Review Article

Method development and validation: Skills and tricks

Abstract

Because of the introduction of a lot of drugs in the market every year, it becomes necessary to develop newer analytical methods for such drugs. Method development can take a number of forms. At one extreme, it involves adapting an existing method or making minor changes so as to make it suitable for the new application, as for developing a method for the estimation of drugs using a complex analytical technique like HPLC. After the development, there is a need of method validation. Method validation is defined as the process of proving that an analytical technique is acceptable for the intended use and this is an important requirement for analytical purpose. Validation is done according to the guidelines of ICH and FDA. Here, in this review, we have discussed method development and the various parameters used for method validation, namely accuracy, precision, limit of detection, limit of quantification, specificity, robustness, ruggedness, and range.

Key words:
Analytical, FDA, high performance liquid chromatography, ICH

Introduction

In industries, new measurement technologies can only be adopted if a sound scientific rationale for the application has been developed, proven, and justified and the developed method has been approved by internal company procedures.[1] A number of drugs are being introduced in the market every year, and these are either new drugs or the modification of the existing moieties. Because of the possible uncertainties in the continuous use of these drugs, appearance of some new toxicities and patient resistance or introduction of some better drug leads to a time lag from the date of introduction of drug in the market to the date of its inclusion in pharmacopoeias. So there is a need to develop and validate newer analytical techniques for such drugs as it may not be available in pharmacopoeias.[2]

It is internationally recognized that a developed method should necessarily be validated as these validation methods also show the qualification and competency of the analytical laboratory.[3] Analytical measurements are associated with every aspect of society, and there are innumerable reasons for making these measurements. Clearly, it is important to determine the correct result and be able to show that it is correct. Therefore, a method validation is required.[2] For e.g., the expanding use of innovative botanical ingredients in dietary supplements and foods has resulted in a flurry of research aimed at the development and validation of analytical methods for the accurate measurement of active ingredients.[4]

Method Development

Method validation often evolves from method development. Method development can take a number of forms.[5] At one extreme, it may involve adapting an existing method, making minor changes so that it is suitable for a new application.[6] It requires a lot of effort, and there is a degree of doubt initially to whether the method will be successful. It involves working on various ideas simultaneously and then finally picking one of those. Various steps involved in method development and validation are:[7]

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Method development plan definition
↓
Background information gathering
↓
Laboratory method development
↓
Generation of test procedure
↓
Methods validation protocol definition
↓
Laboratory methods validation
↓
Validated test method generation
↓
Validation report

A well-developed method is always to validate.[7] While using any analytical technique for the estimation of the drug it needs a proper method to be developed. Let us take an example of a most complex analytical technique, i.e., high performance liquid chromatography (HPLC) which is complex in the sense that there are a wide variety of equipments, columns, eluents, and other parameters for operation which makes it so. There are various aspects which should be kept in mind while developing a method for HPLC.[8] They are explained as follows:

- Selection of the HPLC method which includes choosing either of the two, reverse phase or normal phase HPLC depending upon the nature of the sample, for example, for polar analytes we use reverse phase HPLC so that we obtain better retention and resolution and for low or medium polarity samples we generally prefer normal phase chromatography
- Then, choosing a proper mobile phase for the given analyte is the most crucial stage in developing a method for HPLC. A mobile phase which has the capability of pulling the analyte from the column is chosen. When dealing with weak acids and bases, we have to adjust the pH also as it affects the retention
- A stationary phase is generally C_{18} bonded in the case of reverse phase HPLC and cyano-bonded in the normal phase
- Then, the detectors are selected based on the nature of the analyte. We observe that whether it has chromophores which will enable their detection in UV while using UV-detectors. Fluorescence detectors are used in the case of trace analysis and in preparative HPLC refractive index detectors are used.

Therefore, these criteria are kept in mind while developing a method for HPLC and this developed method is further being validated using various parameters which are defined later in this review; for example, development and validation for the determination of the level of EDTA in nonalcoholic drinks using HPLC. In this a reverse phase C_{18} column was used and the column was eluted in 0.01 M ammonium phosphate monobasic: acetonitrile:40% tetrabutylammonium hydroxide (90:10:0.2).[9] Another example is reverse phase HPLC for the estimation of ezitimibe in its tablet dosage form,[10] a method for this drug was developed and validated. Reverse phase HPLC has been used extensively for various drug combinations such as cefixime and dicloxacilin, ropinirole hydrochloride, paracetamol and tramadol, ofloxacin and ornidazole, and so on.[11-14]

Problems in method development

1. Stored samples are initially accurate but slowly become inaccurate with low bias
2. Absorption issue: A serially diluted curve is concave. The response factors drop with decreasing concentration. An increased exposure due to number of dilutions, surface area contact, and time may cause this problem
3. Homogeneity: the sample to be analysed gets partitioned.

These problems can be overcome by adding a surfactant to the sample under test.

Method Validation

The word validation originated from the Latin word *validus* meaning strong, and suggests that something has been proved to be true, useful, and of an acceptable standard.[15] Method validation can be defined as the process of proving that a particular developed analytical method is acceptable for its intended use.[7,16-19] Validation is an important requirement in the practice of an analytical process. Method validation can be interpreted as the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with that the application requires.[5] In connection with biotechnological synthesis of pharmaceutical drugs, validated methods for quantification of both the product and the substrate at different time intervals are essential for proper calculation of rate coefficients.[20] The current trend is in the direction of phase-dependent methods development and validation. Analytical methods are progressively optimized, and a preliminary validation package is furnished as part of the IND application before Phase I safety trials are initiated. All analytical methods should be fully optimized and validation should be completed before the NDA is submitted at the end of Phase III studies.[7,21] Method validation is a continuous process, and the final goal of validation of an analytical method is to ensure that every future measurement in routine analysis will be close enough to the unknown true value for the content of the analyte in the sample.[22,23] Because of the ongoing advances in analytical chemistry technologies, analytical methods are updated over time; thus, validation and cross validation of methods become important for accurate interpretation of data collected over years.[24-29]

Validation is performed with a formal, approved, and signed methods validation protocol in quality assurance (QA) unit.

Validation is complete when we:

1. Demonstrate that you have met all the acceptance criteria
2. Clearly document the results in a cGMP compliant fashion
3. Show how you met the acceptance criteria in a final methods validation report, including references to raw data, all of which have been reviewed and approved by the appropriate personnel including peers, management, and QA.

Validation guidelines

1. ICH Q2A text on validation of analytical procedures: definitions and terminology (March 1995)[30]
2. ICH Q2B validation of analytical procedures: methodology (June 1997)
3. FDA (Draft) guidance for industry: analytical procedures and methods validation
4. Pharmacopoeias USP and European Pharmacopoeia.

Why is analytical method validation required?

Method validation is required for the following reasons:[19]

1. For assuring the quality of the product
2. For achieving the acceptance of the products by the international agencies
3. It is a mandatory requirement for accreditation as per ISO 17025 guidelines
4. A mandatory requirement for registration of any pharmaceutical product or pesticide formulation.

Validated methods are only acceptable for undertaking proficiency testing.

Validation not only improves the processes, but also confirms that the process is properly developed. For the manufacturer method validation is important in the following aspects:

- It deepens the understanding of processes and decreases the risk of preventing problems
- It decreases the risk of defect costs
- It decreases the risk of regulatory noncompliance
- A fully validated process may require less in-process controls and end product testing.[31,32]

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 a drug substance.

Identification tests are intended to ensure the identity of an analyte in a sample. This is 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. The test is intended to 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.[33]

Steps in the methods validation process

Method evaluation and further method development
↓
Final method development and trial method validation
↓
Formal method validation and report generation
↓
Formal data review and report issuance

Total time for method evaluation, validation, data/documentation review, and reporting is approximately 6–10 months.[43]

Criteria that must be satisfied by a validation process

1. The whole method must be validated. It is quite usual to focus on the detection technique or the instrumental measurement, which often means that just this stage is validated. However, the previous steps of sample pretreatment, extraction, or preconcentration also belong to the method of analysis and are of utmost importance. Therefore, they must all be validated
2. The whole range of concentrations must be validated. It is difficult to comply with this condition because a method may work very well in one particular concentration range but not in others
3. The whole range of matrices must be validated. It is well known that the matrix can have a decisive effect on the analysis. Therefore, and for the sake of representativeness, several matrices must be submitted to method validation.[35]

Performance characteristics for method validation

- Accuracy
- Precision
- Specificity
- Limit of detection
- Limit of quantification
- Linearity
- Range
- Robustness
- Ruggedness

Accuracy

It is the closeness to the true value, measured by % recovery

of sample spikes or % error in the analysis of a reference sample.[36] Accuracy is normally studied as two components: 'trueness' and 'precision'.[6,37-41] The 'trueness' (of a method) is an expression of how close the mean of a set of results (produced by the method) is to the true value. Trueness is normally expressed in terms of bias.

Trueness

Two techniques are available and if possible both should be performed.

a. Method comparison: If you are introducing a new method into the department, which has already been validated, compare the results from the two methods for the same samples. It is recommended that a minimum of 10 samples are compared; however, more the results better the comparison. If an analyser is being used then this number should be at least 30 samples. Use a statistical package to compare the two methods using linear regression, thereby calculating any bias[5]
b. Reference comparison: Obtain reference material from the relevant External Quality Assessment scheme along with the statistical results showing all method mean, or if available a certified reference material. Run the EQA samples as many times as possible (depending on available sample) up to a maximum of 10 and determine the mean and standard deviation of these replicate tests. Compare the results with the method mean obtained nationally.[5]

Accuracy should be established across a specified range of analytical procedure. It should be assessed using a minimum of three concentration levels, each in triplicate (total of nine determinations). Results of the accuracy study should be reported as:

- Percent recovery of the known amount added or
- The difference between the mean assay result and the accepted value.

A set of data showing the accuracy study is given in Table 1. Data give the information about the amount of analyte added and the percentage recovery of the same.

Precision

The degree of agreement between replicate analyses of a homogenous sample, usually measured as the relative standard deviation (RSD) of a set of replicates.[36] The measured standard deviation can be subdivided into three categories: repeatability, intermediate precision, and reproducibility.[42] Repeatability is obtained when one operator using one piece of equipment over a relatively short time-span carries out the analysis in one laboratory. At least five or six determinations at two or three different concentrations should be done and the RSD calculated.[19] Precision is usually stated in terms of standard deviation or RSD. Both repeatability and reproducibility are generally dependent on analyte concentration, and so should be determined at a number of concentrations and if relevant, the relationship between precision and analyte concentration should be established.[5]

- Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time.[42] From the repeatability standard deviation or sr it is useful to calculate the 'repeatability limit '*r*'', which enables the analyst to decide whether the difference between duplicate analyses of a sample, determined under repeatability conditions, is significant[6]
- Reproducibility: Reproducibility expresses the precision between laboratories.[42] From the reproducibility standard deviation or sr it is useful to calculate the 'reproducibility limit *R*', which enables the analyst to decide whether the difference between duplicate analyses of a sample, determined under reproducibility conditions, is significant. These calculations can be performed directly with the built-in statistics function of the instrument, if available, or by using a pocket calculator or a Personal Computer (PC) with a suitable software package (e.g. spreadsheet program).[6]

Specificity

It is the ability to measure the desired analyte in a complex mixture.[7] It is the degree of bias (or lack thereof) caused by expected sample components and common interferences, determined by measuring the analyte with and without anticipated interferences.[36] For example, it has been recommended that when developing an analysis for a drug in blood or plasma, that at least six independent sources of blank matrix be tested for interferences.[36,43] 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.[42]

Table 1: Recovery studies for the determination of an analyte in tablet dosage form by initial rate method

Formulations	Initial rate method				
	Amount taken (μg/ml)	Amount added (μg/ml)	Amount found (μg/ml)±SD	% Recovery	% RSD
Analyte	5	4	9.01±0.018	100.46	0.200
		5	9.98±0.057	99.68	0.571
		6	11.08±0.016	101.38	0.144

Values are mean±SD for 3 determinations

Specificity relates to the ability of the test to identify negative results. Consider the example of the medical test used to identify a disease. The specificity of a test is defined as the proportion of patients who do not have the disease who will test negative for it. This can also be written as:

$$\text{specificity} = \frac{\text{number of true negatives}}{\text{number of true negatives} + \text{number off alse positives}}$$

If a test has high specificity, a positive result from the test means a high probability of the presence of disease.

Limit of detection

The limit of detection (LOD) is the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified.[6,19,42] Several approaches for determining the detection limit (DL) are possible, depending on whether the procedure is a noninstrumental or instrumental. Approaches other than those listed below may be acceptable.[42]

- Noninstrumental methods are based on the visual evaluation. The DL is determined by the analysis of samples with known concentrations of the analyte and by establishing the minimum level at which the analyte can be reliably detected
- Based on signal-to-noise, this approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of the 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 DL
- The DL may be expressed as: DL = $3.3\sigma/S$, where $\sigma\sigma$ is the standard deviation of the response, S is the slope of the calibration curve, the slope S may be estimated from the calibration curve of the analyte. The estimation of σ may be carried out in a variety of ways, for example:
  (i) Based on the standard deviation of the response, the blank measurement of the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and calculating the standard deviation of these responses
  (ii) When determining the data from a calibration curve, a specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.[42]

Limit of quantification

It is the concentration level above which the concentration can be determined with acceptable precision [usually relative standard deviation (RSD) < 10–25%] and accuracy.[6,36,42,44,45] Several approaches for determining the quantitation limit (QL) are possible, depending on whether the procedure is a noninstrumental or instrumental. Approaches other than those listed below may be acceptable.[42]

- Limit of quantification is evaluated based upon the visual evaluation which is a type of noninstrumental method. Using this method, QL is determined by the analysis of the samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision
- Based on the 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 the 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
- The QL may be expressed as: QL = $10\sigma/S$, where σ is the standard deviation of the response, S is 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 including:
  (i) Measurement of the magnitude of analytical background response is performed by analysing an appropriate number of blank samples and calculating the standard deviation of these responses
  (ii) Based on the calibration curve, a specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.[42]

Linearity

The linearity of an analytical method is its ability to elicit test results that are (directly or by means of well-defined mathematical transformations) proportional to the concentration of analytes in samples within a given range or proportional by means of well-defined mathematical transformations.[19,46,47] Linearity may be demonstrated directly on the test substance by preparing a series of dilution of a standard stock solution or by using separate weighing of synthetic mixtures of the test product components, using the proposed procedure.[19,42,48] Test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.[42]

Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of >0.999 is generally considered

as evidence of acceptable fit of the data to the regression line. The *y*-intercept should be less than a few percent of the response obtained for the analyte at the target level. Figure 1 shows the linearity of an analyte over a concentration range of 2–20 mg/ml with a correlation coefficient of 0.999.[49]

Aspects

- Test across the range (at least five concentrations)[30]
- Evaluate linearity by visual inspection of the plot and by statistical techniques
- Calculate corr. coefficient, *y*-intercept, slope, and res. sum of squares.

Range

It is the concentration, upper and lower levels, which meets the linearity, precision, and accuracy performance characteristics.[36,42] The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method.[19]

The following minimum specified ranges should be considered: [42]

- for the assay of an active substance or a finished product: normally from 80 to 120% of the test concentration;
- for content uniformity, covering a minimum of 70–130% 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% 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 1–120% 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.

To demonstrate an acceptable linear range, it is generally suggested to prepare five different standard solutions from 50% to 150% of the target analytical concentration.[15,47]

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit; 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 1–120% of the assay specification.[42]

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.[36,42,50] For the determination

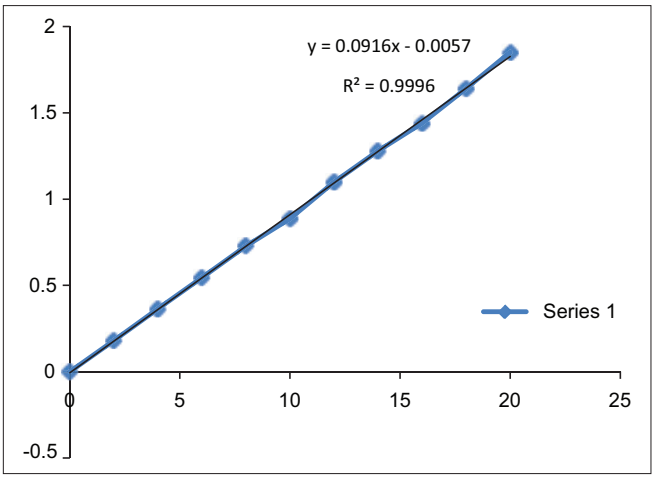

Figure 1: Figure shows the linearity of an analyte over a concentration range of 2-20 μg/ml with a correlation coefficient of 0.999

of a method's robustness, a number of method parameters, for example, pH, flow rate, column temperature, injection volume, detection wavelength, or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range.[19,51]

Ruggedness

Ruggedness is normally evaluated during method development, typically by the originating laboratory, before collaborating with other laboratories and is a measure how well a method stands up to less than perfect implementation. In any method there will be certain stages, which, if not carried out with sufficient care, will have a severe effect on method performance, and may even result in complete loss of the activity of the method. These stages should be identified, usually as a part of method development, and if possible, their influence on method performance evaluated using 'ruggedness tests'.[6] It is the variance in the analysis of homogenous samples between analysts and laboratories. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst.[19,51,52]

As we discussed method development of HPLC, after developing a method it needs proper validation and all the above-mentioned parameters are studied for these developed methods.

Validation characteristics and requirements given by WHO

There are a number of 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.

For all these classes given by WHO, characteristics that should be considered for them are given in Table 2.[53]

Classification of analytical methods as per USP

- 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.[51]

Characteristics required to be validated according to USP are given in Table 3.

Revalidation

Revalidation means the repetition of the validation process. A validation status once reached is not static, but is subject to everyday dynamics. A revalidation is necessary whenever a method is changed and the new parameter is outside the operating range. Processes and procedures should undergo periodic critical re-evaluation to ensure that they remain capable of achieving the intended results. As in industrial manufacturing, process validation is carried out on the basis of approved manufacturing instructions. If at a later stage, these manufacturing instructions are intended to be changed, it is necessary to check, document, and possibly show through revalidation that this change has no effect on the product quality. For example, if ingredients such as APIs or critical excipients are changed or even their percentage of quantity is changed, the original validation results are no longer relevant.[54] Another example is that if the operating range of column temperature is changed from the specified one, i.e., 35–40°C to 42°C then the method is required to be revalidated.

Conclusion

Method development and validation play an important role in the pharmaceutical industry. Methods resulted are used in the QA of the drug entity. Validating a developed method is important as it is meaningless if the method cannot be

Table 2: 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 quantification	–	Yes	–	–	–

Table 3: Characteristics required for assay validation as per USP

Analytical performance characterisitics	Category I	Category II		Category III	Category IV
		Quantitative tests	Limit tests		
Accuracy	Yes	Yes	*	*	
Precision	Yes	Yes		Yes	
Specificity	Yes	Yes	Yes	*	Yes
Limit of detection			Yes	*	
Limit of quantification		Yes		*	
Linearity	Yes	Yes		*	
Range	Yes	Yes	*	*	

*Indicates that may be required depending upon the nature of the specific test

reproduced. Validation is always a balance between costs, risks, and technical possibilities. Therefore, a strong training will ensure successful method development and validation.

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