A Guide to Analytical Method Validation

INTRODUCTION

Method validation is the process by which it is established, through laboratory studies, that the performance characteristics of the method meet the requirements for its intended purpose (1–5). It is a part of the overall validation process that also includes software validation (6), instrument qualification (7,8), and system suitability (9). Typical analytical characteristics used in method validation are highlighted in Figure 1. Although all analytical procedures or methods used in a regulated laboratory must be validated, this chart focuses specifically on liquid chromatography.

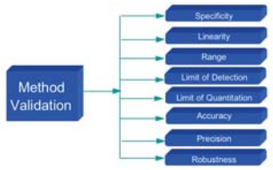

Figure 1: Typical analytical characteristics used in method validation, commonly referred to as the "Eight Steps of Method Validation."

ROBUSTNESS

Robustness is the capacity of a method to remain unaffected by small, deliberate variations in method parameters; a measure of the reliability of a method.

- Robustness should be evaluated in late development, or early in the method validation process. If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.
- Robustness can be used to establish system suitability parameters.
- Normally, after implementing a validated method, it can be adjusted within the confines of the robustness study without triggering a revalidation. However, method changes, outside the range of parameters validated, would require at least some revalidation to show equivalency of results.

Methodology

- Purposely vary method parameters over a known range, and determining the effect (if any) on the method results.
- Multivariate statistical experimental design can be used to control method variables (for example, Factorial, Fractional Factorial, or Plackett-Burman designs).
- Theoretical modeling software can also be used to predict robustness and then verified experimentally.

Documentation

- Robustness can be illustrated by many different means, using summary tables, bar, and control charts, effect and probability plots, and other means of result comparisons.

Table I: Example robustness factor selection and limits for an isocratic LC method. These limits are examples only and should be chosen according to expected laboratory and instrument variations.

Factor	Limit Range (+/–)
Organic Solvent Concentration	2–3%
Buffer Concentration	1–2%
Buffer pH (if adjusted)	0.1-0.2 pH units
Temperature	3°C
Flow rate	0.1-0.2 mL/min.
Wavelength	2–3 nm for 5 nm bandwidth
Injection Volume	Injection type and size dependant

Table II: Example robustness factor selection and limits for a gradient LC method. Factors and limits listed here are in addition to many of the factors considered in an isocratic method. *It is increasingly common for gradient methods to have an initial hold time to accommodate transfer to instruments with different dwell volumes.

Factor	Limit Range (+/–)
Initial hold time*	10–20% of segment time
Slope and length	The slope is set by the initial %B and the final %B, as well as the gradient length. It is recommended to adjust the lengths by 10–20% and allow the slope to vary.
Final hold time	Adjusted according to the last eluting compound and varied accordingly

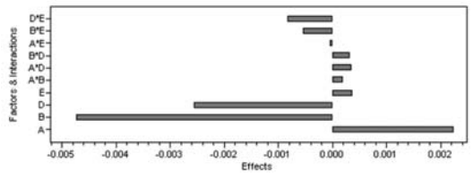

Figure 2: Example Effects Plot. Factor effects can be either positive or negative. The bar indicates the magnitude and the bias of the effect. The effect is the change in response due to the change of a factor. It is the average response at the high level minus the average response at the low level. There are both main effects (due to the change of a single factor) and interaction effects (due to the change of more than one factor).

SPECIFICITY

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix.

Identification tests

- Specificity ensures the identity of the analyte of interest.

Purity tests

- Specificity ensures that the method allows for an accurate statement of the impurity content (that is, in related substances tests, heavy metals and organic volatile impurity limits)

Assays

- Specificity provides an exact result for a determination of the content or potency of the analyte.

Methodology

- Identification (qualitative analyses)
  - Specificity is demonstrated by the ability to discriminate between compounds of closely related structures, or by comparison to known reference materials.
- Assays
  - Specificity is demonstrated using spiked samples to show that the method results are unaffected by the presence of impurities or excipients.
- Impurity tests
  - Impurities available
    - Specificity is demonstrated by spiking the drug substance or product with the appropriate levels of impurities and determining them with the appropriate accuracy and precision.
  - Impurities not available
    - Compare results to a second well-characterized procedure.
    - Include samples stored under relevant stress conditions, (for example, light, heat, humidity, acid/base hydrolysis, and oxidation). For assay, the two results are compared. For impurity tests, the impurity profiles are compared head-to-head.

Documentation

- For chromatographic procedures, representative chromatograms with peaks labeled should be included. Resolution, plate count (efficiency), and tailing factor should be measured and documented.
- Peak purity tests using advanced detection such as photodiode array or mass spectrometry should be used to show that the response is not due to more than one component.

LINEARITY AND RANGE

Linearity

- The ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range.

Range

- The interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the method as written.

Methodology

- Linearity
  - Demonstrate across the entire range of the analytical procedure.
  - A minimum of five concentrations is recommended.
- Range
  - Verify that the method provides acceptable precision, accuracy, and linearity when applied to samples at the extreme as well as within the range.
  - Recommended minimum Ranges:
    - Assay of Drug Substance or Finished Product
      - From 80–120% of the test concentration.
    - Determination of an Impurity
      - From 50–120% of the specification.
    - Content Uniformity
      - A minimum of 70–130% of the test con centration unless a wider or more appropriate range is justified based upon the dosage form.
    - Dissolution Testing
      - +/– 20% over the specified range of the dissolution test.

Documentation

- The report should include:
  - The slope of the regression line.
  - The correlation coefficient.
  - *y*-intercept.
  - The residual sum of squares.

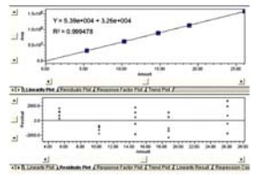

Figure 3: Chromatography data system linearity plot, showing *y*-intercept, slope, and coefficient of determination, and residual plot. Each residual is an estimate of the error in the data and displays how far the data points fall from the regression line. Each residual is the difference between the observed (or actual) response and the response of the regression line.

DETECTION LIMIT (DL OR LOD)

Characteristic of limit tests, the LOD is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value.

Methodology

- Noninstrumental methods
  - Determine LOD by analyzing samples at known concentrations and establishing the minimum level at which the analyte can be reliably detected.

Instrumental methods

- LOD can be determined as a signal to noise ratio, usually 2:1 or 3:1, Or,
- LOD can be calculated at levels approximating the LOD according to the formula: LOD = 3.3(SD/S)
  - (SD) = standard deviation of the response based on either the standard deviation of the blank, the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines.
  - (S) = slope of the calibration curve

Documentation

- Express the LOD as the concentration of the analyte.
- Document and support the method used to determine LOD.
- An appropriate number of samples should be analyzed at the limit to validate the level. In practice, it is almost never necessary to determine the actual LOD. Instead, the detection limit is shown to be sufficiently low (for example, 0.1%) to be able to reliably detect at the level specified.

ACCURACY

Accuracy is the closeness of test results to the true value.

Methodology

- Drug substance
  - Comparison of the results with the analysis of a standard reference material.
  - Comparison to a second, well-characterized method.
- Drug product
  - Evaluate by analyzing synthetic mixtures of known amounts or samples spiked with known quantities of components.
  - Comparison to a second, well-characterized method.
- Quantitation of impurities
  - Analyze samples (drug substance or drug product) spiked with known amounts of impurities. (If impurities are not available, see specificity.)
- Data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates of each concentration).

Documentation

- Reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals.

QUANTITATION LIMIT (QL OR LOQ)

LOQ is the lowest concentration of an analyte in a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational conditions of the method.

Methodology

- Noninstrumental methods
  - Determine LOQ by analyzing samples at known concentrations and establishing the minimum level at which the analyte can be reliably detected.

Instrumental methods

- LOQ can be determined as a signal to noise ratio, usually 10:1, Or,
- LOD can be calculated at levels approximating the LOD according to the formula: LOD = 10(SD/S).
  - (SD) = standard deviation of the response based on either the standard deviation of the blank, the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines.
  - (S) = slope of the calibration curve

Documentation

- Express LOQ as a concentration, with the precision and accuracy of the measurement.
- Documented and support the method used to determine LOD.
- An appropriate number of samples should be analyzed at the limit to validate the level. In practice, it is almost never necessary to determine the actual LOQ. Instead, LOQ is shown to be sufficiently low (e.g. 0.1%) to be able to reliable quantitate at the level specified.

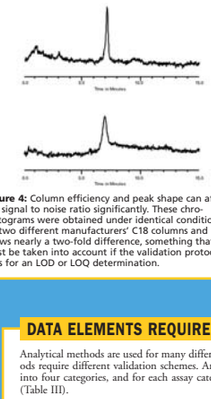

Figure 4: Column efficiency and peak shape can affect the signal to noise ratio significantly. These chromatograms were obtained under identical conditions on two different manufacturers' C18 columns and shows nearly a two-fold difference, something that must be taken into account if the validation protocol calls for an LOD or LOQ determination.

PRECISION

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple samplings of a homogeneous sample.

Repeatability

- Results of the method operating over a short time interval under the same conditions (interassay precision).
- Generally the criteria of concern in *USP* procedures.

Intermediate precision (formerly ruggedness)

- Results from within-laboratory variations due to random events such as different days, analysts, equipment, etc. Experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

Reproducibility

- Results of collaborative studies between laboratories.

Methodology

- The precision of a method is determined by assaying aliquots of a homogeneous sample to be able to calculate statisti cally significant estimates of standard deviation or relative standard deviation (coefficient of variation). Assays should be of samples that have all gone through the entire analytical procedure from sample preparation through final analysis.
- A minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or a minimum of six determinations at 100% of the test or target concentration is recommended.

Documentation

- Precision is expressed as the standard deviation or the relative standard deviation (coefficient of variation) for a statisti cally significant number of measurements and confidence interval. Statistical tables, bar charts, and other types of graphs are commonly used to document precision.

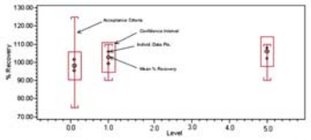

Figure 5: An example of a chromatography data system Whisker Plot, a common way of documenting precision and accuracy. The box represents the upper and lower confidence intervals, the whiskers with up-tics and down-tics represent the user-defined upper and lower acceptance criteria. The small points are the individual data points of % Recovery (at each concentration level); the large points are the mean % recovery at each concentration level.

SYSTEM SUITABILITY

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. System suitability tests are an integral part of chromatographic methods, and are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations, and samples constitute an integral system that can be evaluated as a whole. System suitability parameters are established as a direct result of robustness studies.

Methodology

- Plate count (N), tailing factor (T), resolution (R_s) and reproducibility (%RSD) are determined from replicate injections of a standard (an analyte peak and an internal standard, related compound, excipient, and/or impurity, etc.) compared against method specifications.
  - If the %RSD specification is below 2.0%, five replicates are used.
  - If the %RSD specification above 2.0%, six replicates are used.
- System suitability must be demonstrated at appropriate intervals before, during, and after the analysis of unknown samples, or whenever there is a significant change in instrumentation, or in a critical reagent.

Documentation

- Documentation of system suitability is accomplished by summarizing data on reproducibility, efficiency, tailing and resolution for the replicate injections. Results can also be used to troubleshoot the method. Results stored in a relational database can be compared and summarized on a peak-by-peak or system-by-system basis to provide additional feedback necessary to determine system performance. No sample analysis is acceptable unless system suitability specifications have been met.

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(8) *Pharmacopeial Forum*, 31(5), Sept-Oct 2005, p. 1453-1463.
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DATA ELEMENTS REQUIRED FOR ASSAY VALIDATION

Analytical methods are used for many different purposes, and different test methods require different validation schemes. Analytical Test Methods can be divided into four categories, and for each assay category, different information is needed (Table III).

Category 1: Analytical methods for the quantitation of major components of bulk drug substances or active ingredients in finished pharmaceutical products.

Category 2: Analytical methods for the determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products, including quantitative assays and limit tests.

Category 3: Analytical methods for the determination of performance characteristics (for example, dissolution, drug release).

Category 4: Identification tests.

Table III: Data elements required for validation.

Analytical Performance Characteristics	Category 1	Category 2 Quantitative	Category 2 Limit Tests	Category 3	Category 4
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection Limit	No	No	Yes	*	No
Quantitation Limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

* May be required depending on the nature of the test.

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