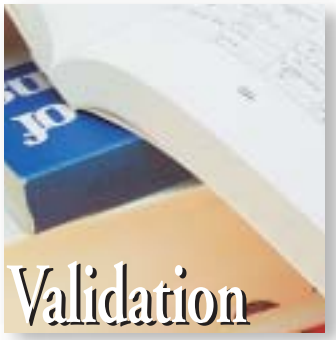

Viewpoint

Validation of Bioanalytical Methods — Highlights of FDA's Guidance

Bioanalytical methods are used in human clinical pharmacology, bioavailability, and bioequivalence studies that require pharmacokinetic evaluation in support of various drug applications to regulatory agencies such as the U.S. Food and Drug Administration. In a regulated laboratory, bioanalytical methods, as any other analytical method, must be validated to demonstrate that they are reliable and reproducible for their intended use. This "Validation Viewpoint" column explores and highlights recently published guidelines for bioanalytical method validation.

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Validation Viewpoint Editors

Chemists in regulated pharmaceutical laboratories have the responsibility of developing and validating various methods. Analytical methods for finished products, raw materials, or active pharmaceutical ingredients have their own development and validation challenges. However, bioanalytical methods in support of pharmacokinetic studies arguably are among the more challenging types of methods to develop and validate. The complexity of the sample matrices, the trace levels of drug and metabolites usually encountered, and even the complexity of the instrumentation used for the analyses combine to challenge analysts charged with validating a bioanalytical method in a unique way.

Bioanalytical methods are defined as methods that are used to quantify drugs and their metabolites in a biological matrix such as blood, plasma, serum, or urine. The sensitivity and selectivity of these methods are essential to the success of preclinical and clinical pharmacology studies. As with any other analytical method, the performance characteristics of a bioanalytical method must be shown, by documented laboratory data, to be reliable and reproducible for the method's intended use. At least two conferences have been held to discuss this topic during the past several years (1,2). As a result, the U.S. Food and Drug Administration (FDA) issued a guidance document in May 2001 about validating bioanalytical methods (3). Although this "Validation Viewpoint" column will discuss the FDA guidance in some detail, we encourage readers to consult the references for additional details. The guidance addresses immunological and microbiological procedures; however, our discussion will be limited to instrumental procedures such as gas chromatography (GC), high performance liquid chromatography (HPLC), and hyphenated GC and LC mass spectrometry (MS) procedures.

For the most part, regulated bioanalysis is the realm of the triple quadrupole MS. By using selective multiple-reaction monitoring to focus on the analyte of interest and MS–MS to filter the background, users can realize a level of sensitivity and specificity unavailable from any other analytical technique. Usually, rapid, high-throughput techniques are required in a clinical study because of the large number of samples involved. In addition to the quadrupole mass spectrometer, a typical LC–MS system includes a binary gradient HPLC system that can provide fast gradient cycle times using short columns (30 mm × 2.1 mm) and autosamplers that can inject from microtiter plates with low carryover and fast injection cycle times.

The Bioanalytical Method Validation Process

In a regulated laboratory, the process of validating any type of analytical method doesn't start with the method itself. The instrument and any software used first must be qualified or validated according to existing standard operating procedures, and any data generated must be maintained compliant with FDA's electronic records and signature rules. After the stage is set, the process of validating a bioanalytical method can be divided into three parts: reference standard preparation, bioanalytical method development and establishment of the validated assay procedure, and application of the validated method to routine drug analyses.

Reference standard preparation: When analyzing biological samples, it is common practice to use quality control samples or samples spiked with calibration or reference standards. Reference standards can be one of three types: certified reference standards (for example, *United States Pharmacopeia [USP]* compendial standards), commercially obtained reference standards, or cus-

tom standards of documented purity that have been synthesized by an analytical laboratory or other noncommercial source. Whenever possible, standards should be identical to the analyte — or at least an established chemical form such as a free acid or base, salt, or ester — and of known purity. Supporting documentation such as the lot number, expiration date, certificates of analysis, and evidence of identity and purity should be on hand. Indeed, reference standards often are subjected to more scrutiny than the analyte of interest or the drug substance or product itself.

Bioanalytical Method Development and Establishment of the Validated Assay Procedure

The basic bioanalytical performance parameters that must be validated for each analyte of interest in the matrix include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. In practice, to develop the method and to establish the validated assay procedure, analysts investigate four areas: selectivity; accuracy, precision, and recovery; calibration curve; and stability.

Selectivity: Analytical chemists always seem to debate whether selectivity or specificity is the proper term. The two terms really do have separate and distinct definitions, but the purpose of this column is not to extend the debate. Whatever the term du jour, the objective is to accurately measure the analyte of interest free of interferences from other components in the sample. Interferences can take the form of endogenous matrix components, metabolites, degradation products, concomitant medications, or other analytes of interest. FDA guidelines recommend analyzing blank samples of the appropriate biological matrix from at least six different sources. However, when using hyphenated MS methods six independent matrices usually are not necessary, but matrix effects still should be investigated. Each blank sample should be tested for potential interferences to concentrations at the lower limit of quantification.

Accuracy, precision, and recovery: The *accuracy* of a bioanalytical method is defined as the closeness of test results to the true value as determined by replicate analyses of samples that contain known amounts of the analyte of interest, and it is reported as the deviation of the mean from the true value. FDA guidelines recommend using a minimum of five determinations per concentration and a minimum of three concentrations throughout the expected range. The guidelines also recommend that the mean value be within 15% of the actual value, except at the lower limit of quantification, where 20% is acceptable.

The *precision* of a bioanalytical method is defined as the measure of the amount of agreement among test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. As in recent International Conference on Harmonization of Technical Requirements for the Registration of Drugs For Human Use (ICH) guidelines, precision can be divided into *repeatability determinations* (within-run or intrabatch) and *intermediate precision* (between run or interbatch) (4,5). Again, FDA guidelines recommend using a minimum of five determinations per concentration and a minimum of three concentrations throughout the expected range. The precision measured at each concentration level should not exceed 15% of the coefficient of variation except for the lower limit of quantification, which should not exceed 20% of the coefficient of variation.

The assay *recovery* relates to the extraction efficiency, and it is determined by comparing the response from a sample extracted from the matrix with the reference standard. Recovery of the analyte does not need to be 100%, but it must be quantitative; that is, it should be consistent, precise, and reproducible. Recovery experiments should be performed at three concentrations (low, medium, and high) by comparing the results for extracted samples with unextracted samples representing 100% recovery.

Calibration or standard curve: A calibration or standard curve illustrates the relationship between the instrument response and the known concentration of the analyte, and it should be proportional to the response within a given range chosen upon the basis of expected values. The simplest model that describes the proportionality should be used. At least four of six non-zero standards should fall within a 15% deviation from the nominal concentration (20% at lower limit of quantification). The calibration curve should be generated for every analyte in the sample and prepared in the same matrix as the samples by spiking the matrix with known concentrations of the analyte. FDA guidelines suggest that a calibration curve be constructed from a blank sample (a matrix sample processed without an internal standard), a zero sample (a matrix processed with an internal standard), and six to eight non-zero samples covering the expected range, including the lower limit of quantification. Two conditions must be met to determine the lower limit of quantification: the analyte response at the lower limit of quantification should be five times the blank response; and the analyte peak should be identifiable, discrete, and reproducible with a precision of 20% and an accuracy of 80–120%.

Figure 1 shows an example calibration curve for an LC–MS–MS experiment.

Stability: Many different factors can affect bioanalytical sample stability, including the chemical properties of the drug, the storage conditions, and the matrix. Studies must be designed to evaluate the stability of the analyte during sample collection and handling, under long-term (at the intended storage temperature) and short-term (benchtop, controlled room temperature) storage conditions, and through any freeze-and-thaw cycles. The conditions used in the studies should reflect the actual conditions the sample could experience during routine analysis in working and stock solutions. Stock solutions should be prepared in appropriate solvents at known concentrations. Analysts should ascertain the stability for stock solutions at room temperature for at least 6 h, and they should evaluate the storage condition stability as well. Because the processed samples — drugs and internal standard — also will be on a benchtop or in an autosampler for some period of time, it is important to establish their stability for the anticipated run time for a sample batch. Working standards should be prepared from freshly made stock solutions of the analyte in the appropriate matrix. Analysts

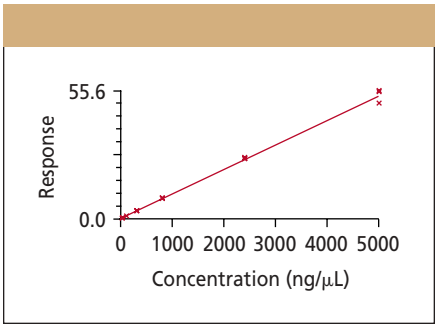

Figure 1: Example calibration plot obtained for the LC–MS–MS analysis of standard at 10, 30, 100, 300, 800, 2400, and 5000 ng/μL. Column: 100 mm × 2 mm C18; mobile phase: 55% acetonitrile, 45% water, 0.1% formic acid; flow rate: 0.3 mL/min; injection volume: 20 μL. MS detection: Quatro Ultima (Waters Corp.) MS–MS with a positive-ion electrospray Z source; cone voltage: 100 V; collision energy: 18 eV; collision gas pressure: 2.5 mbar argon.

should follow appropriate standard operating procedures for the experimental studies, as well as for the poststudy statistical treatment of the data.

FDA guidelines recommend a minimum protocol that includes the following:

- For *freeze-and-thaw stability,* workers should expose three aliquots at each of the low and high concentration samples to freeze-and-thaw cycles. The samples should be kept at the storage temperature for 24 h and then thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12–24 h, and then the process should be repeated twice more. Analysis then can proceed after the third freeze-and-thaw cycle.
- For *short-term temperature stability,* analysts should thaw three aliquots (at each of the low and high concentrations) and keep them at room temperature for a period of time that is representative of how long the samples will be maintained at room temperature in the actual study for analysis.
- The storage time in a *long-term stability* evaluation should bracket the time between the first sample collection and the last sample analysis, and the sample volume reserved should be sufficient for at least three separate analyses. Workers should test at least three aliquots (at each of the low and high concentrations) stored under the same conditions as the study samples. In a long-term stability study, the results for all samples should be compared with the mean of the results for the standards backcalculated to the first day of the study.

Routine Application of the Bioanalytical Method

After the bioanalytical method has been validated for routine use, system suitability and quality control samples are used to monitor the system and ongoing accuracy and precision and to accept or reject runs. Quality control samples are prepared separately and analyzed with true unknowns at intervals according to the number of unknown samples to be analyzed. The minimum number of quality control samples (in multiples of three) is recommended to be at least 5% of the number of unknown samples or six, whichever is greater. Duplicate quality control samples (prepared from the matrix spiked with the analyte) at three concentrations (low [near the lower limit of quantification], mid-range, and high) normally are used. At least four of every six quality control sample results should be within 15% of their respective nominal value.

Table I lists data representative of typical results obtained for the analysis of quality control samples (at 10, 35, 1000, 4400, and 5000 ng/μL). As mentioned above, the precision at each concentration level measured by the coefficient of variation, and the accuracy measured by bias, must be 15% or less for acceptable method validation. The coefficients of variation (≤4.1%) and biases (≤ 10.8%) at all concentration levels were within the validation guidelines.

System-suitability samples, acceptance criteria, and guidelines for repeat analysis or data reintegration also should be performed according to an established standard operating procedure. The rationale for repeat analyses, data reintegration, and the reporting of results should be clearly documented. System suitability should be performed both before and after the analysis of unknowns to ensure the system — instrument and method — is performing properly. Problems from inconsistent replicate analysis, sample processing errors, equipment failure, or poor chromatography can cause the reanalysis of samples.

Documentation

Good record keeping and documented standard operating procedures are essential parts of any validated method. After the validity of a bioanalytical method is established and verified by laboratory studies, analysts must provide pertinent information in an assay validation report. Although data generated during method development and quality control should be available for audit and inspection, documentation for submission to FDA should include summary information, method development and establishment, reports of the application of the method to routine sample analysis, and other miscellaneous

Table I: Example quality control results*

	Quality Control Sample 1 (10 ng/μL)	Quality Control Sample 2 (35 ng/μL)	Quality Control Sample 3 (1000 ng/μL)	Quality Control Sample 4 (4400 ng/μL)	Quality Control Sample 5 (5000 ng/μL)
Run 1	11.8	35.7	1009.8	4670.3	5425.0
Run 2	11.1	37.1	1036.0	4796.4	5334.5
Run 3	11.4	35.4	1047.2	4684.9	5180.9
Run 4	10.4	36.0	975.8	4964.3	5241.6
Run 5	10.8	34.6	1047.8	4628.6	5285.6
Run 6	10.9	34.9	986.5	4564.3	5049.0
Run 7	10.9	33.6	971.8	4491.9	5009.2
Run 8	10.8	32.6	960.4	4404.1	4883.7
Run 9	11.3	33.2	956.7	4539.5	5170.8
Run 10	11.4	34.4	977.8	4558.6	4802.7
n	**10**	**10**	**10**	**10**	**10**
Target	**10.0**	**35.0**	**1000.0**	**4400.0**	**5000.0**
Mean	**11.1**	**34.8**	**997.0**	**4630.3**	**5138.3**
Standard deviation	**0.401**	**1.41**	**35.45**	**160.48**	**199.45**
Coefficient of variation (%)	**3.6**	**4.1**	**3.6**	**3.5**	**3.9**
Bias (%)	**+10.8**	**−0.7**	**−0.3**	**+5.2**	**+2.8**

*Data representative of typical results obtained for the analysis of quality control samples at 10, 35, 1000, 4400, and 5000 ng/μL. For experimental conditions, see Figure 1. The coefficient of variations (≤4.1%) and biases (≤ 10.8%) at all concentration levels were within the validation guidelines.

information such as standard operating procedures, abbreviations, and references.

The *summary information* should include a tabular listing of reports, protocols, and codes used. The documentation for *method development and establishment* should include a detailed operational description of the experimental procedures and studies, purity and identity evidence, method validation specifics (results of studies to determine accuracy, precision, and recovery), and any protocol deviations with justifications. Documentation of *the application of the method to routine sample analysis* is quite extensive. It should include

- summary tables describing sample processing and storage;
- detailed summary tables of analytical runs of preclinical or clinical samples;
- calibration curve data;
- quality control sample summary data, including raw data, trend analysis, and summary statistics;
- complete serial chromatograms — unknowns, standards, and quality control samples — for as many as 20% of the subjects;
- reasons and justifications for any missing samples or any deviations from written protocols or standard operating procedures; and
- documentation for any repeat analyses or reintegrated data.

Summary

Validation is a constantly evolving process, and a bioanalytical method can undergo many modifications during the course of a typical drug development program. After a full validation study, laboratories often implement partial validation to modify existing validated methods and cross validation to transfer methods or to compare two or more methods. But the bottom line is that using laboratory data to document that a method is suitable and reliable for its intended application never changes. It is the basic tenet of method validation, no matter what type of analytical method is under scrutiny.

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The columnists regret that time constraints prevent them from responding to individual reader queries. However, readers are welcome to submit specific questions and problems, which the columnists may address in future columns. Direct correspondence about this column to "Validation Viewpoint," LCGC*, 859 Willamette Street, Eugene, OR 97401, e-mail lcgcedit@lcgcmag.com.*