USP <1225/1226>
ICH Q2(R1)
GxPcGMP
PIC/S SOP
ISO 17025
QA/QC
LOD/LOQ
API EP
FDA
OECD
GCP

A Primer

Validation of Analytical Methods

Agilent Technologies

Validation of Analytical Methods

Ludwig Huber

Contents

Preface

This primer is intended to guide managers and analysts from (bio)pharmaceutical, food, chemical, environmental and other regulated laboratories through all aspects of analytical methods validation. Quality managers and staff as well as regulatory affairs professionals will also benefit through extensive discussions of relevant regulations, quality standards and guidelines. The primer will give strategies and specific recommendations for the validation of new methods that are developed internally as well as for the verification of compendial and standard methods.

In less than one day readers will get:

- An overview of regulatory and quality standard requirements
- A literature overview
- Strategies for implementation
- Information on test parameters, acceptance criteria and test conditions
- Recommendations for special applications, such as validation of bioanalytical methods, verification of compendial methods and transfer of analytical methods

The concepts and ideas expressed in this primer are my own and do not necessarily reflect official Agilent or Labcompliance policies.

Some text information and figures in this primer have been taken from Dr. Ludwig Huber's reference book with permission from the publisher INFORMA HEALTHCARE, New York.

- *L. Huber, Validation and Qualification in Analytical Laboratories, Interpharm, Informa Healthcare, New York, USA, 2007*

Regulations and quality standards are dynamic. They are updated every few years. Implementation guidelines, as developed by regulatory task and industry task forces, are published more frequently since state-of-the art today may not be considered as such in the future.

A timely update of all information is important and only possible using on-line information tools, such as the Internet. Therefore, I recommend the following websites with regular updates related to quality standards in laboratories:

http: / / www.fda.gov
Regulations and specific guidelines for the validation of analytical methods and procedures.

http: / / www.agilent.com / chem / pharmaqaqc
The Agilent Website for pharmaceutical QA/QC with monthly newsletter for regular updates.

http: / / www.labcompliance.com
A website with regular updates including tutorials and many references related to all quality and compliance issues in laboratories.

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Chapter 1

Introduction

1

Introduction

The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice. Validation of analytical methods is also required by most regulations and quality standards that impact laboratories.

Analytical methods need to be validated, verified, or revalidated in the following instances:

- Before initial use in routine testing
- When transferred to another laboratory
- Whenever the conditions or method parameters for which the method has been validated change (for example, an instrument with different characteristics or samples with a different matrix) and the change is outside the original scope of the method.

Method validation has received considerable attention in literature from industrial committees and regulatory agencies. This chapter outlines how method validation helps to achieve high quality data (there are other elements, as it will be explained later).

1.1 Literature Overview

The scope of this primer is to provide concepts and strategies for implementing method validation. It does not describe details of method validation. However, there is a wide variety of information and guidance available, published as a result of work done in task forces and by private authors. This chapter presents an overview. Additional literature references with information about specific aspects such as transfer of analytical methods, and specific parameters can be found in related chapters.

- The Laboratory of the Government Chemist (LGC) developed a guide for internal method validation[1]. It includes a discussion of related laboratory accreditation requirements.
- The United States Food and Drug Administration developed two industry guidelines: one for the validation of analytical methods[2] and one for the validation of bioanalytical methods [3].
- ICH published two guidelines for method validation. Q2A[4] describes terminology and definitions for eight validation parameters that should be considered for validation. Q2B[5] includes methodology but allows flexibility through the statement "It is the responsibility of the applicant to choose the validation procedure and the protocol most suitable for their product".
- IUPAC[6] published "Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis".
- EURACHEM[7] published a detailed guide for method validation. This is the most detailed official guide for theory and practice of method validation. It has been primarily developed for ISO/IEC accredited laboratories but because of its completeness it is also a good source for (bio)pharmaceutical laboratories.
- Huber[8] authored a validation reference book for the analytical laboratory with a chapter on method validation.
- AOAC[9] has published a technical document for the verification of analytical methods for the ISO 17025 accreditation.
- Viswanathan and co-authors[10] developed an overview for validation of bioanalytical methods.

1.2 Terminology: Validation vs. Verification

There have been discussions about the terms validation and verification of analytical methods and both terms are interchangeably used. The difference is best explained by referring to USP Chapters <1225> and <1226>. Chapter <1225>[11] is titled: "Validation of Compendial Methods". It describes the validation of analytical methods with all validation parameters from introduction. The result is a validated method for a specific sample. This procedure is recommended for the validation of methods developed internally.

Chapter <1226>[12] is titled "Verification of Compendial Methods." It provides recommendations of compendial methods that demonstrate a laboratory's ability to successfully run the method. Methods are also verified during method transfer by the receiving laboratory. Details of verification of compendial methods and method transfer will be discussed later in this primer.

1.3 Elements of Data Quality in Laboratories

The objective of validating an analytical procedure is to demonstrate "suitability for its intended purpose". The intent of analytical measurement is to generate accurate and reliable data. Method validation alone cannot guarantee this, but it should be part of integrated quality assurance for analytical measurement. This has been clearly spelled out by USP in Chapter <1058> on analytical instrument qualification[13]. The chapter starts with a discussion on the importance of various elements of data quality in laboratories. It also explains why system suitability testing or the analyses of quality control charts are not enough to ensure valid analytical test results. Instrument qualification and method validation are equally important.

Figure 1 illustrates the different components of data quality: analytical instrument qualification, analytical methods validation, system suitability testing and analytical quality control through quality control samples.

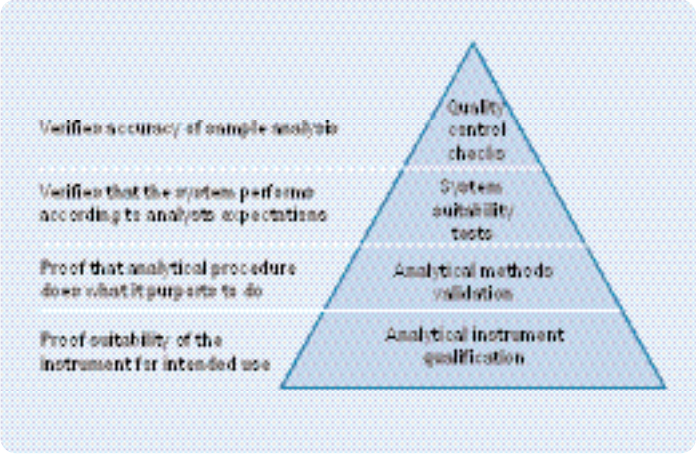

Figure 1
Components of analytical data quality.

Instrument qualification means that the specifications are defined, tested and confirmed so that the instrument is suitable for the methods to be validated. The analytical methods are then validated on qualified instruments to prove that the method works as intended. This is independent of any specific instrument. If we want to use the method with instruments from different vendors, the method should be validated on those instruments as well.

A specific instrument is then combined with a specific method to run system-suitability tests. System suitability parameters should be selected during method validation. Successful system suitability test runs ensure that the complete system meets the analyst's expectations under the specific conditions of the tests.

The highest level of testing is the analysis of quality control samples. Standards or samples with known amounts are analyzed and the results compared with the known amounts.

Method validation occurs between analytical instrument qualification and system suitability testing and is linked to all other quality elements. Methods should be validated using qualified instruments. During method validation, parameters and acceptance criteria for system suitability checks and quality control checks should be defined.

Chapter 2

Regulations and Quality Standards

2

Regulations and Quality Standards

Regulations and quality standards that have an impact on analytical laboratories require analytical methods to be validated. The regulation may use the word "validation" directly, list specific parameters, or imply validation with statements such as: "test methods should be appropriate for their intended use". This chapter reviews the most important regulations, quality standards and official guidelines. The extent of guidelines for validation requirements provided by different organizations varies widely, but the objective of validation is always to achieve valid analytical test results. This is important to ensure the quality and safety of products that are measured using the analytical method.

2.1 United States Food and Drug Administration (FDA)

Analytical method validation is essential for adherence to Current Good Manufacturing Practice (cGMP)[14] and Good Laboratory Practice (GLP) regulations. The US cGMPs spell out the requirements for validation in sections 211.165 (e) and 211.194:

- 165(e): "The accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented. Such validation and documentation may be accomplished in accordance with 194(a)(2).
- 194(a)(2): Laboratory records should include a statement of each method used in the testing of the sample. The statement shall indicate the location of data that establish that the methods used in the testing of the sample meet proper standards of accuracy and reliability as applied to the product tested. The suitability of all testing methods used shall be verified under actual conditions of use.
- 194(b): Complete records shall be maintained of any modification of an established method employed in testing. Such records shall include the reason for the modification and data to verify that the modification produced results that are at least as accurate and reliable for the material being tested as the established method.
- The FDA GLP regulation 21 CFR Part 58[15] itself does not mention the word validation but inspectors want to see validation studies by referring to Part 58.113 which states: "Tests shall be conducted by appropriate

analytical methods", where the word "appropriate" implies validation. Also the FDA Guidance on Validation of Bioanalytical Methods[3] defines Preclinical Toxicology as one of its scopes.

- FDA's regulation for Bioavailability and Bioequivalence Requirements 21 CFR 320[16] states in section 29:
  (a) The analytical method used in an in vivo bioavailability study to measure the concentration of the active drug ingredient or therapeutic moiety, or its metabolite(s), in body fluids or excretory products, or the method used to measure an acute pharmacological effect shall be demonstrated to be accurate and of sufficient sensitivity to measure, with appropriate precision, the actual concentration of the active drug ingredient or therapeutic moiety, or its metabolite(s), achieved in the body.

2.2 Pharmaceutical Inspection Cooperation Scheme (PIC/S) and Europe

The Pharmaceutical Inspection Cooperation Scheme's (PIC/S) mission is "to lead the international development, implementation and maintenance of harmonized Good Manufacturing Practice (GMP) standards and quality systems of inspectorates in the field of medicinal products".

This is achieved by developing and promoting harmonized GMP standards and guidance documents; training competent authorities, in particular inspectors; assessing (and reassessing) inspectorates; and facilitating the co-operation and networking for competent authorities and international organizations. As of November 2009 there are 36 participating authorities in PIC/S, including all EU member countries. Authorities from more countries have applied for PIC/S membership, such as the U.S. FDA. Most likely new member countries that don't have their own GMP regulations will accommodate PIC/S GMPs[17], which are very similar to the EU GMP directives[18].

For example, the requirement for analytical method validation is stated in both documents in Part 6.1.5 with identical text:

- Analytical methods should be validated. All testing operations described in the marketing authorization should be carried out according to the approved methods.

More details on inspectors' expectations are laid down in the PIC/S Laboratory Inspection Guide, section 8.7[19]. The guide has a list of questions that inspectors should ask when inspecting quality control laboratories. They include:

- Is method validation part of the validation master plan?
- Is there a general SOP on method validation available and is the validation report formally approved?
- Is the purpose of validation specified?
- Is validation completed and documented in each protocol for parameters defined in ICH[5]?
- Are acceptance criteria in each protocol defined and met?
- Is there an SOP for transfer of analytical methods?

2.3 International Conference for Harmonization (ICH)

The International Conference for Harmonization (ICH) was initiated in 1990 to bring together the regulatory authorities of Europe, Japan and the United States and experts from the pharmaceutical industries in the three regions to discuss scientific and technical aspects of product registration.

ICH publishes guidelines that are either signed into law by member countries, (for example, those in Europe) or recommended as guidelines by national authorities such as the US FDA.

One of the most important ICH documents is the GMP Guide for Active Pharmaceutical Ingredients[20]. Requirements for method validation are specified in Chapter 12:

- Analytical methods should be validated unless the method employed is included in the relevant pharmacopoeia or other recognized standard reference. The suitability of all testing methods used should nonetheless be verified under actual conditions of use and documented.
- The degree of analytical validation performed should reflect the purpose of the analysis and the stage of the API production process.
- Appropriate qualification of analytical equipment should be considered before starting validation of analytical methods.

2.4 Unites States Pharmacopeia (USP)

The United States Pharmacopeia (USP) develops methodology for specific applications and general chapters on different analytical aspects of FDA-regulated industry. According to section 501 of the Federal Food Drug and Cosmetic act, USP methodology constitute legal standards. USP has developed two general chapters related to method validation and another one with information on allowed method changes without the need for revalidation.

- Chapter <1225> on "Validation of Compendial Methods"[11]. This chapter describes parameters as they are used for validation of new methods. Recommendations can be used to validate methods developed by pharmaceutical laboratories.
- Chapter <1226> on "Verification of Compendial Methods"[12]. This chapter has been written for laboratories implementing compendial and standard methods. The recommendations are also useful for laboratories implementing validated methods from other laboratories.
- Chapter <621> on "Chromatography"[21]. This chapter has useful recommendations on how much GC and HPLC methods can be adjusted or changed without the need for revalidation.

2.5 ISO / IEC 17025

ISO/IEC 17025 is the most relevant ISO Standard for chemical analytical laboratories[22]. It specifies general requirements for the competence to carry out tests or calibrations or both. The standard is widely used as a quality system in environmental, food, chemical and clinical testing laboratories. It is used to assess laboratories that seek accreditation status.

The standard has many requirements related to the subject of this primer. The most important ones can be found in Chapter 5.4.5:

- The laboratory shall validate nonstandard methods, laboratory-designed and developed methods, standard methods used outside their intended scope, and amplifications and modifications of standard methods to confirm that the methods are fit for their intended use.
- The laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations.

- When some changes are made in the validated nonstandard methods, the influence of such changes should be documented and, if appropriate, a new validation should be carried out.
- If standard methods are available for a specific sample test, the most recent edition should be used.
- Validation includes specification of requirements, determination of method characteristics, a check that the requirements can be fulfilled by using the method, and a statement on validity.
- The following parameters should be considered for validating in-house developed methods: limit of detection, limit of quantitation, accuracy, selectivity, linearity, repeatability or reproducibility, robustness, and linearity.

Unlike regulations, this standard is quite specific. Even though this standard is not widely accepted currently by pharmaceutical laboratories, validation experts are advised to consult it when developing a method validation process and take recommendations appropriate for specific applications.

Chapter 3

Parameters and Tests for Method Validation

3

Parameters and Tests for Method Validation

According to USP <1225> analytical methods should be validated through laboratory tests: "Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications". The required laboratory tests for method validation have been defined in different working groups of national and international committees and are described in the literature. Unfortunately, some of the definitions vary between the different organizations. Therefore, laboratories should have a glossary with definitions on their understanding of the terms. In an attempt to standardize, representatives from the industry and regulatory agencies from the United States, Europe and Japan defined parameters, requirements and methodology for analytical methods validation through the ICH. The parameters, as defined by the ICH and other organizations and authors, are summarized in Figure 2 and are described briefly in the following paragraphs.

Parameter	Comment
Specificity	USP, ICH
Selectivity	ISO 17025
Precision	USP, ICH
Repeatability	ICH, ISO 17025
Intermediate precision	ICH
Reproducibility	ICH, defined as ruggedness in USP, ISO 17025
Accuracy	USP, ICH, ISO 17025
Linearity	USP, ICH, ISO 17025
Range	USP, ICH
Limit of detection	USP, ICH, ISO 17025
Limit of quantitation	USP, ICH, ISO 17025
Robustness	USP, Included in ICH as method development activity, ISO
Ruggedness	USP, defined as reproducibility in ICH

Figure 2
Parameters for method validation with reference to ICH, USP and ISO 17025.

3.1 Specificity / Selectivity

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc."USP <1225> refers to the same definition but also comments that other reputable authorities such as IUPAC and AOAC use the term "selectivity" for the same meaning. This reserves the use of "specific" for those procedures that produce a response for a single analyte only. ISO/IEC most likely has the same understanding because it requires a method to be "selective" rather than specific. Our goal is to distinguish and quantify the response of the target compounds from the responses of all other compounds.

Analytical techniques that can measure the analyte response in the presence of all potential sample components should be used for specificity validation. It is not always possible to demonstrate that a single analytical procedure is specific for a particular analyte. In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination. A frequently used technique in pharmaceutical laboratories is high performance liquid chromatography (HPLC) and to some extent gas chromatography (GC). In practice, a test mixture is prepared that contains the analyte and all potential sample components. The result is compared with the response of the analyte. In pharmaceutical test mixtures, components can come from synthesis intermediates, excipients and degradation products. Generation of degradation products can be accelerated by putting the sample under stress conditions, such as elevated temperature, humidity or light.

Specificity in liquid chromatography is obtained by choosing optimal columns and setting chromatographic conditions, such as mobile phase composition, column temperature and detector wavelength. Besides chromatographic separation, the sample preparation step can also be optimized for best selectivity.

It is a difficult task in chromatography to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. The analyst should know how many compounds are in the sample which is not always possible. Therefore, the target compound peak should be evaluated for purity.

Although in the past chromatographic parameters such as mobile phase composition or the column were modified, the current practice is the use of spectroscopic detectors coupled online to the chromatograph. UV-visible diode-array detectors and mass spectrometers acquire spectra on-line throughout the entire chromatogram. The spectra acquired during the elution of a peak are compared. If the spectra are different, the peak consists of at least two compounds.

The principles of using spectral detectors for specificity evaluation is shown in Figure 3 using a high performance liquid chromatography (HPLC) UV-visible diode array detector as an example. The figure shows examples for a specific and non-specific chromatographic method. The two peaks look very similar. From the peak shape it is not obvious whether the peak consists of one or more compounds.

For both examples UV spectra are taken at the peak upslope and down-slope, normalized and compared. In the example on the left, the spectra are identical indicating that the peak consists of single compound, or the peak is spectrally pure. In the example on the right, the peak is clearly impure which is demonstrated by two different UV spectra. Modern diode array detectors compare the spectra automatically and print a

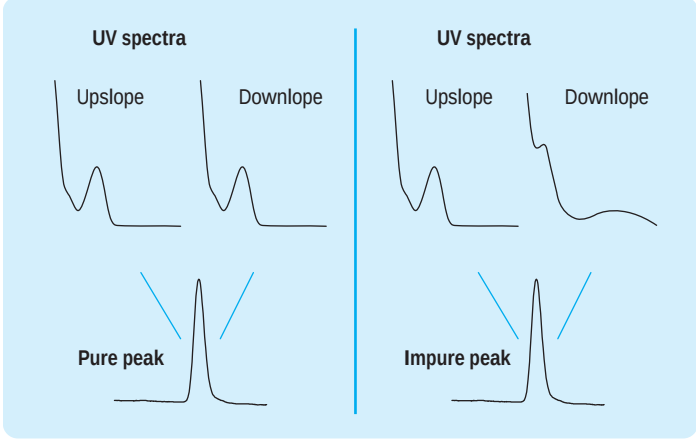

Figure 3
Identical spectra acquired across the peak indicate a pure peak (left). Different spectra indicate an impure peak (right).

match factor for each peak. This, in combination with the graphical visualization helps to assess selectivity without any additional workload.

UV-visible diode array detectors have limitations if the spectra of the co-eluting compounds are very similar which can happen especially when comparing metabolites with parent compounds. In this case a mass selective detector coupled to an HPLC is preferred. Mass spectra are typically more specific than UV spectra and generally should be used for chromatographic selectivity assessment. An example for using a mass spectrometer coupled to an HPLC is shown in Figure 4. Different mass spectra acquired in different sections of the peak at 4.86 min indicate peak impurity.

Selectivity studies should also assess interferences that may be caused by the matrix, such as urine, blood, soil, water or food. Optimized sample preparation can eliminate most of the matrix components. The absence of matrix interferences for a quantitative method should be demonstrated by the analysis of at least five independent sources of control matrix.

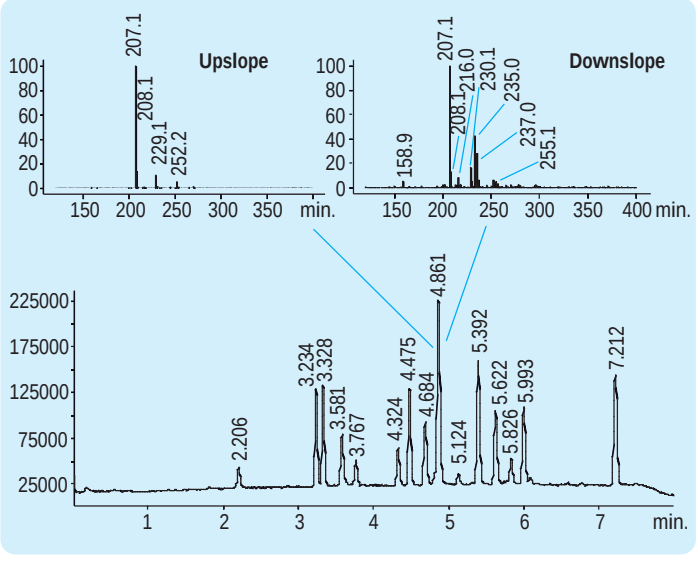

Figure 4
Mass spectra acquired in different sections of the peak at 4.86 min indicate peak impurity.

3.2 Precision

ICH defines the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

- **Repeatability** expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.
- **Intermediate precision** expresses variations within laboratories, such as different days, different analysts, different equipment, and so forth.
- **Reproducibility** expresses the precision between laboratories (collaborative studies usually applied to standardization of methodology).

The ICH[5] requires repeatability to be tested from at least six replications measured at 100 percent of the test target concentration or from at least nine replications covering the complete specified range. For example, the results can be obtained at three concentrations with three injections at each concentration.

Intermediate precision is determined by comparing the results of a method run within a single laboratory over a number of days. A method's intermediate precision may reflect discrepancies in results obtained from:

- different operators
- inconsistent working practice
- different instruments
- standards and reagents from different suppliers
- columns from different batches
- a combination

The objective of intermediate precision validation is to verify that in the same laboratory the method will provide the same results once the development phase is over.

The objective of reproducibility is to verify that the method will provide the same results in different laboratories. The reproducibility of an analytical method is determined by analyzing aliquots from homogeneous lots in

different laboratories with different analysts. In addition, typical variations of operational and environmental conditions that may differ from, but are still within, the specified parameters of the method are used. Validation of reproducibility is important if the method is to be used in different laboratories. Factors that can influence reproducibility include differences in room temperature and humidity, or equipment with different characteristics such as delay volume of an HPLC system, columns from different suppliers or different batches and operators with different experience and thoroughness.

3.3 Accuracy and Recovery

ICH defines the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found.

Accuracy can also be described as the extent to which test results generated by the method and the true value agree.

The true value for accuracy assessment can be obtained in several ways. One alternative is to compare the results of the method with results from an established reference method. This approach assumes that the uncertainty of the reference method is known.

Secondly, accuracy can be assessed by analyzing a sample with known concentrations (for example, a control sample or certified reference material) and comparing the measured value with the true value as supplied with the material. If certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration by weight or volume.

After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in a pure solvent. Because this accuracy assessment measures the effectiveness of sample preparation, care should be taken to mimic the actual sample preparation as closely as possible. If validated correctly, the recovery factor determined for different concentrations can be used to correct the final results.

The concentration should cover the range of concern and should include concentrations close to the quantitation limit, one in the middle of the range and one at the high end of the calibration curve. Another approach is to use the critical decision value as the concentration point that must be the point of greatest accuracy.

The ICH document on validation methodology recommends accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations with three replicates each). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value, together with the confidence intervals.

3.4 Linearity and Calibration Curve

ICH defines linearity of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Linearity may be demonstrated directly on the test substance (by dilution of a standard stock solution) or by separately weighing synthetic mixtures of the test product components.

Linearity is determined by a series of five to six injections of five or more standards whose concentrations span 80–120 percent of the expected concentration range. The response should be directly proportional to the concentrations of the analytes or proportional by means of a well-defined mathematical calculation. A linear regression equation applied to the results should have an intercept not significantly different from zero. If a significant nonzero intercept is obtained, it should be demonstrated that this has no effect on the accuracy of the method.

Frequently, the linearity is evaluated graphically, in addition to or as an alternative to mathematical evaluation. The evaluation is made by visually inspecting a plot of signal height or peak area as a function of analyte concentration. Because deviations from linearity are sometimes difficult to detect, two additional graphical procedures can be used. The first is to plot the deviations from the regression line versus the concentration or versus the logarithm of the concentration if the concentration range

covers several decades. For linear ranges, the deviations should be equally distributed between positive and negative values.

Another approach is to divide signal data by their respective concentrations, yielding the relative responses. A graph is plotted with the relative responses on the y-axis and the corresponding concentrations on the x-axis, on a log scale. The obtained line should be horizontal over the full linear range. At higher concentrations, there will typically be a negative deviation from linearity. Parallel horizontal lines are drawn on the graph corresponding to, for example, 95 percent and 105 percent of the horizontal line. The method is linear up to the point where the plotted relative response line intersects the 95 percent line. Figure 5 shows a comparison of the two graphical evaluations using HPLC.

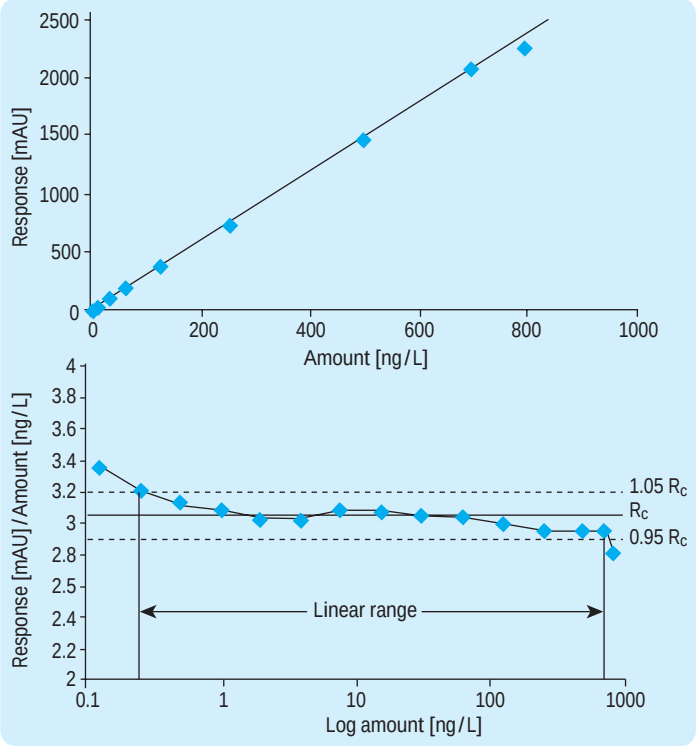

Figure 5
Graphical presentations of linearity plot using HPLC.

Plotting the sensitivity (response/amount) gives a clear indication of the linear range. Plotting the amount on a logarithmic scale has a significant advantage for wide linear ranges. Rc designates the line of constant response.

ICH recommends the linearity curve's correlation coefficient, y-intercept, slope of the regression line and residual sum of squares for accuracy reporting. A plot of the data should be included in the report. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. In order to establish linearity, a minimum of five concentrations is recommended. Other approaches should be justified.

3.5 Range

ICH defines the range of an analytical procedure as the interval from the upper to the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The range of an analytical method is the interval from the upper to the lower levels (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results (for example percentage, parts per million) obtained by the analytical method.

For assay tests, ICH requires the minimum specified range to be 80 to 120 percent of the test concentration. It also requires the range for the determination of an impurity to extend from the limit of quantitation or from 50 percent of the specification of each impurity, whichever is greater, to 120 percent of the specification. Figure 6 shows graphical definition of linearity and range.

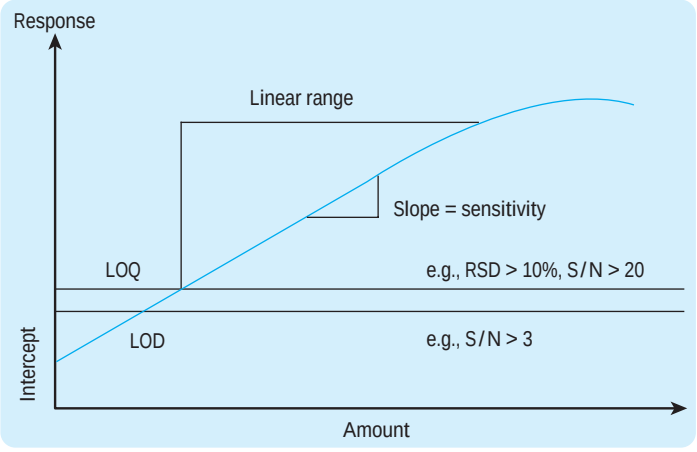

Figure 6
Definition for linearity and range.

3.6 Limit of Detection

ICH defines the detection limit of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

The limit of detection (LOD) is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified. The limit of detection is frequently confused with the sensitivity of the method. The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass.

In chromatography, the detection limit is the injected amount that results in a peak with a height at least two or three times as high as the baseline noise level. Besides this signal-to-noise method, the ICH[5] describes three more methods:

Visual evaluation: The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Standard deviation of the response based on the standard deviation of the blank: Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

Standard deviation of the response based on the slope of the calibration curve: A specific calibration curve is studied using samples containing an analyte in the range of the limit of detection. 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. Figure 7 illustrates the graphical evaluations of LOD and LOQ via signal-to-noise.

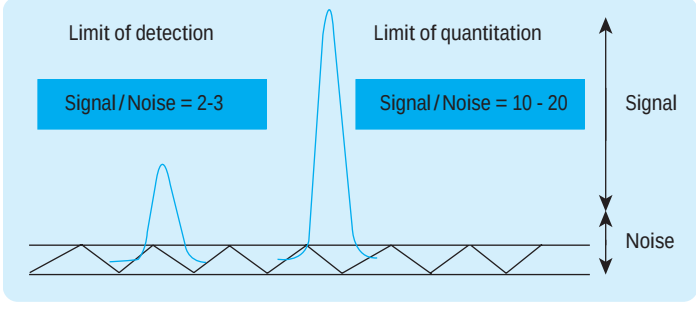

Figure 7
Limit of detection and limit of quantitation via signal-to-noise.

3.7 Limit of Quantitation

ICH defines the limit of quantitation (LOQ) of an individual analytical procedure as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities or degradation products.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. If the required precision of the method at the limit of quantitation has been specified, 5 or 6 samples with decreasing amounts of the analyte are injected six times. The amounts range from the known LOD as determined above to 20 times the LOD.

The calculated relative standard deviation (RSD) percent of the precision of six repetitive injections is plotted against the analyte amount. The amount that corresponds to the previously defined required precision is equal to the limit of quantitation. It is important to use not only pure standards for this test but also spiked matrices that closely represent the unknown samples. Figure 8 shows required experimental steps and a typical graph.

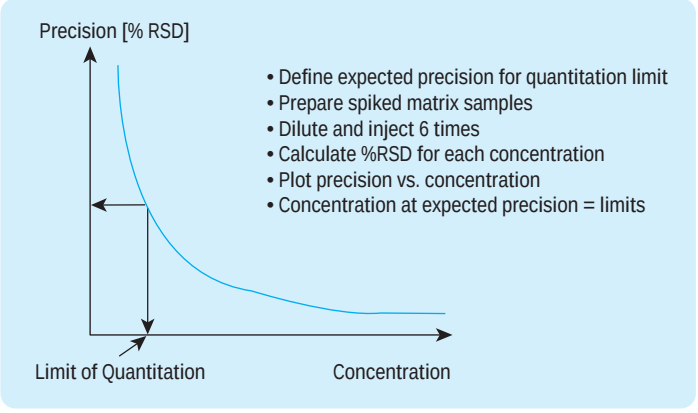

Figure 8
Limit of quantitation based on selected precision.

For chromatographic methods the LOQ can also be determined through comparing measured signals from samples with known low concentrations of analyte with those of blank samples. This establishes the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

Any results of limits of detection and quantitation measurements must be verified by experimental tests with samples containing the analytes at levels across the two regions. It is equally important to assess other method validation parameters, such as precision, reproducibility and accuracy, close to the limits of detection and quantitation. Figure 6 illustrates the limit of quantitation (along with the limit of detection, range, and linearity). Figure 7 illustrates both the limit of detection and the limit of quantitation based on signal-to-noise.

3.8 Ruggedness

Ruggedness is not addressed in the ICH documents[4,5]. Its definition has been replaced by reproducibility, which has the same meaning. Ruggedness is defined by the USP as the degree of reproducibility of results obtained under a variety of conditions, such as different laboratories, analysts, instruments, environmental conditions, operators and materials. Ruggedness is a measure of the reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

3.9 Robustness

ICH defines the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the procedure's reliability during normal usage.

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, a number of method parameters, such as 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.

Obtaining data on these effects helps to assess whether a method needs to be revalidated when one or more parameters are changed, for example, to compensate for column performance over time. In the ICH document[5], it is recommended to consider the evaluation of a method's robustness during the development phase, and any results that are critical for the method should be documented.

3.10 Stability

Chemical compounds can decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Under these circumstances, method development should investigate the stability of the analytes

and standards. It is a measure of the bias in assay results generated during a preselected time interval, for example, every hour up to 46 hours, using a single solution.

Stability testing is important for estimating the allowed time span between sample collection and sample analysis. It is also important to evaluate an analytical method's ability to measure drug products in the presence of its degradation products. Experiments should be conducted under real sample storage conditions because the stability of drug substances is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system stability. The studies should evaluate the stability of the analytes during sample collection and handling after typical storage scenarios such as long term storage (when frozen at intended storage temperatures), short term storage (during a series of sample analyses at room temperature), and after freeze and thaw cycles. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling, storage and analysis.

All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free matrix. Stock solutions of the analyte for stability evaluation are prepared in an appropriate solvent at known concentrations.

The stability of the stock solutions of the drug and the internal standard should be evaluated at room temperature for at least six hours. After completion of the desired storage time, the stability is tested by comparing the instrument response with that of freshly prepared solutions. System stability is determined by replicate analysis of the sample solution and calculation of the RSD of the responses. System stability is considered appropriate when the RSD does not exceed more than 20 percent of the corresponding value of the short term system precision. If the value is higher on plotting the assay results as a function of time, the maximum duration of the sample solution usability can be calculated.

To force degradation, ICH[4] also recommends conducting stress studies, in conditions such as elevated temperature, humidity or light to demonstrate the specificity of the assay in presence of degradation products. The goal is to generate typical degradation products that may be expected. As a rule of thumb, stress conditions should be selected so that 5-20 percent of the drug substances are degraded.

In addition, it is recommended to measure the stability under different freeze and thaw cycles, both short and long term. Below are example conditions suggested for bioanalytical studies[3]. Exact conditions depend on application-specific storage conditions.

Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature.

Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Stability of Processed Samples

The stabilities of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Chapter 4

Method Validation Process

4

Method Validation Process

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to unknown samples analyzed routinely. The preparation and execution should follow a validation protocol, preferably written in a step-by-step instruction format. This chapter describes the approach and steps to validate nonstandard analytical procedures and methods. The proposed procedure assumes that the type of instrument has been selected and the method has been developed. It meets criteria such as ease of use; ability to be automated and to be controlled by computer systems; costs per analysis; sample throughput; turnaround time; and environmental, health and safety requirements.

4.1 Lifecycle Concept

As with equipment qualifications or computer system validations, method validation is not a single event. It begins when somebody wants to implement a new method in a laboratory and ends when the method is no longer in use. The process is broken down in phases because of the length of time and complexity. The process is illustrated in Figure 9.

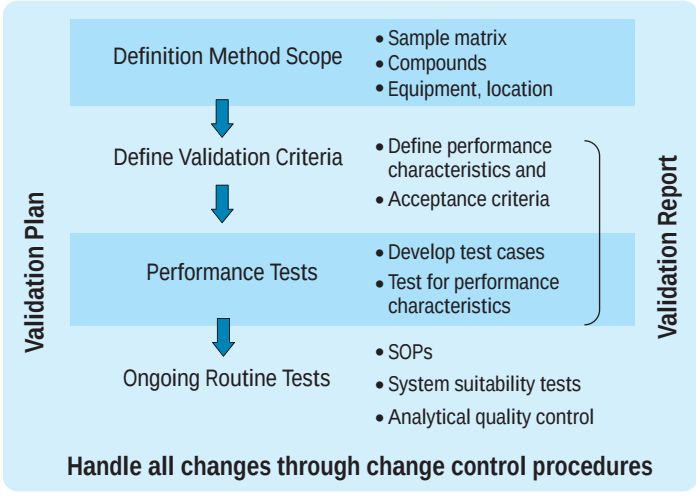

Figure 9
Validation phases.

A validation plan is developed, including owners, responsibilities and deliverables. The first step is to define the scope of the method. This includes the compounds with concentration range, the sample matrix, the specific equipment that should be used and the location where the method should be performed for sample analysis. Once we know what should be analyzed we define performance characteristics, performance tests and acceptance criteria. Test protocols are then developed with all experimental details and the tests are executed according to protocols. Tests results are compared with acceptance criteria. Finally, routine method procedures are developed to verify continual system performance at the time of analysis. Tests may include system suitability testing and the analysis of quality control samples. All experimental conditions and validation results are documented in a validation report.

4.2 Validation Planning

Successful validation requires the cooperative efforts of several departments of an organization including regulatory affairs, quality assurance, quality control and analytical development. Therefore, it is important that the process follows a well-defined validation master plan for analytical methods. The plan documents a company's approach and steps for method validation and serves two purposes. When implemented correctly, it ensures consistent and efficient execution of validation projects. In addition, it answers an inspector's questions regarding the company's approach for all aspects of analytical method validation. The master plan is also an ideal training tool for all employees affected by method validation. The master plan should include:

1. Purpose and scope
2. Glossary
3. Responsibilities, such as user departments, management, QA
4. Method performance characteristics and approaches for testing
5. Steps for method validation
6. Selection of tests and acceptance criteria
7. Approach and parameters for system suitability testing

8. Modification and revalidation of methods
9. Verification of compendial and standard methods
10. Transfer of analytical methods
11. List of required Standard Operating Procedures
12. Approval process, documentation and archiving
13. Templates for project plans, test protocols and validation reports

For each individual validation project a project plan should be developed. It outlines what is to be done in order to get a specific method or procedure validated. This plan should be derived from the master plan. The plan should include a time table with specific tasks, deliverables and owners.

4.3 Scope and Method Specifications

The scope of the method and its validation criteria should be defined early in the process. Defining a scope is a cooperative effort of several departments including business development, analytical development, quality control and quality assurance. Questions include:

- What samples should be analyzed?
- What analytes should be measured?
- What are the expected concentration levels?
- What are the sample matrices?
- Are there interfering substances expected, and, if so, should they be detected and quantified?
- Are there any specific legislative or regulatory requirements?
- Should information be qualitative or quantitative?
- What are the required detection and quantitation limits?
- What is the expected concentration range?

- What precision and accuracy is expected?
- How robust should the method be?
- Which type of equipment should be used? Is the method for one specific instrument, or should it be used by all instruments of the same type?
- Will the method be used in one specific laboratory or should it be applicable in all laboratories at one side or around the globe?
- What skills do the anticipated users of the method have?

Defining the scope of a method is important to find the optimal effort for testing. For example, including equipment from different vendors will increase the test effort but also a laboratory's flexibility to use different instruments in routine analysis. If the method is to be run only on a specific instrument, there is no need to use instruments from other vendors in the validation experiments. In this way, the experiments can be limited to what is really necessary. Similarly, including different locations in the validation study will increase the test effort but it will also allow easy transfer of the method to sites that have been part of the validation studies.

4.4 Selecting Validation Parameters and Limits

For an efficient validation process, it is important to specify the right validation parameters and acceptance criteria. The method's performance parameters and limits should be based on the intended use of the method. It is not always necessary to validate all analytical parameters available for a specific technique. 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 limit of quantitation, or the linearity over the full dynamic range of the equipment. The more parameters, the more time it will take to validate. It is not always essential to validate every analytical performance parameter, but it is necessary to define the ones that are required. The selection of validation parameters and acceptance criteria should be based on business, regulatory and client requirements and should be justified and documented.

Analytical task	Identification	Impurity testing Quantitative	Limit tests	Assay
Accuracy	No	Yes	No	Yes
Precision				
Repeatability	No	Yes	No	Yes
Intermediate precision	No	Yes	No	Yes
Reproducibility	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
Limit of detection	No	No	Yes	No
Limit of quantitation	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

Figure 10
ICH validation characteristics.

Analytical Task	Assay Category 1	Assay Category 2 Quantitative	Limit tests	Assay Category 3
Accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
Limit of detection	No	No	Yes	*
Limit of quantitation	No	Yes	No	*
Linearity	Yes	Yes	No	*
Range	Yes	Yes	*	*
Ruggedness	Yes	Yes	Yes	*

Category 1: Quantitation of major components
Category 2: Impurities
Category 3: Performance characteristics
* May be required, depending on the nature of the specific test

Figure 11
USP validation characteristics.

Both the USP[11] and the ICH[4] contain chapters on parameters to be validated for different analytical tasks (Figures 10 and 11). ICH defines different types of analytical procedures to be validated:

- Identification test
- Quantitation tests for impurities content
- Limit test for the control of impurities
- Quantitative tests of the active ingredient or other main components of the drug

According to the ICH, accuracy, any type of precision and limits of detection and quantitation are not required if the analytical task is for identification purposes. For assays in USP Category 1, the major component or active ingredient to be measured is normally present at high concentrations; therefore, validation of limits of detection and quantitation is not necessary.

Acceptance criteria for the specifications also depend on the intended use of the method. For example, the FDA Guidance on bioanalytical methods validation (3) suggests that for biological samples a precision of 20% RSD to be acceptable at the limit of quantitation. On the other hand precision of drug substances in routine quality control is expected to be 2% RSD or better.

For limits of detection and quantitation of drugs a set of specifications and acceptance criteria should be available for each compound based on data collected during drug development, on product release specifications and shelf life acceptance criteria, and on Pharmacopeial Tests. The specifications and acceptance criteria should be reviewed and updated if necessary as more solid information is available during manufacturing of the product.

Recommendations for setting specifications and limits for detection and quantitation can be obtained from ICH guidelines. For example, ICH Q3A(R) guideline on impurities[23] states: "For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures should be commensurate with the level at which the impurities should be controlled". For establishing acceptance criteria and control limits the guideline makes reference to ICH Q6A Guideline on Specifications[24]. All decisions should be justified and documented.

For contract analyses a method`s performance specifications and acceptance criteria are subject to a formal agreement between the client and the contract laboratory.

4.5 Testing for Performance Characteristics: Preparation and Execution

Once tests and acceptance criteria have been defined, experiments for testing should be thoroughly prepared, executed and documented according to a validation protocol.

Preparation

Good preparation work is important for efficient experiment execution. Most important are the use of qualified material, qualified equipment, trained people and well documented procedures.

- Any chemicals used to determine critical validation parameters, such as reagents and reference standards, should be available in sufficient quantities, accurately identified, sufficiently stable and checked for exact composition and purity according to specifications.
- Any other materials and consumables, for example, chromatographic columns, should be new and qualified to meet the column's performance criteria. This ensures that one set of consumables can be used for most experiments and avoid unpleasant surprises during method validation.
- Analytical equipment should be clearly defined, well characterized, qualified or calibrated to make sure that it meets the functional and performance specifications as required by the analytical method. The selected equipment should have average performance rather than selecting best performing equipment. Otherwise there may be problems with intermediate precision and reproducibility studies to meet acceptance criteria with different equipment.
- Operators should be sufficiently familiar with the technique and equipment. This will allow them to identify and diagnose unforeseen problems more easily and to run the entire process more efficiently.

If there is little or no information on the method's performance characteristics, it is recommended that the suitability of the method be proven for its intended use either during development or in initial validation experiments. These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, the method itself, the specifications and the equipment or the analysis technique should be changed. Method development and validation are, therefore, an iterative process. For example, in liquid chromatography, selectivity is achieved through the selection of mobile phase composition. For quantitative measurements, the resolution factor between two peaks should be 2 or higher. If this value is not achieved, the mobile phase composition needs further optimization. The influence of operating parameters such as pH, mobile composition, or flow rate on the method's performance should be assessed at this stage if this was not done during development and optimization of the method.

Test Execution

There are no official guidelines for the sequence of validation testing. The optimal sequence may depend on the method itself. Based on the author's experience, for a liquid chromatographic method, the following sequence has proven to be useful:

1. Specificity/selectivity
2. Repeatability of retention times and peak areas
3. Linearity, limit of quantitation, limit of detection, range
4. Accuracy at different concentrations
5. Intermediate precision
6. Reproducibility

The more time-consuming experiments, such as intermediate precision and reproducibility, are included towards the end. Some of the parameters, as listed under points 2-4, can be measured in combined experiments. For example, when the precision of peak areas is measured over the full concentration range, the data can be used to validate the linearity.

4.6 Developing a Quality Control Plan for Routine Analysis

The objective of analytical method validation is not only to ensure valid analytical data during initial use of the method but during its entire lifetime. Appropriate checks should be included in the routine sample analysis to verify that the method and the system perform as initially specified at the time of sample analysis. Checks do not need to cover all initial tests but should focus on the most critical performance characteristics, especially those that are most likely to change over time. Such check procedures for execution should be developed and documented as part of the method validation processes because information about the critical items is probably most readily available at this time.

Common on-going tests are system suitability tests (SST) and the analysis of quality control samples. For chromatographic methods system suitability tests are described in Pharmacopeias[21,25] and typically include resolution between two peaks, repeatability of peak areas, tailing factor, and number of theoretical plates. System suitability testing is recommended as a component of any analytical procedure, not just those that involve chromatographic techniques. For example, titration-based analytical procedures should always include the evaluation of a blank.

Analysis of quality control samples is required by quality and accreditation standards such as ISO/IEC 17025. Criteria should indicate when the method and system are beyond statistical control. The aim is to optimize these experiments so that, with a minimum number of control analyses, the method and the complete analytical system will provide long-term results to meet the objectives defined in the scope of the method. The percentage of control samples relative to the number of unknown samples depends on the criticality of samples and stability of the system. It is typically 5 to 20 percent. Regulations and quality standards don't require a specific number but expect that the frequency is defined, justified and documented by company procedures. Once the frequency of control checks is defined, inspectors will check if the procedure is adequate and followed. The procedure should also give instructions on what to do if acceptance criteria are not met.

4.7 Validation Report and other Documentation

Once the method has been developed and validated, a validation report should be prepared. The report should include sufficient information so that an experienced analyst can repeat the validation study. Typically it should include the following:

- Purpose and scope of the method (applicability, type)
- Summary of methodology
- Responsibilities
- Type of compounds and matrix
- All chemicals, reagents, reference standards, QC samples with purity, grade, their source, or detailed instructions on their preparation
- Procedures for quality checks of standards and chemicals used
- Safety precautions
- A plan and procedure for method implementation from the method development lab to routine analysis
- Critical parameters taken from robustness testing
- Detailed parameters and conditions on how the experiments were conducted, including sample preparation and method parameters
- Statistical procedures and representative calculations
- Procedures for QC in routine analyses, such as system suitability tests
- Representative plots, such as chromatograms, spectra and calibration curves including raw data
- Method acceptance limit performance data
- Expected uncertainty of measurement results
- Criteria for revalidation
- Qualification records of the individuals who developed and validated the method

- References, if necessary
- Deviations from the validation plan and protocol
- Summary and conclusions
- Approval with names, titles, date and signatures of those responsible for the review and approval of the analytical test procedure.

Chapter 5

Method Adjustments, Changes, Revalidation and Verification of Standard and Compendial Methods

5

Method Adjustments, Changes, Revalidation and Verification of Standard and Compendial Methods

Most likely analytical methods have to be changed or adjusted during the life of the method. For example, method parameters may need modification back to their original specifications if the method is no longer meeting performance requirements. Method revision would also be required if the scope of a method changes, such as the addition of new target compounds or alteration of the sample matrix. This chapter discusses both scenarios and recommends when revalidation is required and how it is documented.

5.1 Method Adjustments

Frequently, validated analytical methods do not perform as expected. For example, chromatographic peaks are not separated as the method predicts. This may happen when:

- A method is transferred from a development laboratory to a routine lab or between routine laboratories.
- A compendial or standard method is introduced into a laboratory.
- Different instrument models with different characteristics either from the same or different vendors are used, such as HPLCs with different delay volumes.
- The column performance changes over its lifetime.
- New column batches with different characteristics are used.
- New technology is introduced to optimize analyses; for example, save operating costs, reduce column inside diameter to reduce mobile phase consumption, or reduce particle size to minimize analysis time.

Typically analysts try to modify method parameters such as the mobile phase composition, column temperature or flow rate, to bring the performance back to original specifications. It is unclear if the method has to be revalidated after these types of changes.

Answers to this question have been addressed for chromatographic methods by Pharmacopeias in Europe[25] and the United States[21]. The chapters list performance characteristics for liquid and gas chromatography with changes that do not require revalidation as long as system suitability parameters are met.

The recommendations from USP and EP for HPLC and for GC are shown side by side in Figure 12. Recommendations from USP and EP are the same or similar. For example, the parameters for the column are identical for USP and EP.

High Performance Liquid Chromatography	USP	EP
Column length	±70%	±70%
Internal diameter	Can be adjusted if linear velocity is kept constant	±25%
Particle size	Reduction of 50%, no increase	Reduction of 50%, no increase
Flow rate	±50% or more as long as linear velocity is kept constant	±50%
Column temperature	±10 C	±10% Max 60 C
Injection volume	May be decreased (if LOD and repeatability ok.)	May be decreased (if LOD and repeatability ok.)
pH	±0.2 units	±0.2 (±1% for neutral substances)
UV wavelength	No adjustment permitted	No adjustment permitted
Conc. of salts in buffer	±10%	±10%
Composition of mobile phase	Minor components (<50%) ±30% relative or ±10% absolute whichever is smaller	Minor components ±30% relative or ±2% absolute whichever is larger

Gas Chromatography	USP	EP
Column length	±70%	±70%
Column internal diameter	±50%	±50%
Particle size	Changes allowed SST must pass	-50%, no increase
Film thickness	-50 to +100%	-50 to +100%
Flow rate	±50%	±50%
Oven temperature	±10 %	±10%
Injection volume	May be decreased (if LOD and repeatability ok.)	May be decreased (if LOD and repeatability ok.)

Figure 12
Allowed modifications for HPLC and GC.

The given limits should not be interpreted as saying that any method can be changed up to the limit as long as it meets all performance characteristics. The recommendation is that system suitability tests be performed after any modification. Revalidation is not required if all system suitability criteria are met. In other words, the performance of the method should be verified, but does not need to be revalidated. The baseline point is always the last revalidation, not the last parameters before the method changes are implemented. For example, if the flow rate at initial validation was 1.0 min, 1.4 at the first modification (40%) and then 1.7 (20% from last change but 70% from baseline point) the method needs to be revalidated even if the system suitability test (SST) passed. Figure 13 shows a flow chart that can be followed in case chromatographic methods required modification.

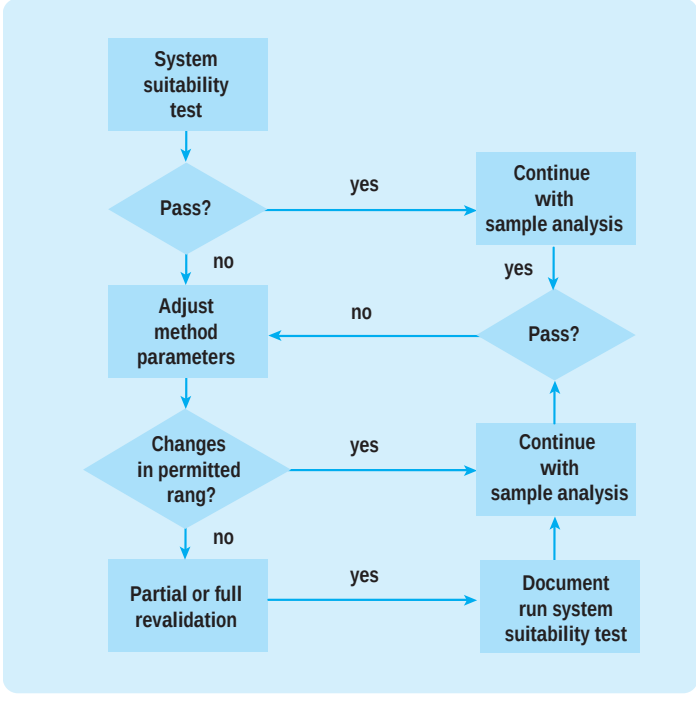

Figure 13
Flow chart for method modifications.

The USP originally defined the same variations for the internal diameter of the HPLC as the EP (±25%). The USP has changed this with USP 32 Second Supplement (Dec 1, 2009). The column diameter changes are allowed, provided that the linear velocity is kept constant according to the formula:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

where F, l, and d are the flow rates, the column lengths, and the column diameters, respectively, before the change (subscript 1) and after the change (subscript 2).

This allows a reduction in mobile phase consumption through reduced column diameters and flow rates, as long as the method's performance is verified under the new conditions.

Otherwise, the chapter does not allow changes to the column specified in the monograph. For example, switching to a column with different particle size and dimensions may provide a more rapid separation with equivalent, chromatographic performances. However, both these situations currently require revalidation. USP is aware of this and may edit their requirements to address it. A committee, lead by USP's H. Pappa, published a stimuli paper in the Pharmacopeial Forum[26]. The article proposes a new approach that will both preserve the quality of the separation as well as expand the changes in particle size beyond the current twofold decrease. The intent of this proposal is to allow the chromatographer a reduction in analysis time without the need for revalidation as long as chromatographic performance is maintained.

5.2 Revalidation

Analytical methods require revalidation if:

- The method parameters have to be changed to maintain the original performance and the change is outside the tolerance allowed by USP
- New compounds are analyzed that are not within the method's original scope
- The sample matrix changes.

Any such modification should be documented following a change control procedure and the method should be revalidated. As part of the procedure the reason for the change should be defined and the change should be authorized for implementation and documented. Performance tests should be justified and documented, and the change formally released.

5.3 Verification of Standard and Compendial Methods

Laboratories working in regulated or quality standard environments are recommended to use the official methods developed by organizations such as the EPA, American Society for Testing and Materials (ASTM), AOAC, ISO or the USP. For example, the US Food, Drug and Cosmetic Act requires FDA-regulated industries to use compendial methods or demonstrate equivalency. ISO/IEC 17025 states: "Methods published in international, regional or national standards shall preferably be used." These methods are validated; therefore many analysts incorrectly assume that the methods can be used as they are without any further validation, verification or testing done in the laboratory. The US FDA cGMP regulation states in 21 CFR 211.194 (a)(2); "If the method employed is in the current revision of the United States Pharmacopoeia, or in other recognized standard methods, or is detailed in an approved new drug application and the referenced method is not modified, a statement indicating the method and reference will suffice. The suitability of all testing methods used shall be verified under actual condition of use. "ISO/IEC 17025 has similar requirements as stated in Par. 5.4.2: "The laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations. If the standard method changes, the confirmation shall be repeated".

This makes it clear that official methods don't need to be validated as long as they are not changed, but the laboratory should demonstrate that it is capable of successfully running the method. Issues arise when determining the best way to do this. It is unclear whether or not some or all validation experiments need to be repeated, or if successful system suitability tests or the analysis of quality control samples are enough.

USP answers these questions in Chapter <1226>: Verification of compendial methods[12]. The given recommendations apply to implementation of compendial methods and standard methods.

The key recommendations are:

- Demonstrate the performance of the laboratory and system through system suitability tests
- Assess the criticality and complexity of the method
- Select most critical performance characteristics of the method
- Depending on the criticality and complexity of the method, repeat one to three most critical validation experiments.

Similar to the validation of methods developed internally, the evaluation and verification of standard methods should also follow a documented process such as a validation plan or an SOP. Results should be documented in the validation protocol. Both documents will be the major source for the validation report.

The process for verification of compendial/standard methods is illustrated in Figure 14.

1. Define the scope of the analytical tests to be carried out in the laboratory.
2. Verify that the scope of the compendial/standard procedure is identical to the scope defined.
3. If the scope as defined is not identical, modify the existing method or develop a new method and validate for characteristics that are not the same.
4. If the method is identical, perform system suitability tests and run one to three validation experiments, depending upon the criticality of the method. If the tests pass acceptance criteria, document the scope, tests, and test results and write a statement that the method is ready for use.

5. If the test results are not acceptable try to find the reason or root cause of the problem. This could be inadequate equipment or reference material.If the cause is obvious, correct the problem and test again. This can be an iterative process.
6. If the root cause cannot be found change the method or develop a new one.
7. If the required changes are outside acceptable limits as defined in Pharmacopeias, the method needs to be revalidated.

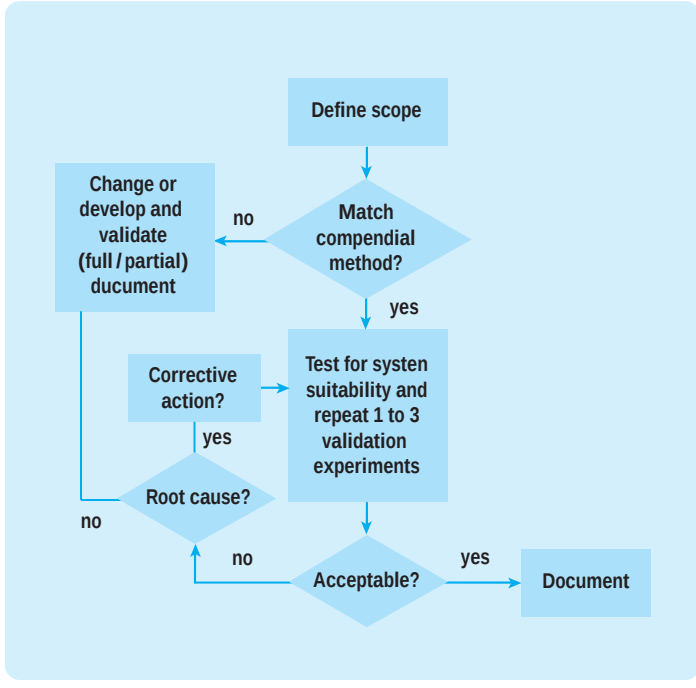

Figure 14
Process for verification of analytical methods.

The selected verification tests are application specific. Figure 15 shows recommended verification tests for selected pharmaceutical applications.

Example #	Application	Recommended tests
1	Quantitation of major compounds of drug substances in finished drugs or APIs	Precision, specificity, linearity
2	Quantitative determination of impurities in drug substances or degradation products in finished drugs	Precision, specificity, limit of quantitation
3	Limit tests of impurities in drug substances or degradation products in finished drugs	Specificity, limit of detection

Figure 15
Verification tests recommended by USP <1226> for selected applications.

AOAC has published recommendations for verification activities when standard methods are implemented for ISO/IEC 17025. Examples for different applications are listed in Figure 16.

Example #	Application	Recommended tests
1	Confirmation of identity	• Specificity
2	Quantitative analysis at low concentrations	• Accuracy • Precision • Specificity • LOQ / LOD
3	Analyte is present above or below specified low concentration (Limit Test)	• Specificity • LOD
4	Quantifying an analyte at high concentrations	• Accuracy • Precision • Specificity

Figure 16
Verification tests recommended by AOAC for selected applications (extract from Tables 2 to 5 in reference 9).

Chapter 6

Transfer of Analytical Methods

6

Transfer of Analytical Methods

When validated methods are transferred between laboratories the receiving laboratory should demonstrate that it can successfully perform the method. Typical instances when method transfer occurs are from the Research and Development (R&D) laboratory to the Quality Control (QC) laboratory, Site A to Site B when a product line is moved, from a sponsor company to a contract laboratory and from Company X to Company Y when a product is purchased by another company. Currently, there is no official document available that can be used as a guide for performance demonstration of the receiving laboratory. Most promising is a Stimuli article published by a USP General Chapter Expert Committee under the lead of USP's H. Pappa with the title "Transfer of Analytical Methods - A Proposal for a New General Information"[27]. The article describes the most common practices of method transfer which are: comparative testing, co-validation between two laboratories or sites, complete or partial method validation or revalidation, and the omission of formal transfer, sometimes called the transfer waiver.

6.1 Transfer Procedures

Comparative testing

Comparative testing is the most common form of method transfer in testing laboratories. Well-characterized representative samples are analyzed in the transferring and receiving laboratories. Before the method is transferred, care should be taken that the method and its critical parameters are well understood by the workers in the receiving laboratory. A detailed transfer protocol, a documented procedure for method implementation, and good communication between the transferring and receiving departments are equally important. The transfer protocol outlines both the testing to occur and the roles of the two laboratories, and defines the acceptable values for the transfer. If the results conform to previously defined acceptance criteria the method can be used in the receiving laboratory. The number of samples to be analyzed depends on the criticality of the method, on the complexity of the method, and whether the receiving laboratory is experienced with similar methodologies. Considerations for comparative testing are:

- Number of samples, lots, batches (for example, 2 to 5)
- Number of concentrations (for example, 1 to 3)

- Number of repetitive analyses / sample (for example, 4 to 6)
- Number of analysts (for example, 1 to 2)
- Duration of time (for example, 2 to 5 days)
- Equipment from one or more manufacturers (1 to all available)

Co-validation between two or more laboratories

An alternative to comparative testing is the involvement of the receiving laboratory in the validation of the method to be transferred. For example, the receiving laboratories can participate in interlaboratory tests for ruggedness testing. In principle this is a special form of comparative testing. The advantage is that the resources typically required for method validation and transfer can be shared. However, this only makes sense if all participating laboratories plan to implement the method at around the same time. The transfer should be officially documented by following a pre-approved protocol and predefined acceptance criteria.

Transfer waiver

Under certain circumstances, conventional transfer qualification experiments may be omitted altogether. When a transfer waiver is applied, the receiving laboratory can use the analytical test procedures under discussion without generating comparative testing or validation data. This option should be handled with care and, if used, should be justified and documented. Justifications for a waiver could be that a very similar method is already in use with good experience, or the person who originally developed and validated the method moved from the transferring to the receiving laboratory.

6.2 Steps for Method Transfer

Transfer of Analytical Methods should be thoroughly planned, executed and documented. This section presents the steps necessary to complete a successful method transfer by means of the comparative testing.

- Develop a transfer plan. This includes activities, a time schedule, owners and deliverables. This project-specific plan should be based on a master policy that describes a company's approach to transferring analytical methods.
- Develop a transfer protocol. The protocol describes the objective of the transfer, the scope, the responsibilities of transferring and receiving

laboratories, the materials, the equipment and method parameters. The transfer protocol also includes details of tests, acceptance criteria, justifications for the tests, documentation requirements, and supporting materials such as chromatograms and spectra. In addition, the protocol also includes information on the number of batches, replicates per batch, injection sequences and how deviations will be handled.

- Develop SOPs for executing the tests. The SOPs include preparation of the sample, reference material, and reviewing and documenting test results.
- Train workers. Workers from the transferring laboratory train analysts from the receiving laboratories. Training includes protocol detail, methodology and all issues that have arisen in the past.
- Execute the protocol in both laboratories. Actual results are compared with expected results and acceptance criteria.
- Identify the root cause of any issues. If acceptance criteria are not met, the root cause is indentified and the issue resolved.
- Generate documentation specified in the plan. All documents as specified in the plan are generated, reviewed and signed by laboratory and QA management of the transferring and receiving units.

Chapter 7

Validation of Bioanalytical Methods

7

Validation of Bioanalytical Methods

Bioanalytical methods are used for the quantitative determination of drugs and/or metabolites in biological matrices such as blood, serum, plasma or urine. Analytical results are important in pharmaceutical development for the evaluation and interpretation of preclinical and clinical studies, such as bioavailability, bioequivalency, pharmacokinetic and toxicokinetic experiments. These studies generally support regulatory filings.

Because of the nature of the sample, the bioanalytical methods are more complex and more difficult to validate than methods used for quality control of drug products and drug substances. This is because bioanalytical methods include more complex matrices, a large variety of possible interferences from metabolites, and typically low sample volumes. In addition, bioanalytical data needs to be accurate and reliable because they are used for critical applications including the calculation of pharmacokinetic parameters, that are critical to the review of the performance of a drug or a formulation. Therefore, bioanalytical methods need to be thoroughly validated.

This chapter describes the most important references for bioanalytical method validation, the validation parameters, acceptance criteria, quality control procedures for routine analysis and required documentation. Validation recommendations in this chapter are based on the FDA Guide[3] and on the AAPS workshop report from 2006.[10]

7.1 Validation Guidelines from Conferences and FDA

The importance of bioanalytical methods and their validation has been recognized by regulatory organizations and scientists from industry and universities since the late 1980's. At that time there was no guidance at all, official or unofficial, that bioanalysts could use to validate bioanalytical methods. Since then several workshops and conferences were held with conference reports serving as the first practical validation guidelines. The most important events have been organized by the American Association of Pharmaceutical Scientists (AAPS) and were co-sponsored by the FDA in 1990, 2000 and 2006.

During the first workshop/conference[28] consensus was reached on which bioanalytical method parameters should be evaluated. Essential parameters such as accuracy, precision, selectivity, sensitivity, reproducibility, limit of detection and stability were identified. There was also agreement on some acceptance criteria.

In the second conference in 2000[29] delegates mainly discussed experiences and progress made since the first conference. The workshop report "Bioanalytical Method Validation – A Revisit with a Decade of Progress" was the basis of the FDA Guidance published in 2001.

The main purpose of the third AAPS conference in 2006 was to review the scope and applicability of bioanalytical procedures for the quantitative analysis of samples from bioequivalence, pharmacokinetic and comparability studies. Another purpose was to evaluate validation and implementation requirements for chromatographic and ligand-based quantitative bioanalytical assays for macromolecules.

The most important references available at the time this primer was written are the FDA Guidance on Bioanalytical Method Validation[3] and the AAPS workshop report of the conference in 2006: "Quantitative bioanalytical methods validation and implementation: Best practices for chromatographic and ligand binding assays"[10]. While the FDA guidance mainly focuses on methodology and remains valid, the conference report also provides clarification and best practice recommendations to enhance the quality of bioanalytical work. For example, the workshop clarified issues related to placement of QC samples, determination of matrix effects, stability considerations, use of internal standards and system suitability tests.

In addition, the workshop report gives clear guidelines regarding which validation study records should be retained. Records generated during the course of method validation and study sample analysis are source records and should be retained to demonstrate the validity of the method. For example, chromatograms and run preparation, extraction, and run summary sheets are considered source data. This includes sample runs as well as failed runs. Furthermore, reintegrated chromatograms should be explicitly identified. The reason for the reintegration and the mode of reintegration should be documented. The original and reintegrated chromatogram should be retained ideally as electronic records. Electronic audit trail functionality that record changes to integration parameters must not be disabled.

7.2 Validation Parameters

The fundamental validation parameters for a bioanalytical method validation are not much different from parameters as described earlier in the chapter. They include: selectivity/specificity, precision, recovery, linearity and stability of the analyte. In this section we discuss parameters with specific recommendations for bioanalytical methods. The principles of the stability studies are the same as discussed in the chapter "Parameters and Tests for Method Validation" and are not repeated here.

Selectivity / Specificity

For selectivity/specificity, analysis of blank samples of the appropriate biological matrix should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantitation (LLOQ).

Precision

In line with ICH guidelines[4, 5] precision is subdivided into short term (within-run precision or intrabatch) and intermediate precisions (between-run or interbatch) which measure precision with time, and may involve different analysts, equipment and reagents. Intrabatch precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

For interbatch precision, intrabatch experiments are repeated on four different days by different analysts. Precision from the four-day experiments is compared with the intrabatch precision.

Accuracy

Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

Recovery

Recovery is similar to accuracy but includes the extraction efficiency of an analytical method. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

Calibration / Standard Curve

This validation parameter as defined in the FDA guidance is equivalent to linearity as defined earlier in this primer. It examines the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample and the response should be proportional to the analyte concentration.

A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ. The lowest standard on the calibration curve should be accepted as the limit of quantification if the analyte response is at least five times the response compared to the blank response and if the analyte response is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80 to 120%.

The simplest model that adequately describes the concentration response should be used. At least four of the six non-zero standards should fall within a 15% deviation from the nominal value except at LLOQ, where it should not deviate by more than 20%.

Lower Limit of Quantitation (LLOQ)

LLOQ is the lowest amount of an analyte that can be quantitated with suitable precision and accuracy. The recommended precision at the LLOD is equal or better than 20%. There are different approaches for the determination of the LLOQ. The most practical approach is to determine the amount where the precision and accuracy is at least 20%.

7.3 Quality Control in Bioanalytical Routine Analysis

Biological samples can be analyzed with well validated methods and a single determination, as long as the variability of precision and accuracy routinely falls within acceptable tolerance limits. If this is not the case, duplicate or even triplicate analyses should be performed.

Procedures should be established to maintain optimal conditions and monitor accuracy and precision during routine analysis in order to ensure the defined quality of analytical data. The following considerations are critical:

- Multilevel calibrations should be applied with calibration standards ranging from the lowest to highest expected sample concentrations.
- The calibration (standard) curve should cover the expected unknown sample concentration range, with a calibration sample at the LLOQ.
- Estimation of concentration in unknown samples by extrapolation of standard curves below the LLOQ or above the highest standard is not recommended. Instead, the standard curve should be redefined or samples with higher concentration should be diluted and re-assayed.
- Each analytical batch should include analysis of a blank matrix (sample without internal standard), a zero standard (matrix with internal standard) and a minimum of six non-zero calibration standard points.
- A number of QC samples, matrix spiked with analyte, should be analyzed with processed test samples at intervals based on the total number of samples.
- The minimum number of QC samples should be 5% of the number of sample runs.
- QC samples should be analyzed in duplicate at three concentrations. For example, they should be analyzed once at three times the LLOQ, once in midrange and once approaching the high end of the range.
- At least four of every six QC samples must be within 15% of their respective nominal value. Two of the six QC samples may be outside the 15%, but not both at the same concentration.
- Qualified and properly maintained instruments should be used for implementation of bioanalytical methods. System suitability (SST) parameters, acceptance criteria and frequency should be defined to ensure proper operation of the system. System suitability tests do not replace the required run acceptance criteria with QC samples.

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Glossary

AOAC	Association of Official Analytical Chemists
API	Active Pharmaceutical Ingredients
ASTM	American Society for Testing and Materials
CFR	Code of Federal Regulations
EP	European Pharmacopeia
EU	European Union
FDA	Food and Drug Administration
GC	Gas Chromatography
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HPLC	High Performance Liquid Chromatography
ICH	International Conference for Harmonization
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LGC	Laboratory of the Government Chemist
LOD	Limit of Detection
LOQ	Limit of Quantitation
NATA	National Association of Testing Authorities, Australia
PIC/S	Pharmaceutical Inspection Cooperation Scheme
QA	Quality Assurance
QC	Quality Control
SOP	Standard Operating Procedure
SST	System Suitability Testing
USP	United States Pharmacopeia