



VALIDATION OF ANALYTICAL METHODS – STRATEGIES & IMPORTANCE

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ABSTRACT

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc. In this review article we discussed about the strategy and importance of validation of analytical methods.

Keywords: Validation; Analysis; Accuracy; Precision

INTRODUCTION

Analytical chemistry, which is both theoretical and practical science, is practical in a large number of laboratories in many diverse ways. The analytical procedure refers to the way of performing the analysis. Analytical method validation is required for herbal procedure, new process and reaction, new molecules, active ingredients, residues, impurity profiling and component of interest in different matrices. An analytical methodology consists of the techniques, method, procedure and protocol. This methodology includes the required data for a given analytical problem, required sensitivity, required accuracy, required range of analysis and required 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 17025 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 word validation was not mentioned in the current Good Manufacturing Practices (cGMP's) of 1971, and precision and accuracy were stated as laboratory controls. The need for validation was implied only in the cGMP guideline of March 1979. It was done in two sections: (1) Section 211.165, where the word 'validation' was used and (2) section 211.194, in which the proof of suitability, accuracy and reliability was made compulsory for regulatory submissions.

The World Health Organization (WHO) published a guideline under the title, 'Validation of analytical procedures used in the examination of pharmaceutical materials'. It appeared in the 32nd report of the WHO expert committee on 'specifications for pharmaceutical preparations' which was published in 1992.

The International Conference on Harmonization (ICH), which has been on the forefront of developing the harmonized tripartite guidelines for adoption in the US, Japan and EC, has issued two guidelines under the titles-'Text on validation of Analytical procedures (Q₂A) and validation of Analytical procedure Methodology (Q₂B)'.¹

Among the pharmacopoeias, USP XXII 1225 (1995) has a section which describes requirements of validation of compendia methods. The British Pharmacopoeia includes the definition of method validation in latest editions, but the term is completely missing from the Indian Pharmacopoeia (1996).

The United States Environmental Protection Agency (US EPA) prepared a guidance for methods development and validation for the Resource Conservation and Recovery Act (RCRA).¹ The pharmaceutical industry uses methodology published in the literature.² The most comprehensive document was published as the

'Conference Report of the Washington conference on analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies held in 1990 (sponsored by the American Association of Pharmaceutical Scientists, the AOAC and the US FDA, among others).³ The report gives guiding principles for validation of studies in both human and animal subjects that may be referred to in developing future formal guidelines. Hokanson applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods.^{4,5}

Green gave a practical guide for analytical method validation with a description of a set of minimum requirements for a method.⁶ Renger and his colleagues described the validation of a specific analytical procedure for the analysis of theophylline in a tablet using high performance thin layer chromatography (HPTLC).⁷ The validation procedure in that article is based on requirements for European Union multistate registration. Wegscheider has published procedures for method validation with special focus on calibration, recovery experiments, method comparison and investigation of ruggedness.⁸

The Association of Official Analytical Chemists (AOAC) has developed a Peer-Verified Methods validation program with detailed guidelines on what parameters should be validated.⁹ This article gives a review and a strategy for the validation of analytical methods for both in-house developed as well as standard methods and a recommendation on the documentation that should be produced during and at the end of method validation.

Types of analytical procedures to be validated

Discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

1. Identification tests
2. Quantitative tests for impurities content
3. Limit tests for the control of impurities
4. Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product

Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a property of the sample (e.g., spectrum, chromatographic behavior, chemical reactivity, etc) to that of a reference standard. Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample. Different validation characteristics are required for a quantitative test than for a limit test. Assay procedures are intended to measure the analyte present in a given sample. In the perspective of this document, the assay represents a

quantitative measurement of the major component(s) in the drug substance. For the drug product, similar validation characteristics also apply when assaying for the active or other selected component(s). The same validation characteristics may also apply to assays associated with other analytical procedures.

The various validation parameters are:

- Accuracy
- Precision (repeatability and reproducibility)
- Linearity and range
- Limit of detection (LOD)/ limit of quantitation (LOQ)
- Selectivity/ specificity
- Robustness/ ruggedness
- Stability and system suitability studies

Advantages of analytical method validation

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

Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

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. Possible steps for a complete method validation are listed below.

Steps in method validation

1. Develop a validation protocol or operating procedure for the validation
2. Define the application, purpose and scope of the method
3. Define the performance parameters and acceptance criteria
4. Define validation experiments
5. Verify relevant performance characteristics of equipment
6. Qualify materials, e.g. standards and reagents
7. Perform pre-validation experiments
8. Adjust method parameters or/and acceptance criteria if necessary
9. Perform full internal (and external) validation experiments
10. Develop SOPs (standard operating procedures) for executing the method in the routine
11. Define criteria for revalidation
12. Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for the routine
13. Document validation experiments and results in the validation.

First the scope of the method and its validation criteria should be defined. These include: compounds, matrices, type of information, qualitative or quantitative, detection and quantitation limits, linear range, precision and accuracy, type of equipment and location. The scope of the method should include the different types of equipment and the locations where the method will be run. The method's performance characteristics should be based on the intended use of the method. For example, if the method will be used for qualitative trace level analysis, there is no need to test and validate the method's linearity over the full dynamic range of the equipment. Initial parameters should be chosen according to the analyst's best judgment. Finally, parameters should be agreed between the lab generating the data and the client using the data.¹⁰

Instruments performance should be verified using generic standards, before an instrument is used to validate a method.^{11,12} These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, either the method itself or the equipment or the analysis technique or the acceptance limits should be changed. In this way method development and validation is an iterative process. For example, in liquid chromatography selectivity is achieved through selection of mobile phase composition. For quantitative measurements the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization. There are no official guidelines on the sequence of validation experiments and the optimal sequence can depend on the method itself.

Validation of standard methods

A laboratory applying a specific method should ensure that they have documentary evidence that the method has been appropriately validated. "The responsibility is with the user to ensure that the validation documented in the method is sufficiently complete to meet his or her needs".¹³ 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

A revalidation is necessary whenever a method is changed and the new parameter is outside the operating range. Operating ranges should be clearly defined for each method based on experience with similar methods, or they should be investigated during method developments. These ranges should be verified during method validation in robustness studies and should be part of the method characteristics. Availability of such operating ranges makes it easier to decide when a method should be revalidated. If, for example, the operating range of the column temperature has been specified to be between 35 and 40°C, if, for whatever reason, the new operating parameter has been selected as 42°C, and then the method should be revalidated. Revalidation is also required if the sample matrix changes and if the instrument type changes.

Key parameters of the analytical method validation

It is important for one to understand the parameters or characteristics involved in the validation process. The various performance parameters, which are addressed in a validation exercise, are grouped as follows.

Accuracy

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. The accuracy of an analytical method may be determined by any of the following ways:

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

In both methods (spiked – placebo recovery and standard addition method), recovery is defined as the ratio of the observed result to the expected result expressed as a percentage.

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

Accuracy may also be determined by comparing test results with those obtained using another validated test method. Dosage form assays commonly provide accuracy within 3-5% of the true value. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated determination for each concentration).¹⁴

Precision

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 a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances.

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.

Determination of repeatability

It is normally expected that at least six replicates be carried out and a table showing each individual result provided from which the mean, standard deviation and co-efficient of variation should be calculated for set of n value. 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). The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

Determination of reproducibility

Reproducibility means the precision of the procedure when it is carried out under different conditions-usually in different laboratories-on separate, putatively identical samples taken from the same homogenous batch of material. 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.^{15,16}

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well defined mathematical transformation) 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. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation $y = ax + b$ together with the correlation coefficient of determination r. For the method to be linear the r value should be close to ± 1 .

Range

The range of an analytical method is the interval between the upper and lower levels of the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

The following minimum specified ranges should be considered.

- For the assay of an active substance or a finished product: normally from 80 to 120 percent of the test concentration;
- For content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider, more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;
- For dissolution testing: ± 20 percent over the specified range; e.g., if the specifications for a controlled released product cover a region from 20%, after 1 h, up to 90%, after 24 h, the validated range would be 0-110% of the label claim.
- For the determination of an impurity: from the reporting level of an impurity to 120% of the specification; for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled.
- If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120 percent of the assay specification.

Limit of detection and limit of quantitation

Limit of detection

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. The LOD of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted. 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. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (S_a) which may be related to LOD and the slope of the calibration curve, b, by $LOD = 3 S_a / b$. 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.

Limit of quantitation

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. 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. Like LOD, LOQ is expressed as concentration, with the precision and accuracy of the measurement also reported. Sometimes a signal-to-noise ratio of 10 to 1 is used to determine LOQ.

It is measured by analyzing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where, the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of

the limit of quantitation. In many cases, the limit of quantitation is approximately twice the limit of detection.

Selectivity and specificity

Selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components in the mixture. The terms selectivity and specificity have often been used interchangeably. 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 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 IUPAC discourages use of the term specificity and instead encourages the use of the term 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.^{10,17-19}

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case, a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

Identification: To ensure the identity of an analyte.

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.

Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample.

Robustness and ruggedness

Robustness

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. One consequence of 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.

Examples of typical variations are stability of analytical solutions and extraction time

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions such as different laboratories, different analysts, using operational and

environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

For the determination of ruggedness, the degree of reproducibility of test result is determined as function of the assay variable. This reproducibility may be compared to the precision of the assay under normal condition to obtain a measure of the ruggedness of the analytical method.¹⁴

Table 1: Characteristic that should be considered for different types of analytical procedure

Parameters	Class A	Class B		Class C	Class D
		Quantitative tests	Limit tests		
Accuracy	-	Yes	-	Yes	Yes
Precision	Yes	Yes	-	Yes	Yes
Robustness	Yes	Yes	Yes	Yes	Yes
Linearity	Yes	Yes	-	Yes	Yes
Range	-	-	-	-	-
Selectivity	Yes	Yes	Yes	Yes	Yes
Limit of Detection	-	Yes	Yes	-	-
Limit of Quantitation	-	Yes	-	-	-

Analytical methods may be broadly classified as Per USP as follows:

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.

Stability and system suitability tests

Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 h stability is desired for solutions and reagents that need to be prepared for each analysis.

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. The nature of the test and the acceptance criteria will be based upon data generated during method development optimization and validation experiments.

Documentation of system suitability can be accomplished by using software specifically designed for the task to provide a review of method development and to summarize the data regarding reproducibility.¹⁸

Validation characteristics and requirements

There are various analytical methods used for the examination of pharmaceutical materials. Not all the characteristics referred above

will need to be considered in all cases. Analytical methods may be broadly classified as Per WHO as follows:

Class A: Tests designed to establish identity, whether of bulk drug substances or of a particular ingredient in a finished dosage form.

Class B: Methods designed to detect and quantitative impurities in a bulk drug substance or finished dosage form.

Class C: Methods used to determine quantitatively the concentration of a bulk drug substance or of a major ingredient in a finished dosage form.

Class D: Methods used to assess the characteristic of finished dosage forms, such as dissolution profiles and content uniformity.

Table 2: Characteristics required for assay validation as per USP

Analytical performance characteristics	Assay category I	Assay category II		Assay category III	Assay category IV
		Quantitative tests	Limit tests		
Accuracy	Yes	Yes	*	*	
Precision	Yes	Yes		Yes	
Specificity	Yes	Yes	Yes	*	Yes
Limit of Detection			Yes	*	
Limit of Quantitation		Yes		*	
Linearity	Yes	Yes		*	
Range	Yes	Yes	*	*	

Where, * indicates that may be required depending on the nature of the specific test.

CONCLUSION

Validation is a constant, evolving process that starts before an instrument is placed on-line and continues long after method development and transfer. In this review article we discussed about the importance and types of validation of analytical methods. From the above discussed matter we concluded that the validation of developed analytical methods is critical elements in the development of pharmaceuticals. Success in these areas can be attributed to several important factors, which in turn will contribute to regulatory compliance.

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