline resolved from each other with $R_s > 2.0$. After the preliminary method development, the HPLC conditions can be further fine-tuned at a later stage to achieve the required specificity for the other related substances.

Key variables in RP-HPLC method development:

a) pH: In RP HPLC sample retention increases when analyte is more hydrophobic. When the $pH = p^{Ka}$ for this concentration equal proportion of ionic and non-ionic species exists. As a general rule pH causes changes in retention time within ± 1.5 pH units

above and below the p^{Ka} to ensure practically 100% unionization for retention purposes.

b) Mobile phase: The mobile phase composition and solvent strength will affect both selectivity and capacity factor. Three organic solvents [methanol, acetonitrile or THF as (%B)] form the basis of solvent selectivity triangle. They exhibit miscibility with water possesses low viscosity and are UV transparent. Each organic solvent in combination with water or water containing buffer or additives (s) comprises mobile phase. Mobile phase polarity and solvent strength determines analyte separation.

c) Role of buffer: The pH of mobile phase can be controlled by use of buffer that provides capacity. The buffer or ionic strength, will affect the selectivity. While selecting given buffer, the additives or even the solvent, sufficient regard for their compatibility with analyte and HPLC system should be considered.

d) Ion pair reagent: Use of ion pair reagent will depend on the nature of analyte, if simple ionic; use of ion pair reagent improves selectivity. Methanol provides better solubility for ion pair reagent as well as buffer and salts. So, it may be preferred over acetonitrile and THF. For selecting proper ion pair reagent, alkyl chain length must be considered. The longer is the chain, more hydrophobic the counter ion and therefore the greater retention due to equilibrium between counter ion and column adsorbent.

e) Temperature and flow rate: It is reported that with an increase in 1°C will decrease the capacity factor by 1 to 2 % and ionic and neutral samples are reported to show significant changes in selectivity with temperature changes. Flow rate more for isocratic than gradient separation, can sometimes be useful and readily utilized for increasing resolution. Aged HPLC Column: An aged HPLC column should

be used to develop the initial HPLC conditions. Usually it is more difficult to achieve the required resolution with an aged column (e.g., column with about 200 injections). This will reflect the worst-case scenario likely to be encountered in actual method uses, and help the long-term method robustness. In general, develop all methods with HPLC columns from the same vendor. The preferred brand of HPLC column should be selected primarily based on the long-term stability and lot-to-lot reproducibility.

Sample preparation:

Selection of Sample Solvent: This stage focuses on the selection of the sample solvent (for extraction) and the proper sample preparation procedures. Investigate the effect of sample solvents of different % organic, pH, extraction volume and extraction procedure on accuracy, precision, sensitivity (LOQ) and the changes in the chromatography (e.g., peak shape, resolution). Whenever possible use the mobile phases in the sample preparation this will ensure that there will not be any compatibility issues between the sample solution and the HPLC conditions.

Accuracy: To investigate the accuracy in sample preparation (i.e., extraction efficiency), we prepare a spiked solution by adding known amounts of related substances into a sample matrix. Compare responses of the spike solutions and the neat standard solutions and the neat standard solutions to assess the recovery from the sample preparation.

Precision: Use the stressed sample to represent the worst-case scenario and perform replicate sample preparations from the same sample composite. Investigate the consistency of the related substance profile (i.e., any missing peaks?) and the repeatability results from these preparations.

Standardization:

Area % method: If the response of the active pharmaceutical ingredient is linear from LOQ to the nominal sample concentration, use the % area approach where the related substance is reported as % area. This is the most straight forward approach, and doesn't require the preparation of standard solutions. It also has the highest precision since preparation-to-preparation variation will not affect the results. However, in order to ensure the concentration is linear within this range, the sample concentration is usually limited and this will reduce the method sensitivity (i.e., increase LOQ). In general, use this approach as long as the desired LOQ can be achieved.

External Standard method: Use the external standard method if the response of the active pharmaceutical ingredient is not linear throughout the

whole range, or the desired LOQ cannot be achieved by the area % method. The concentration of standard solution should be high enough to ensure the standard solution can be prepared accurately and precisely on a routine basis, it should be low enough to approximate the concentration of related substance in the sample solution. In general, the standard concentration should correspond to about 5 % of related substances to perform the final optimization of the method to improve the accuracy, precision.

Method optimization/ robustness: After the individual components of the method are optimized and LOQ. Use an experimental design approach to determine the experimental factors that have significant impact on the method. This is very important in determining what factors need to be investigated in the robustness testing during the method validation. To streamline the method optimization process, use Plackett Burmann Design or similar approach to simultaneously determine the main effects of many experimental factors. Some of the typical experimental factors that need to be investigated are: HPLC conditions: % organic, pH flow rate, temperature wavelength, column age. Sample preparation: % organic, pH, shaking / sonication, sample size, and sample age. Calculation/standardization: integration, wavelength, standard concentration, response factor correction.

Method validation: Method validation should be treated as a "final verification" of the method performance and could not be used as partly of the method development. Some of the typical method validation parameters should be studied thoroughly in the previous steps. In some cases, robustness can be completed in the final method optimization before method validation. At this point, the robustness experiments should be limited only to the most significant factors (usually less than 4 factors). In addition, unlike the final method optimization, the experimental factors should be varied within a narrow range to reflect normal day-to-day variation.

As per USP: Types of analytical methods

Category I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients including preservatives in finished pharmaceutical products.

Category II: Analytical methods for determination of impurities in bulk drugs or for determination of degradation compounds in finished pharmaceutical products.

Category III: Analytical methods for determination of performance characteristics (e.g. dissolution, drug release).

Category IV: Identification tests.

The data elements required for assay validation is shown in Table 4.

Table.4. Data elements required for assay validation

Type of analytical procedure	Identification	Testing for impurities		ASSAY - dissolution (measurement only) - content/potency
		Quantitat.	Limit	
Characteristics				
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm. Precision	-	+	(1) -	+
Specificity (2)	+	+	+	+
Detection Limit	-	-	(3) +	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated

+ signifies that this characteristic is normally evaluated

- (1) In cases where reproducibility (see glossary) has been performed, intermediate precision is not needed
- (2) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
- (3) May be needed in some cases

As per ICH method validation can be defined as (ICH) “Establishing documented evidence, which provides a high degree of assurance that a specific activity will be consistently produced a desired result or product meeting its predetermined specifications and quality characteristics”. An assay for a major component requires a different approach and acceptance criteria than a method for a trace impurity. A final method may be performed at different sites around the world. Differences in HPLC instrumentation, laboratory equipment and reagent sources and variations in the skills and background of personnel may require specific features in the HPLC method. In addition, the development of different formulations of the same drug with varying strengths or physical forms may require flexibility in method procedures. Method validation study include system suitability, linearity, precision, accuracy, specificity, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments.

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 (whose “true value” is known) 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:

1. Comparison to a reference standard
2. Recovery of the analyte spiked into blank matrix
3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. e.g. Weight / weight or area percent in all cases with respect to the major analyte.

Precision: Precision can be defined as “The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample”. A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types:

- 1.Repeatability
- 2.Intermediate precision and
- 3.Reproducibility

Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

Specificity / Selectivity: The terms selectivity and specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method is said to be selective. Since there are very few methods that respond to only one analyte, the term selectivity is usually more appropriate. The analyte should have no interference from other extraneous components and be resolved well from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

Limit of detection: Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low level compounds due to potential gradual loss of sensitivity of detector lamps with age or noise level variation by detector manufacturer. At low levels, assurance is needed that the LOD and LOQ limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detect ability, extraneous peak(s) could "disappear / appear." A crude method to evaluate the feasibility of the extraneous peak detection is to use the percentage claimed for LOD from the area counts of the analyte. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope

The LOD may be expressed as: $LOD = 3.3\sigma/S$

Where, σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

Limit of quantification: Limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable

precision and accuracy under the stated experimental conditions. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise Approach
- Based on the standard deviation of the response and the slope

The LOQ may be expressed as: $LOQ = 10\sigma/S$

Where,

σ = Standard deviation of Intercepts of calibration curves

S = Mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

Linearity: 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.

Range: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations).

It is established by conforming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to the samples containing amounts of analyte within or extremes of the specified range of the analytical procedure.

Stability: To generate reproducible and reliable results, the samples, standards, and reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, one week, and one month, depending on need). Therefore, a few hours of standard and sample solution stability can be required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different storage conditions from a single lot), automated, overnight runs often are performed for better lab efficiency. Such practices add requirements for greater solution stability. The acceptance criteria of validation for HPLC are shown in Table 5.

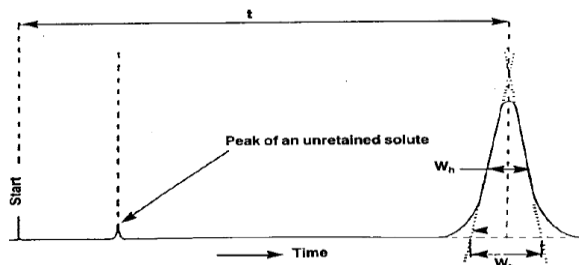
Table.5. Acceptance criteria of validation for HPLC

Characteristics	Acceptance Criteria
Accuracy	Recovery 98-102% with 50, 100, 150%
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate precision	RSD < 2%
Specificity/ selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	$r^2 < 0.999$
Range	80-120%
Stability	>24h or > 12h

System suitability criteria: It is advisable to run system suitability tests in these robustness experiments. During the robustness testing of the method validation, critical method parameters such as mobile phase composition and column temperature are varied to mimic the day-to-day variability. Therefore, the system suitability results from these robustness experiments should reflect the expected range. Consequently, the limits for system suitability tests can be estimated from these experiments. The parameters used in system suitability test report are as follows:

- Number of theoretical plates or Efficiency (N)
- Capacity factor (K)
- Separation or Relative retention (α)
- Resolution (Rs)
- Tailing factor (T)
- Relative Standard Deviation (RSD)

These are measured on a peak or peaks of known retention time and peak width.

**Figure.3. Plate number**

Therefore the column's efficiency can also be quoted as the plate height (h) or the height equivalent to one theoretical plate (HETP).

$$h = L/N$$

Where,

L = length of column N or plates/meter
N = plate number.

Plate number or number of theoretical plates (N):

This is a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP uses the peak width at half the height.

$$BP \dots \dots N = 5.545 \left(\frac{t}{w_h} \right)^2$$

Or

$$USP \dots \dots N = 16 \left(\frac{t}{w_b} \right)^2$$

Where,

W_h = peak width at 1/2 peak height,

W_b = peak width at base (ml, sec or cm)

t = retention time of peak or elution volume (ml, sec or cm)

Therefore the plates number is high the column is more efficient. The plate number depends on column length i.e. the longer the column the larger the plate number.

Capacity factor or Capacity ratio (K): This value gives an indication of how long each component is retained on the column (i.e. how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).

$$K = \frac{t_R - t_M}{t_M}$$

Where,

t_R = unretained peaks retention time,

t_M = retention time of the peak of interest.

K is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (i.e. flow rate) and therefore ensures greater reproducibility from run to run. In practice the k value for the first peak of interest should be > 1 to assure that it is separated from the solvent.

Separation Factor or Relative retention (α): 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. If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition, specified temperature and regardless of the instrument used

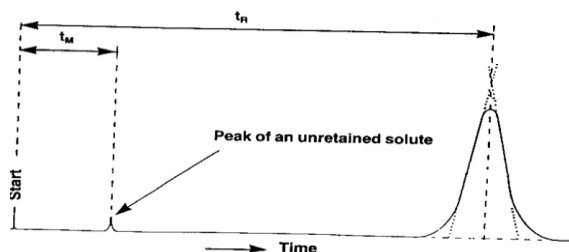


Figure.4.Capacity factor

Tailing factor and Asymmetry factor: If the peak has to be quantified is asymmetric, a calculation of the asymmetry would also be useful in controlling or characterizing the chromatographic system. Peak asymmetry arises from a number of factors. The increase in the peak asymmetry is responsible for a decrease in chromatographic resolution, detection limits, and precision. The peak asymmetry can be calculated by using formula,

$$A_s = \frac{b}{a}$$

Where,

A_s is peak asymmetry factor

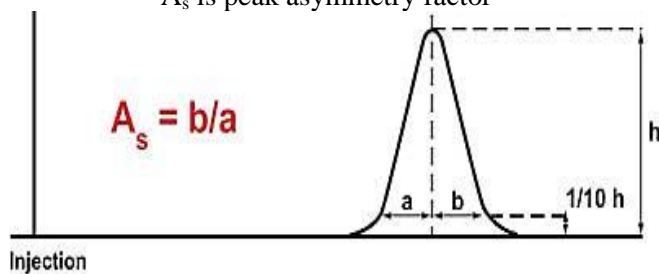


Figure.6(a).Asymmetry factor

Relative standard deviation or precision: For an HPLC system this would involve the reproducibility of a number of replicate injections (i.e. 6) of an

From the peaks A and B,

$$\alpha = \frac{K_B}{K_A}$$

Where,

K_B = capacity factor for B

K_A = capacity factor for A.

Resolution (R_s): The resolution of two neighboring peaks is defined by the ratio of the distance between the two peak maxima. It is the difference between the retention times of two solutes divided by their average peak width. For baseline separation, the ideal value of resolution is 2.0. It is calculated by using the formula,

$$R_S = \frac{R_{t1} - R_{t2}}{0.5(W_1 + W_2)}$$

Where,

R_{t1} and R_{t2} are the retention times of components 1 and 2.

W_1 and W_2 are peak widths of components 1 and 2.

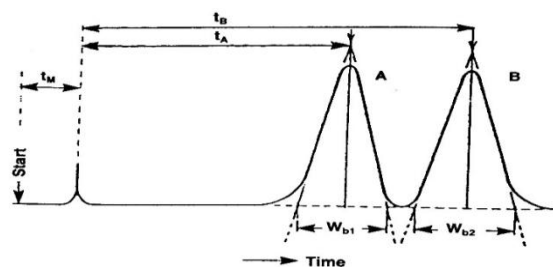


Figure.5.Separation Factor

b is distance from the point at the peak midpoint to the trailing edge

a is distance from the leading edge of peak midpoint to the midpoint

$$T = \frac{(a + b)}{2a}$$

Where,

T is tailing factor (measured at 5% peak height)

b is distance from the point at the peak midpoint to the trailing edge

a is distance from the leading edge of peak midpoint to the midpoint.

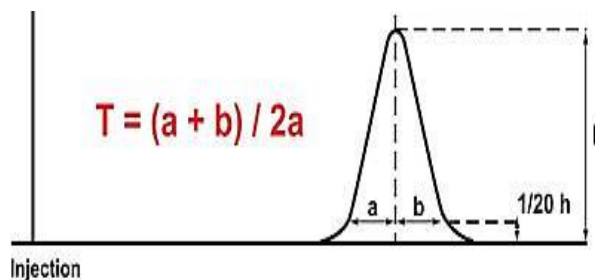


Figure.6(b).Tailing factor

analytical solution. The USP requires that unless otherwise specified by a method: If a relative standard deviation of $<2\%$ is required then five replicate

injections should be used. If a relative standard deviation of >2% is required then six replicate

injections should be used. Limits of system suitability parameters as per USP 2003 are shown in Table 6.

Table 6: Limits of system suitability parameters (usp 2003)

Parameter name	Limit
Number of theoretical plates or Efficiency (N)	> 2000
Capacity factor (K)	< 1
Separation or Relative retention (α)	≥ 1
Resolution (Rs)	≥ 2
Tailing factor or Asymmetry(T)	≤ 2
Relative Standard Deviation (RSD)	≤ 2

CONCLUSION

The main purpose of development of analytical methods are for identification, purification and finally to quatification any required drug etc., The main activities involved in the analytical development of a method are separation and characterization of impurities as well as degraded products, analytical investigations, studies for identification and finally setting up of parameters optimization to specific requirements. Therefore the salient points enumerated in the above review article are immense use to an analyst while estimating the pharmaceutical formulations as well as bulk drugs.

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