REVIEW

Formulation Development and Pharmacokinetic Laboratory, Pharmacy Group, Birla Institute of Technology and Science, Pilani, India

Comparison of various international guidelines for analytical method validation

S. CHANDRAN, R. S. P. SINGH

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Asst. Prof. Dr. Sajeev Chandran, Formulation Development and Pharmacokinetic Laboratory, Pharmacy Group, Birla Institute of Technology and Science, Pilani-333 031, Rajasthan, India
sajeev@bits-pilani.ac.in

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Analytical method validation is the systematic process of establishing that an analytical method is acceptable for its intended purpose. In general the developer or user of the method generates evidence on specificity, linearity range, accuracy, precision, detection limit, quantitation limit, ruggedness and robustness of the method for regulatory submissions or in-house application. The iterative process of method development and validation has a direct impact on the quality of the above data. Such validated analytical methods for qualitative or quantitative testing of drug molecules assume greater importance when they are employed to generate quality and safety compliance data during development and post-approval of drug products. The present paper aims to discuss salient points of the analytical method development and validation cycle. It also attempts to compare and summarize guidelines issued by different agencies for validation of analytical methods used for analysis of drug substances in the pure form and in pharmaceutical formulations.

1. Introduction

Analytical method development and validation procedures are vital in the discovery and development of drugs and pharmaceuticals. Analytical methods are used to aid in the process of drug synthesis, screen potential drug candidates, support formulation studies, monitor the stability of bulk pharmaceuticals and formulated products, and test final products for release. The quality of analytical data is a key factor in the success of a drug and formulation development program. During the post approval commercial production stage of bulk drugs and pharmaceutical products, the official or in-house test methods that have resulted from the analytical method development and validation process cycle become indispensable for reliable monitoring of the integrity, purity, quality, strength and potency of the manufactured products. There is often a need to transfer methodology from one laboratory to another and/or to include it in official compendia. Such exercises include the use of a method by large numbers of people, in various laboratories across the globe and on instruments manufactured by different manufacturers, thereby causing a greater probability of decreased reproducibility and reliability. These problems can be foreseen and avoided by thorough validation of the analytical method (Brown et al. 2001).

For an analytical result to be fit for its intended purpose it must be sufficiently reliable that any decision based on it can be taken with confidence. In the light of this, analytical method validation can be considered as the process of defining the analytical requirements, and confirming that the method under consideration has performance capabilities consistent with what the application requires. The method's performance has to be validated and the uncertainty of the result estimated, at a given level of confidence. It is not sufficient just to determine uncertainty, but it also should be quoted in a way that is widely recognized, internally consistent and easy to interpret. In general, methods for regulatory submission must include studies on specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, and robustness which ensure that the analytical methodology in question gives timely, accurate, reproducible and reliable data which are adequate for the intended purpose of use. Validation of an analytical method cannot eliminate all the problems likely to arise during implementation of the methodology but it does ensure that major problems are prospectively seen and a mechanism to control the variability is suggested (WHO report 1992).

Method validation procedures and acceptance criteria were for a long time matter of personal prudence until various industrial committees and regulatory agencies developed framework guidelines for performing such validations for methods applicable to drugs and pharmaceuticals (Green 1996). The submission of analytical method validation data has been made mandatory for successful submission of New Drug Applications (NDA) and Abbreviated New Drug Applications (ANDA). This requirement has in-

creased the importance given to the validation methodology employed for analytical procedures (Analytical procedures and method validation 2000).
The EN 45000 series of standards (EN 45001 : 1989) and ISO/IEC Guide 25 have proposed general requirements for the competence of calibration and testing laboratories and general criteria for the operation of testing laboratories. The formal recognition that a testing laboratory is competent to carry out specific tests or specific types of tests under the above standards is based on nine validation parameters. Harmonized guidelines between the European Union (EU), Japan and the United States have been developed within the expert working group (Quality) of the International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for human use (ICH Q2A and Q2B). This document defines eight validation parameters (Q2A) and discusses their detailed methodology (Q2B). The United States Environmental Protection Agency (USEPA) has prepared guidance for method development and validation for the Resource Conservation and Recovery Act. The United States Pharmacoepia (USP 2003) has published specific guidelines for method validation for evaluation of compounds. The American Association of Official Analytical Chemists (AOAC), USEPA, and other scientific organizations provide multi-laboratory validated methods and have developed their own peer-verified method validation programs with detailed guidelines on parameters to be validated whereas the International Union of Pure and Applied Chemists (IUPAC) has developed guidelines for single laboratory method validation (Green 1996). The United States Food and Drug Administration (FDA) is the first regulatory agency that has understood the need for guidance for analytical method validation involving biological fluids and accordingly issued guidelines in 2001 because of their importance in bioavailability, bioequivalence and pharmacokinetic studies.
Although there is general agreement on the various validation parameters to be evaluated, diversity prevails in the methodology employed for validation and acceptance criteria. The chasm between the EU, the US and Japan has been bridged by ICH but it still exists with other guidance. This review article discusses the various validation parameters and compares guidelines issued by various regulatory bodies.

2. Analytical method development

Pharmaceutical and biopharmaceutical product development is an inherently complex process. Each stage of the drug development process uses a series of analytical methods, which are developed for the specific needs of that particular development stage. Since the common element in each consecutive stage is the drug, the use of previously developed analytical methods is encouraged. This method transfer concept has two major objectives: (a) saving method development resources; and (b) providing experimental data that are comparable with previous results. In most cases the same analytical methods are not applicable throughout the whole drug development pathway. Each drug development stage has a different objective, a different sample environment, different sensitivity requirements, and a different impurity profile. The sample environment and/or sample matrix can affect sample preparation steps and degradation of the drug substance. For example, at the early research stage purification and identification of the drug substance are the primary goals. For toxicology and drug metabolism studies, purification and identification of the parent compound and its metabolites are needed. At the formulation stage the primary concern is the stability of the drug substance. Dissolution studies measure the kinetics of the appearance of the drug substance as a result of derivatization, adsorption and physical encapsulation of the drug substance by the formulation polymers, alteration of the conformational state of protein and peptide based pharmaceuticals, aggregation and hydrolysis of the drug molecule. The challenge here is to understand how these factors influence the analysis, and finding solutions that help in obtaining the required information reliably in the face of these complexities. Similarly, evaluation of the final drug product requires determination of physical characteristics, drug content uniformity and release kinetics.
The quality of an analytical method developed is always appraised in terms of suitability for its intended purpose, recovery, requirement for standardization, sensitivity, analyte stability, ease of analysis, skill subset required, time and cost in that order. It is highly imperative to establish through a systematic process that the analytical method under question is acceptable for its intended purpose. Recovery refers to the ability of the method to give a response for the entire amount of analyte in the sample. It is generally expressed as the percentage of reference/sample material that has been added to the blank. Its importance increases in the absence of a reference material. Standardization is the cardinal point in method development. External standardization is quite common, in which the response of the analyte alone is plotted versus concentration, whereas internal standardization is used when reproducibility is the problem. In internal standardization, a functional or isotopic analogue of the analyte that is similar in physicochemical properties is added to the standards and sample prior to treatments. The ratio of responses of the standard and internal standard is plotted against the concentration of the standard to obtain a calibration curve (Karnes et al. 1991).
Sensitivity of the method is defined as the increase in response with unit increase in concentration. The sensitivity of the method can be increased by manipulating various factors affecting it, like signal to noise ratio, physicochemical properties of the analyte, and the response of the input transducer to analyte and composition of sample matrix (Pasteelnick 1993). It is important to determine the stability of the analyte in its sample environment in order to access the degradative effect of sample components on analyte response. Ease of analysis refers to simplicity in sample preparation, time required for analysis and the chances of error involved in analysis. A minimal skill subset required in personnel and a cost effective method are always desired (Willard et al. 1995). There can be no demarcation between analytical method validation and development. This is an iterative process in which development is followed by validation, further changes and revalidation.

3. Analytical method validation procedure

The steps involved in development, validation and determination of validation parameters, also termed analytical performance characteristics, depend upon the type and nature of the analytical method. Pharmaceutical analytical methods are categorized into five general types, namely, identification tests, potency assays, impurity tests (quantitative), impurity tests (limits) and specific tests. The first four tests are universal tests, but the specific tests such as

particle-size analysis and X-ray diffraction studies are used to determine specific properties of the active pharmaceutical ingredient (API) or the drug product (Pasteelnick 1993).

A method has to be validated when it is necessary to verify whether its performance parameters are adequate for use for a particular analytical problem. For example, (a) when a new method is developed for a specific problem; (b) when indications exist that an established method is changing with time; (c) when an established method is revised to incorporate changes/improvements or to extend it for another purpose; (d) when an established method is used in a different laboratory, or with different analysts or different instrumentation; (e) to demonstrate the equivalence between two methods, e.g. a new method and a standard. The extent of validation or revalidation required would depend on the nature of the changes made in reapplying a method to different laboratories, instrumentation or operators, and the circumstances in which the method is going to be used. Some degree of validation is always appropriate even when using apparently well-characterized standard or published methods.

A well-developed method should be easy to validate. As the development of the method and the validation process advance, the information gathered is captured in the design and subsequent improvement of the method. Ideally, the validation protocol should be written only following a thorough understanding of the method's capabilities and intended use. The validation protocol will list the acceptance criteria that the method can meet. Any failure to meet the criteria will require that a formal investigation is conducted. Various steps involved in a complete method validation program are summarized in Table 1 and brief definitions of various validation performance characteristics are presented in Table 2.

The validated test method is included in the validation report that summarizes the results of the validation studies. Both the validation report and test method are submitted as parts of the NDA or ANDA. The analytical method's performance characteristics should be based on the intended use of the method and the prudent judgment exercised by the analyst. For example, if the method is to 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 laboratory generating the data and the client using the data. Although validation parameters have been suggested by various regulatory agencies, the sequence of validation is still a matter of personal preference based on past experience and the method itself. Many authors have suggested determining selectivity/specificity first, followed by accuracy, precision, linearity and range, LOD, LOQ, robustness and ruggedness (Green 1996). Detailed guidelines on analytical method validation issued by various international agencies are summarized in Tables 3 to 8. These tables include definition of validation parameters, method of determination, recommendations, method of expressing the parameter and the calculations thereof and the acceptance criteria.

4. Validation parameters

4.1. Specificity

Specificity is the ability of the method to measure the analyte response accurately in the presence of all potential sample components, referred to as the sample matrix. These sample components or matrix may include placebo formulation, synthesis intermediates, excipients, degrada-

Table 1: Brief description of various activities to be performed during method validation

Step No.	Activity
1	Define method development plan and gather background information
2	Develop laboratory method and generate test procedure
3	Develop validation protocol or operating procedure for the validation
4	Define application, purpose and scope of method
5	Define performance parameters and acceptance criteria
6	Define validation experiments
7	Verify relevant performance characteristics of equipment
8	Qualify materials, e.g. standards and reagents
9	Perform pre-validation experiments
10	Adjust method parameters or/and acceptance criteria, if necessary
11	Perform full internal (and external) validation experiments
12	Develop standard operating procedures for executing method routinely
13	Define criteria for revalidation
14	Define type and frequency of system suitability tests and/or analytical quality control (AQC) checks for routine use
15	Document validation experiments and approval of validation report

Table 2: Brief definition of various validation performance characteristics

Performance Characteristics	Activity
Specificity	Ability to measure desired analyte in a complex mixture
Accuracy	Agreement between measured and real value
Precision	Agreement between a series of measurements
Linearity	Proportionality of measured value to concentration
Range	Concentration interval where method is precise, accurate, and linear
Detection limit	Lowest amount of analyte that can be detected
Quantitation limit	Lowest amount of analyte that can be measured or quantified
Robustness	Ability to remain unaffected by small changes in parameters
Ruggedness	Reproducibility under normal but variable laboratory conditions

Table 3: Comparison of different guidelines for 'specificity' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Reference document	Guideline for industry-text on validation of analytical procedure (ICH Q2A) (Mar 1995) Guidance for Industry-Q2B validation of analytical procedures: Methodology (ICH Q2B) (Nov 1996)	Guidance for Industry-Bio analytical method validation (May 2001)	AOAC Peer Verified Methods Program, Manual on policies and procedures (Nov 1993)	General Chapter ⟨1225⟩, Validation of compendial methods, *United States Pharmacopeia XXV*, Rockville, MD, The United States Pharmacopeial Convention Inc. p. 2149–2152 (2003)	Harmonized guidelines for single laboratory validation of methods of analysis (Nov 1999)
Specificity					
Definition	Ability to assess unequivocally the analyte in the presence of components, which may be expected to be present.	Ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample.	Ability of a method to measure only what it is intended to measure.	Ability to assess unequivocally the analyte in the presence of components, which may be expected to be present	The degree to which a method can quantify the analyte accurately in the presence of interferants.
Method	• When the impurities are available: Spiking of pure substance (drug or drug product) in appropriate level of impurities/excipients and demonstrate the result is unaffected. • When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition. • In chromatographic method: Peak purity test to be done by diode array and mass spectrophotometry.	• Analysis of blank samples of appropriate biological matrix obtained from at least six sources and test for interference. • Selectivity should be ensured at LLOQ LLOQ- Lower limit of quantitation	• Reagent blanks and field blanks should be run to ensure no interfering compounds are present. • To verify the specificity of the method for the analyte(s) of interest, results should be tested under different experimental conditions, e.g., two different analytical principles or two different detection techniques. • The method should be able to distinguish the analyte from known interfering materials and the behavior of the analyte during analysis should be indistinguishable from the corresponding standard material in the appropriate matrix.	• When the impurities are available: Spiking of pure substance (drug or drug product) in appropriate level of impurities/excipients and demonstrate the result is unaffected. • When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition. • In chromatographic method: Peak purity test to be done by diode array and mass spectrophotometry.	• Selectivity index should be calclated. Selectivity index $= b_{an}/b_{int}$ b_{an} = Slope of calibration curve b_{int} = Slope of response independently produced by a potential interferant.
Expression/ calculation	• Proof of discrimination of analyte in the presence of impurities e.g. for chromatography chromatogram should be submitted. • Peak purity test helps in demonstrating that the peak is not attributable to more than one component. • For assay two results should be compared and for impurity tests two profiles should be compared.	Evidence that the substance being quantified is the intended analyte.	–	• Proof of discrimination of analyte in the presence of impurities, e.g., for chromatography chromatogram should be submitted. • Peak purity test to demonstrate that the peak is not attributable to more than one component. • For assay two results should be compared and for impurity tests two profiles should be compared.	• Selectivity Index
Acceptance criteria	Not specified	Not specified	Not specified	Not specified	Not specified

tion products, process impurities, etc. The response of the analyte in test mixtures containing the analyte and all potential sample matrixes is compared with the response of a solution containing only the analyte. Any contribution of the matrix to the response leads to constant or proportional systematic error and such methods are referred as non-specific. The analyte is exposed to stress conditions sufficient to generate degradants that can be potentially generated during the normal course of analysis if the sample is subjected to harsh environmental conditions. The stress condition selected should be sufficient to cause substantial degradation (approximately 70–90% of its labeled purity). For bulk pharmaceuticals typical stress conditions employed include heat (50 °C), light (600 foot candle), acid

(0.1 N HCl), base (0.1 N NaOH), and oxidant (3% H_2O_2). For formulated products conditions such as heat (40 °C), light and humidity (60–75% RH) are often used. Methods of determining specificity and its expression as prescribed by various international agencies are presented in Table 3.
A statistical approach that tests the intercept against zero using a one-sided t-test has been reported (Shah 1991; Bolton 1997). In the absence of primary standards the matrix effect is tested by comparing the slopes of matrix and non-matrix standards or by a standard addition method (Shah 1991).

4.2. Linearity and range

The linearity of an analytical method refers to the ability to elicit test results that are, either directly or by well-defined mathematical transformations, proportional to the concentration of analyte in the sample over the entire range of interest. This is determined by measuring the response of standard solutions in the range of 50 to 150% of target concentration. An unweighted linear least square line is plotted, which assumes that all the errors occur in the Y-direction, i.e. the error in measuring response is more than the error in preparation of a sample concentration, and that all errors are normally distributed. Though the linearity characteristic of any proposed method is expected to include the origin of the response and the concentration axes, a significantly different intercept may be acceptable if the accuracy of the method is not compromised (Miller et al. 1988; Miller 1991). Homocedasticity is assessed by observing the plot of residuals versus concentration. If an increase or decrease in variance, better known as heterocedasticity, is observed weighted regression is preferred (Miller et al. 1988; Miller 1991). 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 greater than 0.9999 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.
The range of an analytical method is the interval between the upper and lower levels (including these levels) over which acceptable accuracy, linearity, and precision are obtained. In practice, the range is determined using data from the linearity and accuracy studies. To avoid the possibility of misinterpretation, two approaches have been suggested. In the first approach, deviation from the regression line is plotted against concentration or log concentration. Deviation should be equally distributed between positive and negative values. Another approach suggests plotting the ratio of response to concentration versus concentration or log concentration. The line obtained should be linear over the full range. Some analytical procedures may require non-linear calibration but a linear model with univariant regression is preferred (Shah 1991; Miller 1991). The range is normally expressed in the same units as the test results (e.g. amount per unit volume, parts per million or percentage) obtained by the analytical method. A comparison of various guidelines for the linearity and range parameter of analytical method validation is summarized in Table 4.

4.3. Accuracy

The accuracy of an analytical method is the closeness of the result obtained to the true value. Various guidelines regarding determination of accuracy are listed in Table 5. Four approaches have been suggested to determine the accuracy of analytical methods (Green 1996). The accuracy can be determined using a single certified reference material and comparing the measured value with true value. A second approach compares the result of the proposed method with the result of a reference/another method whose accuracy and precision is known. These two approaches are futile if the certified reference material or method is not available. In such a situation a recovery study is performed in which the analyte is spiked by weight or volume into the matrix covering the entire linearity range followed by quantitation where the procedure follows the final proposed sample preparation and response measurement technique. The result then is expressed as percent recovery. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte, for example, with lyophilized material, in which the speciation in the lyophilized material is significantly different when the analyte is absent. Since the accuracy is often expressed as percentage bias it may be helpful to determine whether the bias is because of random error alone. This is done using the t-test to determine whether the mean value differs significantly from the true value. If the deviation is significant, then the ratio of the deviation between the mean and measured results to the mean result is calculated as the estimate of bias (Green 1996; Miller 1991).

4.4. Precision

The precision of the method is defined as the degree of scatter of individual test results of multiple measurements of a homogenous sample. A comparison of various guidelines regarding determination of precision is presented in Table 6. There are four types of precision that can be determined for an analytical method, namely instrument precision or injection repeatability, repeatability or intra-assay precision, intermediate precision and reproducibility. Though all official guidelines describe only the first three types of precision, there are some papers that describe all four types of precision (Green 1996).
Instrument precision is determined by repeated measurement of one sample solution so as to test the performance of the instrument used in the analytical methodology. The repeatability or intra-assay precision is obtained by repeatedly analyzing independently prepared homogenous samples in one laboratory, by one operator, using one piece of equipment and one set of reagents on one day. At least five determinations of three concentrations at the low, medium and high range of calibration are performed and the % relative standard deviation (RSD) is calculated. Precision of less than 1% RSD is easily achieved in compound analysis in pharmaceutical quality control but precision decreases with the complexity of the matrix especially with biological matrices (Green 1996).
Intermediate precision is the precision obtained when analysis involves multiple analysts, multiple equipment, multiple days, and multiple sets of reagents in the same laboratory. The objective of the determination of this precision is to identify the various factors within a single laboratory that will contribute to the variability of the results and to find a mechanism to control them (Green 1996).
Validation of reproducibility is important if the method is going to be used in different laboratories. When a method is transferred from one laboratory to another it invariably

Table 4: Comparison of different guidelines for 'linearity and range' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Linearity and range					
Definition	Linearity: Ability (within range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Range: Interval between the upper and lower concentration (amounts) of analyte in the sample including the concentrations for which suitable level of accuracy, precision, and linearity has been demonstrated.	Calibration/Standard Curve: Relationship between response and known concentration of analyte.	Linearity – Defines the ability of the method to obtain test results proportional to the concentration.	Linearity: Ability (within range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample. Range: Interval between the upper and lower concentration (amounts) of analyte in the sample including the concentrations for which suitable level of accuracy, precision, and linearity has been demonstrated.	Not explicitly defined
Method	Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by • Visual inspection of plot • Appropriate statistical methods	Sufficient number of standards in the expected matrix is taken and response measured. Plot of concentration vs. response is plotted and linearity is demonstrated. Number of standards depends on: • Anticipated range of analytical value • Nature of analyte/ response relationship Conc. of sample depends on expected concentration range	Not specified	Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by • Visual inspection of plot • Appropriate statistical methods	• Visual examination of residual plot • Application of statistical testing (significance testing) • Test for lack of fit can be weighted/ simple regression
Recommendation	• Minimum of 5 concentrations are recommended for linearity For Range: • Assay of drug/finished product: 80–120% of test concentration. • For content uniformity: 70–130% of test concentration • For dissolution testing: ±20% over specified range • For impurity: from reporting level to 120% of specification.	• One blank sample (matrix sample without internal standard (IS) • One zero sample (matrix sample + IS) • 6–8 non-zero samples covering expected range including LLOQ.	4 conc. levels are to be selected. • 1/2× • 1× • 3/2× • 2× } ×3 Days	• Minimum of 5 concentrations are recommended for linearity For Range: • Assay of drug/finished product: 80–120% of test concentration. • For content uniformity: 70–130% of test concentration • For dissolution testing: ±20% over specified range • For impurity: from reporting level to 120% of specification.	• Six or more calibration standards evenly spaced over the range of interest. • Range: 0–150% or 50–150% of target concentration depending on which of this is more suitable • Calibration standard should be run at least in duplicate. Preferably in triplicate or more
Expression/ calculation	• Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.	Not specified	Not specified	• Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.	Not specified
Acceptance criteria	Not specified	At LLOQ: Not more than 20% deviation from nominal value Other than LLOQ: 15% deviation from nominal value At least 4 out of 6 non-zero sample should meet the above criteria including LLOQ and highest concentration.	Not specified	Not specified	Not specified

Table 5: Comparison of different guidelines for 'accuracy' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Accuracy					
Definition	Expresses the closeness of agreement between the value, which is accepted either as a conventional true value or the value found. (Also referred as trueness).	Closeness of mean test results obtained by method to the true value (concentration of analyte).	Closeness of the determined value to the true value.	Closeness of test results obtained by that method to true value.	Trueness is the closeness of agreement between a test result and accepted reference value of the property being measured. Smaller bias means greater trueness
Method	• Application of procedure to analyze synthetic mixture of known purity. • Comparison of result with already established procedure. • Accuracy may be inferred once precision, linearity and specificity have been established.	• Replicate analysis of samples containing known amounts of analyte.	• Use of certified reference materials • Use of reference method of known uncertainty • Use of recovery from spiked samples	• Application of procedure to analyze synthetic mixture of known purity. • Comparison of result with already established procedure. • Accuracy may be inferred once precision, linearity and specificity have been established.	Bias is determined by comparing response of method to a reference like,. • Use of certified reference material. • Use of reference material • Use of reference methods • Use of spiking/ recovery Significance testing is recommended.
Recommendation	Minimum of nine determinations • Low concentration of range × 3 replicates • Medium concentration of range × 3 replicates • High concentration of range × 3 replicates	Three concentration levels covering entire range • Low concentration of range × 5 replicates • Medium concentration of range × 5 replicates • High concentration of range × 5 replicates (According to CDER review for HPLC method: 80, 100, 120% of target concentration × triplicate)	Three concentration levels throughout the range • Low concentration of range • Medium concentration of range • High concentration of range • Amount added for fortification should be a substantial fraction or more than the amount present in the sample.	Minimum of nine determinations • Low concentration of range × 3 replicates • Medium concentration of range × 3 replicates • High concentration of range × 3 replicates	Not specified
Expression/ calculation	• Percent recovery by the assay of known added amount of analyte • Mean – Accepted true value with confidence interval	Mean − True value	% Recovery = (mean concentration fortified − mean concentration unfortified)/Known increment in concentration	• Percent recovery by the assay of known added amount of analyte • Mean − Accepted true value with confidence interval	Not specified
Acceptance criteria	Not specified	• At LLOQ: Mean value should be within ±20% of actual value • Other than LLOQ: Mean value should be within ±15% of actual value.	Not specified	Not specified	Not specified

encounters: analysts with different experience and thoroughness, differences in room temperature and humidity, equipment with different characteristics, and also variations in the nature and quality of supplies, materials, consumables, and instrument conditions (e.g. in HPLC mobile phase composition, pH, flow rate of the mobile phase, column specifications). Reproducibility is determined by measuring a homogenous sample in multiple laboratories with the object of verifying that the same results are obtained when the methodology is transferred to other laboratories. Statistical equivalence and analytical equivalence are used to judge the acceptability of results obtained from different laboratories. In statistical equivalence the results from other laboratories are compared against the primary laboratory, whereas in analytical equivalence a range of acceptable results is chosen prior to study (USFDA 2001).

4.5. Limit of detection

Limit of detection (LOD) is the lowest concentration of analyte that can be reliably detected using the method but not necessarily quantified. The LOD of a method should be established quite early in the method development-validation process and its determination should be repeated using the specific wording of the final procedure. In the

Table 6: Comparison of different guidelines for 'precision' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Precision					
Definition	The precision of analytical procedures expresses the closeness of agreement (degree of scatter) between series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.	Describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of single homogenous volume of biological matrix	Degree of agreement of measurements under specific conditions.	Degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample.	Closeness of agreement between test results obtained under stipulated conditions.
Method	Determination of % relative standard deviation (RSD) of response of multiple aliquots	Analytical procedure is applied repeatedly to multiple aliquots of single homogenous volume of biological matrix.	Determination of % relative standard deviation (RSD) of multiple aliquots	Determination of % relative standard deviation (RSD) of response of multiple aliquots	Determination of % relative standard deviation (RSD) or % coefficient of variation (CV) and F-test for normally distributed error is applied.
Recommendation	Repeatability (Same operating condition over short interval of time): Minimum of nine determinations over the entire range • Low concentration of range × 3 replicates • Medium concentration of range × 3 replicates • High concentration of range × 3 replicates (or) • At target concentration × 6 determinations Intermediate precision (within laboratory variation): • Different Days • Different Analysts • Different Equipment etc.	Three concentration levels covering entire range • Low concentration of range × 5 replicates • Medium concentration of range × 5 replicates • High concentration of range × 5 replicates Intra batch precision (repeatability): Measures precision during single run Inter batch precision (reproducibility): Measures precision with time.	4 conc. levels are to be selected. • 1/2× • 1× • 3/2× • 2× Repeatability: to be established within laboratory • Different days • Different analysts • Different calibration curves • Different batches of reagents • Different matrices. Reproducibility: Between labs.	Repeatability (Same operating condition over short interval of time): Minimum of nine determinations over the entire range • Low concentration of range × 3 replicates • Medium concentration of range × 3 replicates • High concentration of range × 3 replicates (or) • At target concentration × 6 determinations Intermediate precision (within laboratory variation): • Different Days • Different Analysts • Different Equipment etc.	Minimum of two concentrations • At or near highest concentration in range • At or near lowest concentration in range
Expression/ calculation	Standard deviation, RSD and confidence interval	Coefficient of variation	RSD	Standard deviation, RSD and confidence interval	SD $\{(\sigma_{tot} = \sigma_r^2/n + \sigma_{run}^2)^{1/2}$ where n = no. of repeat results} or RSD
Acceptance criteria	Not specified	• At LLOQ: CV $\leq$20% • Other than LLOQ: $\leq$15%	Not specified	Not specified	Not specified

case of methods requiring technology transfer it is important to test the LOD of the method on different instruments of similar models to those used in other laboratories. Depending on the nature of the method, various approaches have been suggested to determine the detection limit.

Simple visual examination may be adequate for a non-instrumental method. In the case of chromatographic methods that exhibit constant background noise, it can be estimated based on signal-to-noise ratio. The LOD in such cases will correspond to the concentration at which the response signal-to-noise ratio is 3. In the case of spectrophotometric methods LOD is determined using the relation $3.3\sigma/S$ where σ is the standard deviation of the response and S is the slope of the calibration curve. The standard deviation of the response can be obtained either by measuring the standard deviation of the blank response or by calculating the residual standard deviation of the regression line or by calculating the standard deviation of the y-intercept of the regression line or $S_{y/x}$, i.e. the standard error of the estimate (ICH Q2A, ICH Q2B, 1996). A comparison of various guidelines for the limit of detection parameter in analytical method validation is summarized in Table 7.

Table 7: Comparison of different guidelines for 'detection limit' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Detection Limit					
Definition	Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions.	Explicitly not described	Lowest content that can be measured with reasonable statistical certainty	Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions.	Smallest amount of conc. of analyte in the sample that can be reliably distinguished from zero.
Method	1. By visual evaluation 2. Based on S/N ratio • Applicable to procedure, which exhibit baseline noise. • Actual lowest concentration of analyte detected in compared with blank response 3. Based on S.D. of response and slope LOD = 3.3σ/s s = Slope of calibration curve σ = S.D. of response; can be obtained by • Standard deviation of blank response • Residual standard deviation of the regression line • Standard deviation of the y-intercept of the regression line • $S_{y/x}$ i.e. standard error of estimate	Not described	Based on more than 20 blank readings.	For non-instrumental: Analysis of sample with known concentration of analyte and by establishing minimum concentration at which analyte can be reliably detected. For instrumental: Process for non-instrumental can be adopted. Detection limit should be sufficiently low for analysis of samples with known concentration of analyte above and below the required detection limit.	Not specified
Expression/ calculation	• If based on visual examination or S/N ratio – relevant chromatogram is to be presented. • If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.	Not described	The mean value of the matrix blank readings ($n \geq 20$) plus three standard deviations of the mean, expressed in analyte concentration.	Not specified	Not specified
Acceptance criteria	S/N ratio > 2–3; Not specified in other cases	Not described	Not specified	Not specified	Not specified

4.6. Limit of quantitation

The limit of quantitation (LOQ) is the concentration at and above which the analyte can be reliably quantitated with a previously defined level of certainty. A summary of various guidelines for determination of the limit of detection parameter of analytical method validation is presented in Table 8. The LOQ is determined by reducing the analyte concentration until a level is reached where the precision of the method is unacceptable. If the required precision at the LOQ is already specified, then the %RSD of six determinations at decreasing concentration is plotted against concentration. The quantitation limit is determined by extrapolating from the graph (ICH Q2A, ICH Q2B, 1996).
Like LOD, the quantitation limit can be determined by different approaches. In case of non-instrumental techniques even a visual examination may suffice. If the method exhibits background noise this can be determined based on a signal-to-noise ratio of 10. This can also be estimated using the relationship LOQ = 10σ/S where σ is the standard deviation of the response and S is the slope of the calibration curve. σ is obtained from the standard deviation of response of blanks or by residual standard deviation or regression line or the standard deviation of the intercept.

4.7. Robustness

Robustness is the ability of the method to remain unaffected by small changes in method parameters carried out deliberately or otherwise during the validation/usage of analytical methodology. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. Obtaining data on the effects of these parameters may allow a range of acceptable values to be included in the final method procedure. The factors that are generally studied are the pH of the mobile phase or solvent, buffer concentration, small changes in solvent

Table 8: Comparison of different guidelines for 'quantitation limit' parameter of analytical method validation

Guidelines	ICH	US FDA	AOAC	USP	IUPAC
Quantitation Limit					
Definition	Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.	The lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy also called LLOQ (Lower limit of quantification).	The limit of quantitation is the lowest amount of analyte in a sample, which can be quantitatively determined with precision and accuracy appropriate to analyte and matrix considered.	Lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.	Not defined
Method	1. By visual evaluation 2. Based on S/N ratio • Applicable to procedure, which exhibits base line noise. • Low conc. of analyte is compared with blank 3. Based on S.D. of response and slope $LOQ = 10\sigma/s$ s = Slope of calibration curve σ = S.D. of response; can be obtained by • Standard deviation of blank response • Residual standard deviation of the regression line • Standard deviation of the y-intercept of the regression line $S_{y/x}$ i.e. standard error of estimate	Preparation of standard curve and lowest conc. on the calibration curve should be accepted as LLOQ if it satisfies following condition. • Response at LLOQ = 5× Response by blank • Analyte peak should be identifiable discrete and reproducible with precision of 20% and accuracy of 80–120%	Not specified	1. By visual evaluation 2. Based on S/N ratio • Applicable to procedure, which exhibits base line noise. • Low conc. of analyte is compared with blank 3. Based on S.D. of response and slope $LOQ = 10\sigma/s$ s = Slope of calibration curve σ = S.D. of response; can be obtained by • Standard deviation of blank response • Residual standard deviation of the regression line • Standard deviation of the y-intercept of the regression line $S_{y/x}$ i.e. standard error of estimate	Not recommended; only recommends expressing uncertainty of measurement as function of concentration.
Recommendation	Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.	Not specified	Not specified	Not specified	Not specified
Expression/ calculation	• Limits of quantitation and method used for determining should be presented. • Expressed as analyte concentration	Not specified	Mean value of the matrix blank reading plus 10 standard deviations of the mean, expressed in analyte concentration.	Expressed as analyte concentration (% or ppb)	Not specified
Acceptance criteria	Not specified	Not specified	Not specified	Not specified	Not specified

system, temperature, and injection volume etc. (ICH Q2B 1996; USP 1995).

4.8. Ruggedness

Ruggedness is not defined by ICH guidelines. It is defined by the USP as the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions. It involves analysis of aliquots of homogenous lots in different laboratories by different analysts under different operational and environmental conditions. The degree of reproducibility of test results is determined as a function of the assay variables. IUPAC considers the effect of change of instrument, operator, brand of reagent, concentration of reagent, pH of solution and time allowed (run time) for completion of the process (AOAC 1993; Thompson et al. 2002).

4.9. System suitability

The system has to be tested for its suitability for the intended purpose. During the early stages of the method development process some of the more sophisticated system suitability tests may not be practical due to the lack of experience with the method. In the early stages of development it may be useful to perform some additional system suitability tests to evaluate system performance under different method conditions. This information will help to develop an appropriate system suitability test strategy in the future. As more experience is acquired for this method, a more sophisticated system suitability test may be necessary. For this, all critical factors that will significantly affect the method performance need to be identified and they should remain within the specified limits or the critical factors should be manipulated to change the sys-

tem performance favorably. Therefore, the system suitability strategy not only consists of the tests and limits but also the approach used to optimize the system performance when the original test result exceeds the limit. In addition, if the method demands high method sensitivity, a detector sensitivity solution may be required to demonstrate suitable signal to noise ratio from the system exhibiting baseline noise (USFDA 1993).

Numerous approaches may be used to set the limits for system suitability tests. This depends on experience with the method, material available and personal preference. Default values for system suitability for HPLC methods are: capacity factor should be more than 2, injection precision should have %RSD less than 1%, resolution factor should be greater than 2, tailing factor should be less than 2% and theoretical plate should be more than two thousand (USFDA 1993).

4.10. Stability

During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phases, standards, and sample solutions. For routine testing in which many samples are prepared and analyzed each day, it is often essential that solutions are stable enough to allow for delays such as instrument breakdowns or overnight analyses using auto-samplers. Stability has not been given due importance in ICH guidelines but the US FDA has discussed stability parameters for biosamples. It is important to determine the stability of an analyte in a particular matrix by comparison with freshly prepared standards. It recommends freeze thaw stability for three freeze thaw cycles. It also recommends short-term temperature stability, which is done at room temperature for 4–24 h based on the expected duration for which samples will be maintained at room temperature for the intended study.

Long-term stability should also be assessed over a time period greater than the time difference between the date of first sample collection and the date of last sample analysis. The concentration of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentration from the first day of long term stability testing.

The stability of stock solutions of drug and the internal standards should be evaluated at room temperature for at least 6 h and if the stock solution is stored then the relevant stability should be checked. The stability of processed samples, including their residence time in an autosampler known as postoperative stability, is to be determined (USFDA 2001).

At this point, the limits of stability should be tested. Samples and standards should be tested over at least a 48 h period, and the quantitation of components should be determined. If the solutions are not stable over 48 h, storage conditions or additives should be identified that can improve stability.

5. Conclusion

The efficient development and validation of analytical methods are critical elements in the development of pharmaceuticals and ensuring regulatory compliance. Analytical method validation is an important tool for ensuring the performance of the method. Various guidelines by different regulatory bodies and organizations disagree on different points. Though ICH guidelines have resolved the differences between Europe, the USA and Japan, organizations like IUPAC and AOAC still have differences on some points. There should be an effort to put forward uniform guidelines for validation throughout the world and to create a similar platform for acceptance criteria.

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