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IN THE LAB - Methods Validation | Perspectives on Method Validation II

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Editor's Note: *This article is the second in a three-part series on method validation. Part three will focus on large-molecule applications.*

A risk-based approach to method validation takes into consideration the phase of drug development when determining the level of validation necessary to show that the method is suitable for its intended use. Both the United States Pharmacopeia (USP <1225>) and the International Conference on Harmonisation (ICH Q2[R1] and Q3B[R2]) specify the validation elements required by method type, focusing mainly on the validation of chromatographic methods.1-3

These same principles are applied to the validation of non-chromatographic methods such as those used to determine pH, moisture, or inorganics. Although each generic validation element is defined in these documents, method type and drug-specific factors, as well as phase of drug development, must be considered when designing the validation experiments for a given small-molecule method.4

Specificity

For every phase of product development, the analytical method must demonstrate specificity. The method must have the ability to unambiguously assess the analyte of interest while in the presence of all expected components, which may consist of degradants, excipients/sample matrix, and sample blank peaks. The sample blank peaks may be attributed to things such as reagents or filters used during the sample preparation.

For identification tests, discrimination of the method should be demonstrated by obtaining positive results for samples containing the analyte and negative results for samples not containing the analyte. The method must be able to differentiate between the analyte of interest and compounds with a similar chemical structure that may be present. For a high performance liquid chromatography (HPLC) identification test, peak purity evaluation should be used to assess the homogeneity of the peak corresponding to the analyte of interest.

For assay/related substances methods, the active peak should be adequately resolved from all impurity/degradant peaks, placebo peaks, and sample blank peaks. Resolution from impurity peaks could be assessed by analyzing a spiked solution with all known available impurities present or by injecting individual impurities and comparing retention to that of the active. Placebo and sample matrix components should be analyzed without the active present in order to identify possible interferences.

If syringe filters are to be used to clarify sample solutions, an aliquot of filtered sample diluent should be analyzed for potential interferences. If the impurities/degradants are unknown or unavailable, forced degradation studies should be performed. Forced degradation studies of the active pharmaceutical ingredient (API) and finished product, using either peak purity analysis or a mass spectral evaluation, should be performed to assess resolution from potential degradant products.

The forced degradation studies should consist of exposing the API and finished product to acid, base, peroxide, heat, and light conditions until adequate degradation of the active has been achieved. An acceptable range of degradation may be 10% to 30% but may vary based on the active being degraded. Over-degradation of the active should be avoided to prevent the formation of secondary degradants. If placebo material is available, it should be stressed under the same conditions and for the same duration as the API or finished product. The degraded placebo samples should be evaluated to ensure that any generated degradants are resolved from the analyte peak(s) of interest.

Evaluation of the forced degraded solutions by peak purity analysis using a photodiode array detector or mass spectral evaluation must confirm that the active peak does not co-elute with any degradation products generated as a result of the forced degradation. Another, more conservative, approach for assay/related substances methods is to perform peak purity analysis or mass spectral evaluation on all generated degradation peaks and verify that co-elution does not occur for those degradant peaks as well as the active peak.

Detection Limit

Determination of the detection limit may be delayed until Phase 3 validation of related substances (residual impurities) or residual solvents methods.3,4 The detection limit is the lowest concentration of analyte in a sample preparation that can be detected but not necessarily quantitated as an exact value. Determination of the detection limit should be completed in the presence of product placebo or matrix component, if available. Various approaches for determining the detection limit of a method as presented in Q2(R1) are listed below.2

- **Visual Evaluation:** This approach is utilized for both non-instrumental and instrumental methods. Samples with known concentrations of analyte are analyzed, and the lowest level at which the analyte can be reliably detected is determined.
- **Signal-to-Noise Ratio:** This approach is utilized for methods with baseline noise. The measured signal from samples with known low concentrations of analyte is compared to the baseline noise from the same retention region of a sample blank. Generally, a signal-to-noise ratio of 2:1 or 3:1 is considered acceptable.
- **Based on Standard Deviation and Slope of Response:**

  $$\textit{Detection Limit} = 3.3\,\sigma / S$$

The standard deviation of the response (σ) is based on either the magnitude of the analytical background response (measurement of blank samples) or residual standard deviation of a regression line or standard deviation of Y intercepts of regression lines for calibration curves generated using samples containing the analyte in the range of the detection limit. The slope (S) is based on the calibration curve of the analyte.

An acceptable approach to determine the detection limit of an HPLC-related substances method or a gas chromatography (GC) residual solvent method involves preparing triplicate placebo samples spiked with the analyte of interest at an estimated detection limit concentration. Duplicate injections are made of each spiked placebo preparation. The analyte must be detected in all six chromatograms with a signal-to-noise ratio of approximately 2:1 or 3:1.

Quantitation Limit

The quantitation limit must be determined for related substances methods and for methods that measure residual levels of solvents or other by-products associated with the manufacture of a drug substance or product. The quantitation limit must also be determined for analytical methods associated with cleaning validations. The quantitation limit is typically defined as the lowest concentration of active in the presence of placebo (or vehicle), where the peak area response is greater than a signal-to-noise ratio of 10:1. However, as per ICH Q2(R1), the quantitation limit can be determined in several ways, including visual evaluation, signal-to-noise ratio, and standard deviation of the response and slope.2

- **Visual Evaluation:** This approach is utilized for both non-instrumental and instrumental methods. Samples with known concentrations of analyte are analyzed, and the lowest level at which the analyte can be quantified with acceptable accuracy and precision is determined to be the quantitation limit.
- **Signal-to-Noise:** This approach is utilized for methods with baseline noise. The measured signal from samples with known low concentrations of analyte is compared to the baseline noise from the same retention region of a sample blank. The minimum level at which the analyte can be reliably quantified is determined. Generally, a signal-to-noise ratio of 10:1 is considered acceptable.
- **Based on Standard Deviation and Slope of Response:**

  Quantitation Limit = 10 σ / S

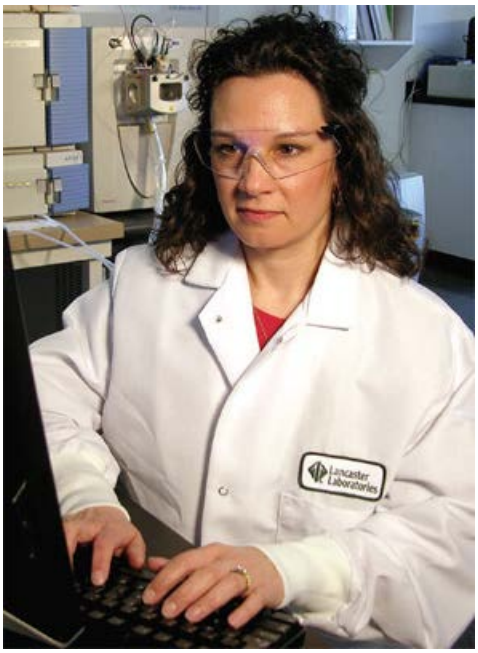

IMAGE COURTESY OF LANCASTER LABORATORIES

Jennifer Roark, Lancaster Laboratories' method development group leader, verifies the linear range of a method developed to test for residual levels of antibiotics using a Thermo Fisher Scientific LTQ-Orbitrap XL mass spectrometer.

The standard deviation of the response (σ) is based on either the magnitude of the analytical background response (measurement of blank samples) or residual standard deviation of a regression line or standard deviation of Y intercepts of regression lines for calibration curves generated using samples containing the analyte in the range of the quantitation limit. The slope (S) is based on the calibration curve of the analyte.

Once the quantitation limit has been identified, further studies are required to verify that it is the lowest concentration that displays acceptable levels of precision, accuracy, and linearity.

An acceptable approach to evaluate the accuracy and precision at the quantitation limit for an HPLC-related substances method or a GC residual solvent method is to prepare six placebo samples spiked with the analyte of interest at the quantitation limit concentration. The analyte should have a signal-to-noise ratio of approximately 10:1 with adequate recovery compared to theoretical content and precision in response (relative standard deviation [RSD] ≤ 20%).5 The determined quantitation limit must not be greater than the reporting level of the method.

Linearity

Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the analyte in the sample. The linear range of the method must be determined regardless of phase of drug development.4 ICH guidelines recommend evaluating a minimum of five concentrations to assess linearity.2 The five concentration levels should bracket the upper and lower concentration levels evaluated during the accuracy study. ICH guidelines recommend the following concentration ranges be evaluated during method validation:2

- **Assay (finished product or drug substance):** 80% to 120% of the sample concentration. This range must bracket that of the accuracy study, however. If accuracy samples are to be prepared at 80%, 100%, and 120% of nominal, then the linearity range should be expanded to a minimum of 75% to 125%.
- **Content Uniformity Method:** 70% to 130% of the sample concentration, unless a wider, more appropriate, range is justified based on the nature of the dosage form (e.g., metered dose inhalers).
- **Dissolution Method:** This requires +/- 20% of the specified range. In cases where dissolution profiles are required, the range for the linearity evaluation should start below the typical amount recovered at the initial pull point to 120% of total drug content.
- **Impurity Method:** Reporting level to 120% of the specification.
- **Impurity and Assay Method Combined:** One hundred percent level standard is used for quantification; reporting level of impurity to 120% of assay specification.

The linearity solutions are prepared by performing serial dilutions of a single stock solution; alternatively, each linearity solution may be separately weighed. The resulting active response for each linearity solution is plotted against the corresponding theoretical concentration. The linearity plot should be visually evaluated for any indications of a non-linear relationship between concentration and response. A statistical analysis of the regression line should also be performed, evaluating the resulting correlation coefficient, Y intercept, slope of the regression line, and residual sum of squares. A plot of the residual values versus theoretical concentrations may also be beneficial for evaluating the relationship between concentration and response.

In cases where individual impurities are available, it is good practice to establish both relative response factors and relative retention times for each impurity compared to the active compound. Response factors allow the end

user to utilize standard material of the active for quantitation of individual impurities, correcting for response differences. This approach saves the end user the cost of maintaining supplies of all impurities and simplifies data processing. To determine the relative response factors, linearity curves for each impurity and the active compound should be performed from the established limit of quantitation to approximately 200% of the impurity specification. The relative response factor can be determined utilizing the following equation based upon the linearity curve generated for each impurity and the active:

Relative Response Factor = Slope of Impurity Linear Curve / Slope of Active Linear Curve

The determined relative response factors can then be utilized for sample analysis to accurately correct for differences between impurity and active response to generate a reliable concentration for each impurity.

Precision and Repeatability

Repeatability reflects the closeness of agreement of a series of measurements under the same operating conditions over a short interval of time. For a chromatographic method, repeatability can be evaluated by performing a minimum of six replicate injections of a single sample solution prepared at the 100% test concentration.

Alternatively, repeatability can be determined by evaluating the precision from a minimum of nine determinations that encompass the specified range of the method. The nine determinations may be comprised of triplicate determinations at each of three different concentration levels, one of which would represent the 100% test concentration.

Intermediate precision reflects within-laboratory variations, such as different days, different analysts, and different equipment. Intermediate precision testing can consist of two different analysts, each preparing a total of six sample preparations, as per the analytical method. The analysts execute their testing on different days, using separate instruments and analytical columns.

Establishment of an appropriate qualification/validation protocol requires the assessment of many factors, including phase of product development, purpose of the method, type of analytical method, and availability of supplies, among others.

The use of experimental design for this study could be advantageous, because statistical evaluation of the resulting data could identify testing parameters (i.e., brand of HPLC system) that would need to be tightly controlled or specifically addressed in the analytical method. Results from each analyst should be evaluated to ensure a level of agreement between the two sets of data. Acceptance criteria for intermediate precision are dependent on the type of testing being performed. Typically for assay methods, the RSD between the two sets of data must be ≤2.0%, while the acceptance criteria for impurities is dependent on the level of impurity and the sensitivity of the method. Intermediate precision may be delayed until full ICH validation, which is typically performed during late Phase 2 or Phase 3 of drug development.4 However, precision testing should be conducted by one analyst for early phase method qualification.

Reproducibility reflects the precision between analytical testing sites. Each testing site can prepare a total of six sample preparations, as per the analytical method. Results are evaluated to ensure statistical equivalence among various testing sites. Acceptance criteria similar to those applied to intermediate precision also apply to reproducibility.

Accuracy

Accuracy should be performed at a minimum of three concentration levels. For drug substance, accuracy can be inferred from generating acceptable results for precision, linearity, and specificity. For assay methods, the spiked placebo samples should be prepared in triplicate at 80%, 100%, and 120%. If placebo is not available and cannot be formulated in the laboratory, the weight of drug product may be varied in the sample preparation step of the analytical method to prepare samples at the three levels listed above. In this case, the accuracy study can be combined with method precision, where six sample preparations are prepared at the 100% level, while both the 80% and 120% levels are prepared in triplicate.

For impurity/related substances methods, it is ideal if standard material is available for the individual impurities. These impurities are spiked directly into sample matrix at known concentrations, bracketing the specification level for each impurity. This approach can also be applied to accuracy studies for residual solvent methods where the specific residual solvents of interest are spiked into the product matrix.

If individual impurities are not available, placebo can be spiked with drug substance or reference standard of the active at impurity levels, and accuracy for the impurities can be inferred by obtaining acceptable accuracy results from the active spiked placebo samples. Accuracy should be performed as part of late Phase 2 and Phase 3 method validations. For early phase method qualifications, accuracy can be inferred from obtaining acceptable data for precision, linearity, and specificity.4

Stability of the compound(s) of interest should be evaluated in sample and standard solutions at typical storage conditions, which may include room temperature and refrigerated conditions. The content of the stored solutions is evaluated at appropriate intervals against freshly prepared standard solutions. For assay methods, the change in active content must be controlled tightly to establish sample stability. If impurities are to be monitored in the method sample, solutions can be analyzed on multiple days and the change in impurity profiles can be monitored.

Generally, absolute changes in the impurity profiles can be used to establish stability. If an impurity is not present in the initial sample (day 0) but appears at a level above the impurity specification during the course of the stability evaluation, then this indicates that the sample is not stable for that period of storage. In addition, impurities that are initially present and then disappear, or impurities that are initially present and grow greater than 0.1% absolute, are also indications of solution instability.

During Phase 3 validation, solution stability, along with sample preparation and chromatographic robustness, should also be evaluated. For both sample preparation and chromatographic robustness evaluations, the use of experimental design could prove advantageous in identifying any sample preparation parameters or chromatographic parameters that may need to be tightly controlled in the method.

For chromatographic robustness, all compounds of interest, including placebo-related and sample blank components, should be present when evaluating the effect of modifying chromatographic parameters. For an HPLC impurity method, this may include a sample preparation spiked with available known impurities at their specification level, or, alternatively, a forced degraded sample solution can be utilized. The analytical method should be updated to include defined stability of solutions at evaluated storage conditions and any information regarding sample preparation and chromatographic parameters, which need to be tightly controlled. Sample preparation and chromatographic robustness may also be evaluated during method development. In this case,

the evaluations do not require repeating during the actual method validation.

Establishment of an appropriate qualification/validation protocol requires assessment of many factors, including phase of product development, purpose of the method, type of analytical method, and availability of supplies, among others. There are many approaches that can be taken to perform the testing required for various validation elements, and the experimental approach selected is dependent on the factors listed above. As with any analytical method, the defined system suitability criteria of the method should be monitored throughout both method qualification and method validation, ensuring that the criteria set for the suitability is appropriate and that the method is behaving as anticipated.

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