Guidance Document

Validation of Analytical Methods

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Introduction

Method validation is the process by which it is demonstrated through documentary evidence that the performance characteristics of the method meet the requirements for the intended application. All the analytical methods that are intended for analyzing any sample need to be validated. The suitability of all test methods used should always be verified under the actual conditions of use and should be well documented.

Validation Process

A well planned process should be followed during validation. Possible steps for a complete method validation are listed below:

1. Develop a Validation Protocol

The first step in method validation is to prepare a written and approved protocol with the instructions in a clear step-by-step format. Following is a step-by-step guide for preparing protocols and performing test methods validation with reference to HPLC. One may use similar criteria for all other instrumental test method validation. Typical method validation protocol should include:

- **Introduction:** Firms validation policy, general description
- **Organizational structure:** Description of all personal responsibilities for all validation activities
- **Process and product description:**Makes a brief description of the process and product or reference to adequate documents
- **Specific process considerations:** describes critical characteristics of the process
- **Key acceptance criteria:** General statement on acceptance criteria for the process
- **Documentation format:** The format used for protocol and report is described
- **Required SOPs:** a list of relevant SOPs should be mentioned
- **Planning and Scheduling:** describes the resources, equipments and chemicals to be used, including time plan of the project
- **Change control:** includes description or reference to the critical parameters variations in the process or product

Analytical Method Validation Protocol-Cover Page

Summary Information

Summary Information	
Organization Name	
Site Location	
Department Performing Validation	
Protocol Title	
Validation Number	
Equipment	
Revision Number	

Project Controller

Project Controller	Name	Signature	Date

Document Approval

Document Approval			
Department / Functional Area	**Name**	**Signature**	**Date**
Technical Reviewer			
End Lab Management			
Health & Safety			
Quality Assurance			
Documentation Control (reviewed and archived by)			

2. Validation Parameters

The analytical methods which need to be validated are as following:

- Identification tests: To ensure identity of an analyte
- Quantitative test for impurities: to accurately and quantitatively reflect the purity of a sample
- Limit test for impurities: to reflect purity characteristics of the sample
- Assay of drug substance and drug products: to measure accurately and quantitatively the analyte present in the sample. These methods also include analysis for content uniformity and measurement of analyte from dissolution samples

The characteristics which need to be validated for the different types of method are summarized in following Table.

Validation Characteristics	Assay	Testing for Impurities		Identification
		Quantitative	Limit	
Accuracy	Yes	Yes	No	No
Precision-Repeatability	Yes	Yes	No	No
Precision-Intermediate Precision	Yes	Yes	No	No
Specificity	Yes	Yes	Yes	Yes
Detection Limit	No	No	Yes	No
Quantitation Limit	No	Yes	No	No
Linearity	Yes	Yes	No	No
Range	Yes	Yes	No	No
Robustness	Yes	Yes	No	No

3. Analytical Performance Characteristics

(i) Specificity

Specificity (or selectivity) of the analytical method is defined as the ability to assess unequivocally the analyte in the presence of other compounds (such as impurities, degradants, matrix, etc.) that are likely to be present. Specificity study of the chromatographic method is performed by the separation of the analyte from the other potential components such as impurities, degradants or excipients etc. The selectivity of chromatographic methods may be assessed by examination of peak homogeneity or peak purity test. Peak purity test shows that there is no co-elution of any sample component. For this, peak purity assessment is done by using PDA or MS detectors. Representative chromatograms with peaks labeled should be included with resolution, plate count and tailing factor reported in the validation report.

- Test procedure
  The specificity of the assay method will be investigated by injecting of the extracted placebo to demonstrate the absence of interference with the elution of analyte.
- Documentation
  Print chromatograms.
- Acceptance criteria
  The excipient compounds must not interfere with the analysis of the targeted analyte.

(ii) Linearity

Linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. For HPLC methods, the linear relationship between detector response (peak area and height) and sample concentration is determined. The relationship can be demonstrated directly on drug substance by dilution of standard stock or by separate weighing of the sample components, using the proposed procedures.
Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is linear relationship, test results should be evaluated by appropriate statistical methods, for example, by regression analysis. Data from the regression line is helpful to provide mathematical estimates of the degree of linearity. It is generally expressed in terms of variance around the slope of regression line. In some cases, the analytical responses should be described by the appropriate function of the analyte concentration. The widely used linearity ranges and acceptance criteria for various pharmaceutical methods are listed in following Table.

Test	Linearity Levels and Ranges	Acceptance Criteria
Assay	Five levels, 50-150% of label claim	Correlation coefficient,R≥0.999
Dissolution	Five to eight levels, 10-150% of label claim	% y intercept NMT 2.0%; R≥0.99
Related Substances	Five levels, LOQ to acceptance criteria	% y intercept NMT 5.0%, R≥0.99

- Test procedure
  Standard solutions will be prepared at six concentrations, typically 25, 50, 75, 100, 150, and 200% of target concentration. Three individually prepared replicates at each concentration will be analyzed. The method of standard preparation and the number of injections will be same as used in the final procedure.
- Documentation
  Record results on a datasheet. Calculate the mean, standard deviation, and RSD for each concentration. Plot concentration (x-axis) versus mean response (y-axis) for eachconcentration. Calculate the regression equation and coefficient of determination (r^2). Record these calculations on the datasheet.
- Acceptance criteria
  The correlation coefficient for six concentration levels will be ≥0.999 for the range of 80% to 120% of the target concentration. The y-intercept must be ≤2% of the target concentration response. A plot of response factor versus concentration must show all values within 2.5% of the target level response factor, for concentrations between 80% and 120% of the target concentration. In general, the coefficient of determination for active ingredients should be ≥0.997, for impurities ≥0.98 and for biologics ≥0.95.

Linearity Data Sheet

Linearity – Data Sheet		Electronic File Name:	
Concentration (mg/ml)	Concentration as % of Analyte Target	Peak Area (mean of three injections)	Peak Area RSD (%)
5 (e.g.)	25		
10	50		
15	75		
20	100		
30	150		
40	200		

(iii) Range

Range of an analytical method is the interval between the upper and lower concentration 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. The range is normally derived from the linearity studies and depends on the intended application of the procedure. The following minimum specified ranges should be considered:

- For the assay method, normally covering from 80% to 120% of the test concentration.
- For content uniformity, covering minimum of 70% to 130% of the test concentration, based on the nature of the dosage form.
- For dissolution testing, ±20% over the specified range.
- For impurity determination, from reporting level of impurity to 120% of the specification.

The range of a method is confirmed when linearity, accuracy and precision criteria are fulfilled.

- Test procedure
  The data obtained during the linearity and accuracy studies will be used to assess the range of the method. The precision data used for this assessment is the precision of the three replicate samples analyzed at each level in the accuracy studies.
- Documentation
  Record the range on the datasheet.
- Acceptance criteria
  The acceptable range will be defined as the concentration interval over which linearity and accuracy are obtained per the above criteria, and in addition, that yields a precision of ≤3% RSD.

Range Data Sheet

Range – Data Sheet	Electronic File Name:
Record Range:	

(iv) Accuracy

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value obtained. Practically no measurement process is ideal, therefore, the true or actual value cannot be exactly known in any particular measurement. The accepted true value for accuracy assessment can be assessed by analyzing a sample with known concentration. The accuracy studies are usually carried out by determining the recovery of the spiked sample of analyte into

the matrix of the sample (a placebo) or by comparing the result to the results of a certified reference material of known purity. If the placebo of the sample is not available, the technique of standard addition is used. In case of methods for quantitation of impurities, the sample with known amount of impurities is assessed. Accuracy should be assessed using minimum of nine determinations over a minimum of three concentration levels covering the specified range (for e.g., three concentrations/three replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the means and the accepted true value together with the confidence intervals. The concentration should cover the range of concern. The expected recovery depends on the sample matrix, the sample processing procedure, and the analyte concentration. The reported limits for accuracy for drug substances and products are 98.0-102.0% and 97.0-103.0% respectively. For the impurity determination, range from 50-150% of average recovery may be accepted.

- Test procedure
  Spiked samples will be prepared at three concentrations over the range of 50 to 150% of the target concentration. Three individually prepared replicates at each concentration will be analyzed. When it is impossible or difficult to prepare known placebos, use a low concentration of a known standard.
- Documentation
  For each sample, report the theoretical value, assay value, and percent recovery. Calculate the mean, standard deviation, RSD, and percent recovery for all samples. Record results on the datasheet.
- Acceptance criteria
  The mean recovery will be within 90 to 110% of the theoretical value for non-regulated products. For pharmaceutical industry, 100±2% is typical for an assay of an active ingredient in a drug product over the range of 80 to 120% of the target concentration. Lower percent recoveries may be acceptable based on the needs of the methods. The required accuracy is a bias of ≤2% for dosage forms and ≤1% for drug substance.

Accuracy Data Sheet

Accuracy – Data Sheet		Electronic File Name:		
Sample	Percent of Nominal (mean of three injections)	Amount of Standard (mg)		Recovery (%)
		Spiked	Found	
1	75 (e.g.)			
2	100			
3	150			
Mean				
SD				
RSD%				

(v) Precision

Precision of an analytical method expresses the closeness of agreement 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.

Repeatability is the precision under the same operating conditions over a short interval of time. It is also termed as intra-assay precision. It is assessed by making six sample determinations at 100% concentration or by preparing three samples at three concentrations in triplicates covering the specified range for the procedure. It involves repeated determination of same sample.

Intermediate precision expresses within laboratories variation: different days, different analyst, different equipments, etc. It is the term synonymous with the term 'ruggedness'. The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. To study intermediate precision, use of an experimental design is encouraged. The intermediate precision is generally studied by multiple preparations of sample and standard solution. Reproducibility is the precision obtained by analysis between laboratories. It is generally assessed during collaborative studies at the time of technology or method transfer. It is assessed by means of an inter-laboratory trial.

The precision data is generally expressed in the form of standard deviation, RSD and confidence interval. To ensure precision of method for major analytes, RSD should be ≤2%. For low level impurities, RSD of 5-10% is usually acceptable.

Precision-Repeatability

- Test procedure

  One sample solution containing the target level of analyte will be prepared. Ten replicates will be made from this sample solution according to the final method procedure.
- Documentation

  Record the retention time, peak area, and peak height on the datasheet. Calculate the mean, standard deviation, and RSD.
- Acceptance criteria

  The RSD should be 1% for drug substances and 2% for drug products. For minor components, it should be ±5% but may reach 10% at the limit of quantitation.

Repeatability Data Sheet

Repeatability – Data Sheet		Electronic File Name:	
Injection No.	**Retention Time (min)**	**Peak Area**	**Peak Height**
Replicate 1			
Replicate 2			
Replicate 3			
Replicate 4			
Replicate 5			
Replicate 6			
Replicate 7			
Replicate 8			
Replicate 9			
Replicate 10			
Mean			
SD			
RSD%			

Intermediate Precision

- Test procedure
  Intermediate precision (within-laboratory variation) will be demonstrated by two analysts, using two HPLC systems on different days and evaluating the relative percent purity data across the two HPLC systems at three concentration levels (50%, 100%, 150%) that cover the analyte assay method range 80 to 120%.
- Documentation
  Record the relative % purity (% area) of each concentration on the datasheet. Calculate the mean, standard deviation, and RSD for the operators and instruments.
- Acceptance criteria
  The assay results obtained by two operators using two instruments on different days should have RSD ≤2%.

Intermediate Precision Data Sheet

Intermediate Precision – Datasheet				**Electronic File Name:**		
Sample	**Relative % Purity (% Area)**					
	Instrument 1			**Instrument 2**		
	S1 (50%)	S2 (100%)	S3 (150%)	S1 (50%)	S2 (100%)	S3 (150%)
Operator 1, Day 1						
Operator 1, Day 2						
Operator 2, Day 1						
Operator 2, Day 2						
Mean (Instrument)						
Mean (Operators)						
RSD%	S1 + S1	S2 + S2	S3 + S3			
Instruments						
Operators						

(vi) Limit of Detection (LOD)

The limit of detection of an individual analytical procedure is the lowest amount of analyte in the sample which can be detected but not necessarily quantified as an exact value. The detection limit can be determined in different ways. The simplest approach is based on the signal to noise ratio. The signal to noise ratio is determined by comparing measured signals from samples with known low concentration of analyte with those of blank samples. The concentration showing signal to noise ratio between 3:1 or 2:1 is generally considered as acceptable detection limit.

The other approach is based on the standard deviation of the response and the slope. The detection limit may be expressed as:

$$LOD = 3.3\ \sigma/S$$

where, σ = the standard deviation of the response; S = the slope of the calibration curve

The slope may be estimated from the calibration curve of the analyte. The σ can be estimated as the standard deviation of the blank. The value of σ can also be estimated based on the calibration curve. For this the specific calibration curve should be studied using sample containing analyte in the range of detection limit. The residual standard deviation of a regression line or the standard deviation of the y-intercept of regression lines may be used as

standard deviation. Another approach for the estimation of the detection limit is based on visual evaluation. This method is applicable to non-instrumental methods but may be applied to the instrumental methods. The LOD is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected. The relevant chromatograms are sufficient for the justification of the detection limit.

- Test procedure
  The lowest concentration of the standard solution will be determined by sequentially diluting the sample. Six replicates will be made from this sample solution.
- Documentation
  Print the chromatogram and record the lowest detectable concentration and RSD on the datasheet.
- Acceptance criteria
  A signal-to-noise ratio of 3:1.

LOD Data Sheet

Limit of Detection – Data Sheet	Electronic File Name:
Record Sample Data Results: (e.g., concentration, S/N ratio, RSD%)	

(vii) Limit of Quantitation (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. It is mainly affected by the detector sensitivity and accuracy of sample preparation. The quantitation limit can be determined in the similar way as that of the detection limit. It is the concentration showing signal to noise ratio of 10:1. Based on the standard deviation of the response and the slope it is calculated by the formula:

$$LOQ = 10\ \sigma/S$$

where, σ = the standard deviation of the response; S = the slope of the calibration curve

The value of S and σ are estimated as for the detection limit. The LOQ can also be established from the visual evaluation as the LOD. The analyte concentration should be quantifiable with acceptable accuracy and precision at LOQ level. Typical acceptance criteria for LOQ are mean recovery at this level between 50 - 150 % with % RSD of ≤25%.

- Test procedure
  Establish the lowest concentration at which an analyte in the sample matrix can be determined with the accuracy and precision required for the method in question. This value may be the lowest concentration in the standard curve. Make six replicates from this solution.
- Documentation
  Print the chromatogram and record the lowest quantified concentration and RSD on the datasheet. Provide data that demonstrates the accuracy and precision required in the acceptance criteria.
- Acceptance criteria
  The limit of quantitation for chromatographic methods has been described as the concentration that gives a signal-to-noise ratio (a peak with height at least ten times as high

as the baseline noise level) of 10:1.2 The quantitation limit is the best estimate of a low concentration that gives an RSD of approximately 10% for a minimum of six replicate determinations.

LOQ Data Sheet

Limit of Quantitation – Data Sheet	Electronic File Name:
Record Sample Data Results: (e.g., concentration, S/N ratio, RSD%)	

(viii) 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. It is partially evaluated during method development stages. The aim of the robustness study is to identify the critical operating parameters for the successful implementation of the method. These parameters should be adequately controlled and a precautionary statement included in the method documentation. In case of an HPLC method, robustness study involves method parameters like pH, flow rate, column temperature and mobile phase composition which are varied within a reasonable range. The system suitability parameters obtained for each condition are studied to check the parameter which significantly affects the method.

Stability of the analytical solution and extraction time are other parameters which are also evaluated as additional parameters during robustness study. Stability of analytical solution is determined by assessing the results obtained by subjecting the analytical solution to the method parameters for longer period of time e.g. 4 hrs, 12 hrs, 24 hrs, 48 hrs etc. The acceptance criteria are based on relative difference between initial value and the value at specified solution stability time. For drug substances and drug products difference should be ≤2.0% and for impurity determination, it should be ≤10%.

When filtration is done during sample preparation filter paper study can be carried out. It involves analysis by filtering sample solution through different types of filter paper.

The chromatography obtained for a sample containing representative impurities, when using modified parameter(s), will be compared to the chromatography obtained using the target parameters. The effects of the following changes in chromatographic conditions will be determined: methanol content in mobile phase adjusted by ±2%, mobile phase pH adjusted by ±0.1 pH units, column temperature adjusted by ±5°. If these changes are within the limits that produce acceptable chromatography, they will be incorporated in the method procedure.

Robustness Data Sheet

Robustness – Data Sheet	Electronic File Name:
Explain / Record Sample Data:	

(ix) System Suitability

System suitability testing (SST) is an integral part of many analytical procedures. The tests are based on the concept that the equipment, analytical operations and samples are the integral part of the system that can be evaluated as such. System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run every time a method is used either before or during analysis.

The results of each system suitability test are compared with defined acceptance criteria and if they pass, the method is deemed satisfactory on that occasion. In case of HPLC methods, system suitability tests ensure the adequacy for performing the intended application on daily basis. The primary SST parameters considered are resolution (Rs), repeatability (% RSD of peak response and retention time), column efficiency (N), and tailing factor (T_f). The other SST parameters include retention factor (k) and separation factor (α). The limits which are considered for the SST parameters are listed following Table.

System Suitability Test	Limits
Resolution (R_S)	>2.0
Repeatability (RSD)	<1.0% for five replicates
Plate count (N)	>2000
Tailing factor (T_f)	≤2.0
Separation factor (α)	>1.0

- Test procedure
  System suitability tests will be performed on both HPLC systems to determine the accuracy and precision of the system by injecting six injections of a solution containing analyte at 100% of test concentration. The following parameters will be determined: plate count, tailing factors, resolution, and reproducibility (percent RSD of retention time, peak area, and height for six injections).
- Documentation
  Print the chromatogram and record the data on the datasheet
- Acceptance criteria
  Retention factor (k): the peak of interest should be well resolved from other peaks and the void volume; generally k should be ≥2.0. Resolution (Rs): Rs should be ≥2 between the peak of interest and the closest eluted peak, which is potentially interfering (impurity, excipient, and degradation product). Reproducibility: RSD for peak area, height, and retention time will be 1% for six injections. Tailing factor (T): T should be 2. Theoretical plates (N): ≥2000.

System Suitability Data Sheet

System Suitability – Data Sheet		Electronic File Name:		
System Suitability Parameter	Acceptance Criteria	Results		Criteria Met/Not Met
		HPLC 1	HPLC 2	
Injection Precision for Retention Time (Min)	RSD ≤ 1%			
Injection Precision for Peak Area (n = 6)	RSD ≤ 1%			
Injection Precision for Peak Height	RSD ≤ 1%			
Resolution (R_s)	Rs = ≥ 2.0			
USP Tailing Factor (T)	T = ≤ 2.0			
Capacity Factor (K)	K = ≥ 2.0			
Theoretical Plates (N)	N = ≥ 2000			

4. Revalidation

Revalidation is necessary whenever a method is changed and the new parameter is outside the operating range. The operating parameters need to be specified with ranges clearly defined. In case of methods for quantitation of impurities, if a new impurity is found that makes the method deficient in its specificity, it needs modification and revalidation. Changes in equipment or chemical quality may also have critical effects on method. So any such change needs revalidation.

Verification of Pharmacopoeial Methods

Method verification is performed for compendial methods to demonstrate their suitability under actual conditions of use for a specific drug substance and/or drug product. As the compendial analytical methods are validated methods, users are not required to validate these methods when first used in their laboratories, but verification of methods shall be performed to establish the objective evidence of suitability of the methods for their intended purpose. Verification consists of assessing selected analytical performance characteristics to generate appropriate data rather than repeating the validation process. If the verification of the compendial method is not successful, it may be concluded that the method may not be suitable for use with the article being tested in that laboratory and an alternate method, as allowed in the General Notices, may be developed and validated.

Verification requirements shall be determined based on an assessment of the complexity of the analytical method as well as the material to which the method is applied. Only those characteristics that are considered to be appropriate for the verification of the particular method need to be evaluated. Although complete revalidation of a compendial method is not required to verify the suitability of a procedure under actual conditions of use, some of the analytical performance characteristics, such as accuracy, precision, andspecificity, may be used for the verification process. Verification should include an assessment of elements such as the effect of the matrix on the recovery of impurities and drug substances from the drug product matrix, as well as the suitability of chromatographic conditions and column, the appropriateness of detector signal response, etc.

References

1. The Indian Pharmacopoeia, 2018