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Validation of Analytical Methods: A Review

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Abstract

The development of analytical method is important during the process of drug discovery, release to market and development, culminating in market approval. The method development, optimization and validation of the drug product from the development stage of the formulation to commercial batch of product is essential. Method development for the finished product or in process tests and sample preparation drug product is to provide practical approaches for determining selectivity, specificity, limit of detection, limit of quantitation, linearity, range, accuracy, precision, recovery solution stability, ruggedness, robustness etc. This review article explains about the strategy and importance of validation of analytical methods.

Keywords: Accuracy; Analytical Method; Linearity; Precision; Range

Introduction

The analytical procedure [1] refers to the way of performing the analysis. Analytical method validation is required to develop new process, new molecules, active ingredients, residues, impurity profiling and component of interest in different matrices. An analytical [2] methodology consists of the techniques, method, procedure and protocol. This methodology includes the required data for a given analytical problem, sensitivity, accuracy, range of analysis and precision to the analyst. It is required for assuring quality, achieving acceptance of products by the international agencies, mandatory requirement purposes for accreditation as per ISO guidelines, mandatory requirement for registration of any pharmaceutical product or pesticide formulation. The main objective is to demonstrate that the procedure is suitable for its intended purpose. The International Conference On Harmonization (ICH) used the forefront of developing the harmonized tripartite guidelines for adoption in the US, Japan and EC issued two guidelines under the titles 'Text on validation [3] of Analytical procedures (Q2A)' and validation of 'Analytical procedure Methodology (Q2B)' [4]. This article gives idea about for the validation of analytical methods for both in-house developed as well as standard methods.

Materials and Methods

Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose.

- **Optimization of an analytical method with acceptance criteria:**

GOAL	COMMENT
Resolution (Rs)	Precise and rugged quantitative analysis requires that Rs be greater than 1.5.

Separation time	< 5-10 min is desirable for routine procedures
Quantitation	≤ % (1SD) for assay; ≤ % 5 for less demanding analysis; ≤15% for trace analysis.
Pressure	< 150 bar is desirable
Peak height	Narrow peaks are desirable for large signal/ noise ratios
Solvent consumption	Minimum mobile phase use per run is desirable

Types of Analytical Procedures to Be Validated

- The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:
- Identification tests;
- Quantitative tests for impurities' content;
- Limit tests for the control of impurities;
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component (s) in the drug product.

Strategy for Validation of Methods

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to the unknown samples analyzed in the routine. The preparation and execution should follow a validation protocol, preferably written in a step by step instruction format.

Steps in Method Validation [5]

- Develop a validation protocol or operating procedure for validation.
- Define the application, purpose and scope of the method.
- Define the performance parameters and acceptance criteria.
- Define validation experiments.
- Verify relevant performance characteristics of equipment.
- Qualify materials, e.g. standards and reagents.
- Perform pre-validation experiments.
- Adjust method parameters or/and acceptance criteria if necessary.
- Perform full internal (and external) validation experiments.
- Develop standard operating procedures for executing the method in the routine.
- Define criteria for revalidation.
- Define type and frequency of system suitability tests and/or Analytical Quality Control (AQC) checks for the routine.
- Document validation experiments and results in the validation.

Validation of Standard Methods

A laboratory applying a specific method should ensure that they have documentary evidence that the method is appropriately validated. "The responsibility is with the user to ensure that the validation documented in the method is sufficiently complete to meet the needs". When standard methods are used, their scope should be in line with the scope of the laboratories, method requirements and the suitability of the entire analytical system in the specific laboratory 's environment should be verified for the method. The laboratory should demonstrate the validity of the method in the laboratories environment. Full validation of a standard method is recommended where no information on type and results of validation can be found in the standard method documentation.

Revalidation

Revalidation is necessary whenever a method is changed and the new parameter is outside the operating range. Operating ranges clearly define for each method based on experience with similar methods or they should be investigated during method developments. These ranges are verified during method validation in robustness studies and may be part of the method characteristics.

Analytical Method Validation [6]

The performance characteristics required to validate various methods by using various guidelines such as USP, ICH, FDA, European guidelines etc.

According to USP

The analytical parameters can be validated are accuracy, precision, specificity, detection of limit, quantitation limit, linearity, range, ruggedness and robustness.

According to ICH

The analytical parameters can be validated are accuracy, precision, specificity, detection of limit, quantitation limit, linearity, range, system suitability and robustness.

According to FDA

The analytical parameters can be validated are accuracy, precision, specificity/selectivity, detection of limit, quantitation limit, linearity, range, system suitability, reproducibility, sample solution stability and robustness.

According to European guidelines

The analytical parameters can be validated are accuracy, precision, specificity, detection of limit, quantitation limit, linearity and range.

Parameters of the Analytical Method Validation [7-9]

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Specificity
- Detection Limit
- Quantitation Limit
- Linearity
- Range

Selectivity and Specificity [10]

Selectivity of a method refers to the extent to which it can determine particular analytes in a complex mixture without interference from other components in the mixture. The terms selectivity and specificity have often been used interchangeably. The term specificity generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that 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 very few analytical methods respond to only one analyte, the use of the term selectivity is more appropriate than specificity. The International Union of Pure and Applied Chemistry (IUPAC) have expressed the view that "Specificity is the ultimate of selectivity'. The selectivity of the analytical method must be demonstrated by providing data to show the absence of interference peaks with regard to degradation products, synthetic impurities and the matrix (excipients present in the formulated product at their expected levels).The selectivity of chromatographic methods may be assessed by examination of peak homogeneity or peak purity test (e.g., diode array, mass spectrometry) to show that the analyte chromatographic peak is not attributable to more than one component.

Identification

Suitable identification tests are used to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. The identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgement with a considerable of the interferences that could occur.

Assay and Impurity Test

Purity 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. For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labeled. Similar considerations should be given to other separation techniques. Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other. In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

Impurities are available

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and or excipients practically, this can be done by spiking pure substances drug substance or drug product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples). For the impurity test, the discrimination may be established by spiking drug substance or drug product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

Impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure e.g. pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

Linearity [11]

Linearity of an analytical method is its ability to elicit test

results that are directly proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte. The linear range of detectability that obeys Beer's law is dependent on the compound analyzed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range.

ICH defines linearity of an analytical procedure as the ability (within a given range) to obtain test results of variable data (e.g. absorbance and area under the curve) which are directly proportional to the concentration (amount of analyte) in the sample. The data variables that can be used for quantitation of the analyte are the peak areas, peak heights, or the ratio of peak areas (heights) of analyte to the internal standard peak. Quantitation of the analyte depends on it obeying Beer's law for the spectroscopic method over a concentration range. Therefore, the working sample concentration and samples tested for accuracy should be in the linear range. There are two general approaches for determining the linearity of the method. The first approach is to weigh different amounts of standard directly to prepare linearity solutions at different concentrations. However, it is not suitable to prepare solution at very low concentration, as the weighing error will be relatively high. Another approach is to prepare a stock solution of high concentration. Linearity is then demonstrated directly by dilution of the standard stock solution. This is more popular and the recommended approach. Linearity is best evaluated by visual inspection of a plot of the signals as a function of analyte concentration. The variable data are generally used to calculate a regression line by the least squares method. At least five concentration levels should be used. Data is used by linear least square regression method of the linear equation $y = mx + c$. Linearity is acceptable with a coefficient of determination (r^2) value should be close to ±1. The slope, residual sum of squares, and *y* intercept should also be reported as required by ICH. The slope of the regression line will provide an idea of the sensitivity of the regression, and hence the method that is being validated. The *y* intercept will provide an estimate of the variability of the method.

Range [12]

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

Method	Range
Assay of drugs	80-120% of the target concentration
Content uniformity	70-130%
Impurity determination	Reporting level of impurity to 120% of the specification
Dissolution testing	±20% Over specified range of the test

Table 1: Recommended range for analytical methods by ICH.

Accuracy [13]

The International Conference On Harmonization (ICH) defines the accuracy of and analytical procedure as the closeness of agreement between the values that are accepted either as conventional true values or an accepted reference value and the value found. The accuracy of an analytical method may be defined as the closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. For drug substance, accuracy may be defined by the application of the analytical procedure to an analyte of known purity (e.g. a reference standard). For the drug product, accuracy will be determined by application of the analytical procedure to synthetic mixtures of the drug product components to which known amounts of analyte have been added within the range of the procedure. The ICH document also recommends assessing a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations/three replicates). Accuracy is usually reported as percent recovery by the assay (using the proposed analytical procedure) of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. The range for the accuracy limit should be within the linear range. Typical accuracy of the recovery of the drug substance is expected to be about 99 -101%. Typical accuracy of the recovery of the drug product is expected to be about 98 - 102%. Values of accuracy of recovery data beyond this range need to be investigated as appropriate. The accuracy of an analytical method may be determined by spiked placebo (product matrix) recovery method and standard addition method.

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 ingredient), 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. accuracy should be established across the specified range of the analytical procedure.

Assay of Drug Substance

Accuracy of drug substance can be determined by

application of an analytical procedure to an analyte of known purity (e.g. reference material) and comparison of the results of the proposed analytical procedure with those of a second well characterized procedure, the accuracy of which is stated. Accuracy may be inferred once precision, linearity and specificity have been established.

Assay of Drug Product

Accuracy of drug product can be determined by application of the analytical procedure to synthetic mixtures of the product components to which known quantities of the substance to be analysed have been added.

It is impossible to obtain samples of all product components, it may be acceptable either to add known quantities of the analyte to the product or to compare the results obtained from a second, well characterized procedure, the accuracy of which is stated. Accuracy may be inferred once precision, linearity and specificity have been established.

Impurities (Quantitation)

Accuracy should be assessed on samples (substance/ product) spiked with known amounts of impurities. it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. 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 [14]

According to USP the precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is usually investigated at three levels repeatability, intermediate precision, and reproducibility.

Repeatability

Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time while reproducibility involves precision study at different occasions, different laboratories and different batch of reagent, different analysts and different equipments. Repeatability is a measure of the precision under the same operating conditions over a short interval of time that is under normal operating conditions of the analytical method with the same equipment. It is sometimes referred to as intra assay precision. Reporting of the standard deviation, relative standard deviation (coefficient of variation), and confidence interval is required. The assay values are independent analyses of samples that have been carried through the complete analytical procedure from sample preparation to final test result.

The ICH recommends that repeatability be assessed using a minimum of nine determinations covering the specified range for the procedure (e.g., three concentrations/ three replicates as in the accuracy experiment) or using a minimum of six determinations at 100% of the test concentration. It is normally expected that at least six replicates be carried out and whatever results obtained are used to calculate the mean, standard deviation and co-efficient of variation for set of n values. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. (RSD below 1% for built drugs, RSD below 2% for assays in finished product).

$$\%RSD=\frac{\text{Standard deviation}}{\text{Mean}}\times 100 \quad \text{(1)}$$

Intermediate Precision

According to ICH intermediate precision is defined as the long term variability of the measurement process. Intermediate precision is the variation within the same laboratory. The extent to which intermediate precision needs to be established depends on the circumstances under which the procedure is intended to be used. Typical parameters that are investigated include day to day variation, analyst variation, and equipment variation. Depending on the extent of the study, the use of experimental design is encouraged. Experimental design will minimize the number of experiments that need to be performed. It is important to note that ICH allows exemption from doing intermediate precision when reproducibility is proven. It is expected that the intermediate precision should show variability that is in the same range or less than repeatability variation. ICH recommends the reporting of standard deviation, relative standard deviation (coefficient of variation) and confidence interval of the data.

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment etc.

Reproducibility

Reproducibility measures the precision between multiple laboratories. This parameter is considered in the standardization of an analytical procedure (e.g., inclusion of procedures in pharmacopeias and method transfer between different laboratories) To validate this characteristic; similar studies need to be performed at different laboratories using the same homogeneous sample lot and the same experimental design. In the case of method transfer between two laboratories, different approaches may be taken to achieve the successful transfer of the procedure. Comparisons

of results obtained by different analysts by the use of different equipments, or by carrying out the analysis at different times can also provide valuable information. The standard deviation, relative standard deviation (coefficient of variation), coefficient of variation and confidence interval should be reported for each type of precision investigated. The most common approach is the direct method transfer from the originating laboratory to the receiving laboratory. The originating laboratory is defined as the laboratory that has developed and validated the analytical method or a laboratory that has previously been certified to perform the procedure and will participate in the method transfer studies. The receiving laboratory is defined as the laboratory to which the analytical procedure will be transferred and that will participate in the method transfer studies. In the direct method transfer, it is recommended that a protocol be initiated with details of the experiments to be performed and acceptance criteria (in terms of the difference between the means of the two laboratories) for passing the method transfer. 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.

Detection Limit/Limit of Detection (LOD) [15]

The Limit of Detection (LOD) of an analytical procedure is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated. It is a limit that specifies whether or not an analyte is above or below certain value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. The detection limit is a characteristic for the limit test only. It is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. The detection is usually expressed as the concentration of the analyte in the sample, for example, percentage; Parts Per Million (ppm) or Parts Per Billion (ppb). There are several approaches to establish the LOD such as visual evaluation, signal to noise and standard deviation methods.

Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. Visual evaluation may be used for non-instrumental (e.g. solution color) and instrumental methods. Detection limit 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. Presentation of relevant chromatograms or other relevant data is sufficient for justification of the LOD.

Based on signal-to-noise

This approach can only be applied to analytical procedures which exhibit baseline noise. For instrumental procedures that exhibit background noise, it is common to compare measured signals from samples with known low concentrations of analyte with those of the blank samples. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

The signal-to noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to-noise ratio.

The signal- to- noise ratio may be expressed as

$$S = H/h \qquad (2)$$

Where His height of paek corresponding to the component, h is absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

Based on the standard deviation

The method used to determine LOD should be documented and supported, and an appropriate number of samples should be analysed at the time to validate the level.

The detection limit may be expressed as

$$LOD = 3.3 \frac{\sigma}{S} \qquad (3)$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways. The IUPAC approach employs the standard deviation of the intercept (Sa) which may be related to LOD and the slope of the calibration curve, b. The detection limit may be expressed as

$$LOD = 3\ Sa / b \qquad (4)$$

Quantitation Limit/Limit of Quantitation [16]

The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy and precision under the stated operational conditions of the method. Limit of Quantitation (LOQ) is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental

or instrumental. There are several approaches to establish the LOQ such as visual evaluation, signal to noise and standard deviation methods.

Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. Visual evaluation may be used for non-instrumental (e.g. solution color) and instrumental methods. Quantitation limit 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. Presentation of relevant chromatograms or other relevant data is sufficient for justification of the LOQ.

Based on signal-to-noise approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Based on the standard deviation

The method used to determine LOQ should be documented and supported, and an appropriate number of samples should be analysed at the time to validate the level.

The quantitation limit may be expressed as

$$LOQ = 10\frac{\sigma}{S} \qquad (5)$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways.

Robustness [17]

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The robustness of a method is evaluated by varying method parameters such as percent organic solvent, pH, ionic strength, temperature and determine the effect (if any) on the results of the method. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. The evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used. In 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 etc. In the case of gas-chromatography examples of typical variations are different columns (different lots or suppliers), temperature, flow rate etc.

Ruggedness [18]

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 of the analytical method.

System Suitability Testing [19]

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. In the final analysis, purpose of validating method is to ensure the procurement of high quality data. 3.0.

Conclusion

Analytical method validation plays a fundamental role in pharmaceutical industry for releasing the commercial batch and long term stability data. Hence the data must be produced to acceptable scientific standards. Therefore, the need to satisfy regulatory authority requirements all analytical methods should be properly validated and documented.

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