Chapter 5

Analytical Method Validation for Biopharmaceuticals

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52561

1. Introduction

Method validation has a long and productive history in the pharmaceutical and now, biopharmaceutical industries, but it is an evolving discipline which changes with the times. Though much has been written about method validation for conventional, small molecule (SM) pharmaceuticals, less has appeared providing an overview of its application for complex, high molecular weight (MW) biopharmaceuticals (or biotechnology) products. This appears to be satisfyingly changing with the times, and this particular chapter has been designed to address this area of method validation. We hope to address herein the important issues of where do analytical method validation guidelines and directives stand today for biopharmaceutical (protein or related) products. Due to the recognized differences and complexity of biopharmaceuticals relative to small molecule drugs, regulatory agencies have accepted that what is expected of all SM, single molecule entities (even enantiomers), cannot be required for complex protein biopharmaceuticals, such as antibodies. While it is quite a simple matter, in most instances, to characterize and validate methods for SM drug substances, this is not always the case for complex biopharmaceuticals. Biotechnology products will always be heterogeneous mixtures of product-related species.

While the chapter below focuses on the principles and practice of method validation for biopharmaceuticals in the biotechnology industry, some comments on the topic of "academic method validation," and if and how that differs from what is required by the industry, seem warranted. In general, academics are not required by any regulatory agency or governmental body to perform any degree of method validation. However, one instance where it might be appropriate to do acceptable (whatever that means) method validation is when a reviewer of a grant proposal or manuscript destined for publication demands that some validation be performed. At times, Journal/Book Editors may suggest that some degree of method validation be performed, but in the final analysis, this requirement is at

the discretion of reviewers. It appears that for the most part, little to no method validation is performed in academic circumstances, but on occasion, attempts are made to validate methods in academic laboratories. However, even there, such efforts do not begin to approach what is expected by regulatory agencies for industrial methods used to release a product for clinical trials use.

At times, in the past, Editors have taken the time to list what is expected in future submissions related to some degree of method validation. However, it was never obvious or clear that a lack of such studies really has ended up in manuscript rejections. Again, to a very large degree, this has depended on the rigorousness of the reviewers, resulting in somewhat a "luck of the draw" approach. Some may view this as frustrating and unfortunate, because in the absence of method validation, there should be no reason to accept the method and its applications, prima facie, or its results/data. However, academics somehow don't believe that method validation is required in order to do "good science" or publish. This situation has been changing for the better, but it is not quite where it really should be today. It will change when all editors, reviewers and manuscript/proposal submitters agree on the importance of doing good science by doing thorough and complete analytical method validation studies. Clearly, practitioners in the pharmaceutical and biopharmaceutical industries have much to offer to academic scientists in this regard.

One publication, years ago, appeared to demonstrate in certain, newer capillary electrochromatography (CEC) studies, unusually high plate counts and efficiencies. However, when others attempted to reproduce such results and data, nobody could come even close to what was in that original publication. Eventually, it was admitted that in the original study, none of those astounding results were reproducible or even replicable in a single lab. The work was never repeatable in their own hands, something that they conveniently forgot to mention anywhere in their papers. How could that happen? Well, it happened because neither the editors nor reviewers were thorough and rigorous in their demand for analytical method validation. They did not ask to see some evidence of repeatability, intermediate precision and other performance characteristics, a situation that would not be permissible in the industrial world due to regulatory requirements for method validation

Method validation in the pharmaceutical and biopharmaceutical industries is designed to help ensure patient safety during clinical trials and later when the drug becomes commercialized. While this reasoning is not applicable to basic research, and basic research in the academic community has at least one self correcting mechanism, peer verification, the lack of a requirement to document the performance characteristics of the methods in the academic world can, at times, lead to the publication of analytical methodologies, as noted above, that may lack scientific integrity.

2. Method validation for the biotechnology industry

The development of biotherapeutics is a complex, resource-intensive and time-consuming process, with approximately 10 years of effort from target validation to commercialization.

This reality, coupled with rapid technological advances and evolving regulatory expectations, impacts the ability of biotechnology companies to rapidly progress with development of their pipeline candidates.

Method validation is a critical activity in biopharmaceutical product development which often causes confusion and, at times, consternation on the part of analytical development teams. Questions surrounding method validation abound: (1) when should we validate our analytical methods? (2) what are the requirements for achieving method validation in a manner that is compliant across multiple regulatory jurisdictions around the world? (3) how can I implement a validation strategy that fits my company's business infrastructure and provides for seamless method transfer activities to other QC organizations in the company as well as contract QC organizations, when required?

Prior to proceeding to a discussion of method validation, it is important to differentiate amongst the categories of analytical methods used in the biopharmaceutical industry for product evaluation. In general, the analytical methods used can be divided into three categories: (1) screening methods; (2) release and stability methods; and (3) characterization methods. Screening methods are used to guide discovery research and process development. These methods, which are often carried out in high-throughput format using automation due to the large volumes of samples tested, do not typically follow any validation guidance, since they are not intended for a QC environment. Nonetheless, it is important to understand the capabilities and limitations of these methods so that the results can be appropriately applied to making decisions during process and product development. This is generally achieved through experience with the method in the analytical development organization. The second class of methods, release and stability methods, are intended for use in a Quality Control environment for product disposition and formal stability studies. In addition, these methods are sometimes used in QC for in-process samples in the form of in-process controls, which are used in the overall control strategy to ensure product quality (and for which the validation strategy should mirror that used for the release and stability methods). Whether used for release, stability, or in-process control applications, these methods are generally validated prior to the validation (conformance) lots to demonstrate that they have acceptable performance according to regulatory guidance (discussed below). The third class of methods, characterization methods, are used to support product characterization studies during reference standard characterization, process characterization, comparability studies, and other product characterization activities, and data from these studies is often submitted to regulatory agencies. Industry practice has recently evolved to meet regulatory expectations that these methods will be qualified according to written company procedures, though no formal written guidance is available, and method validation is not expected for these analytical procedures.

In order to meet current compliance expectations, an analytical method used to support GMP activities must be suitable for its intended use, and appropriate experimental work must be documented that provides this assurance. The demonstration of method suitability can be divided into two sets of activities: qualification and validation. When methods are

new, under development, or subject to process or method changes, this activity is often called qualification, while more formal confirmation of method suitability for commercial applications is called validation (Ritter, Advant et al. 2004; Apostol and Kelner 2008; Apostol and Kelner 2008).

The strategy for method validation involves a continuum of activities that begins at the start of process and product development and carries through to the marketing application and beyond. Typically, analytical method development begins after the biological target has been identified and verified, the protein therapeutic has been defined (primary sequence), and the sponsor has made the decision to develop a manufacturing process that will enable human clinical trials. The initial demonstration that the method is suitable for its intended purpose for use as a release and stability method is generally carried out in the form of method qualification, an activity that generally takes place prior to the release of the material for first-in-human (Phase 1) clinical trials. At the later stage of product development, typically prior to the start of pivotal phase III clinical trials, method developers perform qualification studies which will enable method validation. Finally, method validation generally takes place prior to the release and stability testing of the validation manufacturing lots.

It should be noted that although method qualification, which evaluates the performance characteristics of the method against meaningful target expectations, is a critical development activity that establishes the suitability of the method for release of early to mid-phase clinical materials, this activity is not, to the best of our knowledge, clearly defined in regulatory guidance, which tends to focus on method validation. It is therefore difficult to define the scope of method qualification, though regulatory expectations and industry practices have evolved to define method qualification as a means to assure acceptable method performance during process and product development, prior to the formal validation exercise that occurs before the testing of the validation lots.

The necessity of method validation has been reinforced by a variety of national and international regulations (USP 1994; USP 1999; CDER 2001; ICH 2005) which are subject to user interpretation. For example, current GMP regulations, [21 CFR 211.194 (a)] require that methods used in testing of the samples meet proper standards of accuracy and repeatability. Validation provides assurance that this regulation is met. USP <1225> defines validation of analytical procedures as the process by which it is established by laboratory studies that the performance characteristics of the procedure meet the requirements of the intended analytical application. ICH guideline Q2R1 defines validation of analytical procedures as the demonstration that the method is suitable for its intended purpose. ICH guidance specifies that validation of analytical procedures needs to be included as part of the registration package submitted within the EU, Japan and USA. While the biotechnology industry, in a manner analogous to the pharmaceutical industry, is heavily regulated, the majority of the regulations are targeted at commercial products, leaving a significant gap in available regulatory guidance for earlier stages of product development. While numerous articles have been published to provide the scientific principles and exemplify the types of

associated activities relevant for method validation (Swartz and Krull 1997; Shabir 2003; Ritter, Advant et al. 2004; ICH 2006; Krull and Swartz 2006; Swartz and Krull 2006; Swartz and Krull 2009), the most frequently referenced document, is the ICH guideline Q2R1, "Validation of analytical methods: text and methodology"(ICH 2005), This document covers validation activities targeted at product registration; hence, this guidance is specifically applicable to commercial products. Method qualification has emerged as the typical means of filling the gap for assessing the suitability of analytical method performance at earlier stages of product development.

3. Qualification of characterization methods

Characterization methods typically involve highly specialized technologies which are labor intensive and difficult to perform on a routine basis, which includes, for example, AUC, CD, FTIR, DSC, SEC-LS , and NMR. These methods are often used to supplement lot release methods to provide orthogonal detection/separation modes and/or to verify structural integrity (e.g. primary, secondary, tertiary structure). This is in contrast to Quality Control methods, which typically employ proven technologies to enable in-process controls, lot disposition and GMP stability assessment in the GMP laboratory setting, requiring stringent assessment of performance characteristics that follow ICH guidelines. Therefore, it is important to define an appropriate level of qualification for these complex and non-routine characterization methods. Industry practice has evolved multiple means of defining a qualification path for characterization methods, including:

- Ensuring the adherence to written technical procedures
- Ensuring that the equipment has a documented record of initial equipment qualification (IQ/OQ), preventative maintenance (PM), and/or calibration.
- Ensuring that data are generated by scientists with appropriate technical skills documented through training records and/or academic credentials.
- Ensuring that all experiments are accompanied by proper controls to ensure that the method is capable of measuring the intended attributes of the product. Control experiments should be designed in such a way that the quantitative aspect of the measurement can be clearly demonstrated from the results of the experiments. Properly controlled experiments should be performed to address the precision (repeatability) of the measurements.

Recently Jiang et al. provided an excellent review of the qualification of the biophysical methods including AUC, CD, FTIR, DSC, SEC-LS, MFI and LO based methods. The authors describe how qualification of these methods enables better knowledge of the methods and objective interpretation of the results. The general considerations described there can be applied to other biophysical methods as appropriate as well (Jiang, Li et al. 2012). In most cases qualification of biophysical methods is focused on the determination of precision and demonstration that the methods are suitable for their intended applications. Successful qualification enables the understanding of the method capability and the consistent determination of product attributes.

4. Qualification and validation of release and stability methods

Qualification should be performed prior to method implementation in the Quality laboratories to ensure the integrity of the data provided on the Certificate of Analysis for clinical lots. In most cases, at early stages of clinical development, only one sample type requires qualification because, in general, the drug substance (DS) and drug product (DP), for which specifications are established requiring testing in the Quality labs, often have the same composition (formulation). If this is not the case, a technical assessment should be made of whether differences in the matrix have the potential to impact the qualification results and, if so, a strategy for verifying the qualification status of the two sample types, relative to each other, should be devised. For example, full qualification of the DS can be followed by a matrix verification for the DP, generally in the form of a repeat of the specificity and precision evaluation.

Method validation is typically completed before process validation in adherence with cGMP procedures outlined in ICH Q2R1(ICH 2005) . Method validation for release and stability methods can be considered as the pivotal point in the method lifecycle because it justifies the use of the method in commercial settings to guide decisions about product disposition and lot stability. In addition, the validation activity provides a defined point of transfer of ownership of the methods from the development organization to the commercial (operations) organization. Typically, these activities are initiated after the sponsor has made a commitment to commercialize the drug candidate (which generally occurs after positive feedback from clinical trials).

ICH Q2R1 specifies that method validation has three components: assessment of performance characteristics, demonstration of robustness and system suitability. It should be noted that industry practice dictates that method qualification also evaluates these three components, with a noticeable difference, in that while validation has a formal protocol and pre-defined acceptance criteria for the performance characteristics, method qualification does not. It is a good practice to adopt general target expectations for method qualification as a means of evaluating the outcome of exploratory work on performance characteristics. The expectation should reflect the desired characteristics for the methods with respect to precision, range, QL, etc. If the method does not meet these expectations, the method should be re-developed and/or optimized. Recently, many companies have adopted the practice of developing and qualifying multiproduct methods that can be used for more than one product within specific molecular classes, such as monoclonal antibodies. In such instances, verification of performance could be adequate instead of full qualification studies once the method has undergone full qualification for the first molecule of the specified class.

Standard industry practice dictates that methods used to assess drug substance and drug product stability should show that they are able to detect changes in quality attributes. This can be demonstrated in forced degradation studies on the appropriate sample types using conditions known to impact protein quality, such as elevated temperature, pH extremes, and incubation with oxidizing agents such as hydrogen peroxide to induce molecular changes such as aggregation, deamidation, peptide bond cleavage and protein oxidation.

In addition to the stability indicating properties of the method, the assessment of sample stability can be considered as a pre-requisite to method validation. Sample stability can be divided into two activities – an evaluation of sample storage conditions prior to analysis, and assessment of the stability of prepared samples while waiting for analysis. Samples are often stored frozen after collection and thawed prior to analysis; in these instances, sample integrity should be assessed over a minimum of one freeze-thaw cycle for each sample type (preferably more than one cycle in most cases). Sample stability after preparation and before analysis (e.g., time spent in an auto-sampler) should be evaluated to determine the maximum duration of an assay (sequence). Details of sample handling should be included in the validation protocol.

Method validation confirms the performance characteristics demonstrated during method qualification and demonstrates the suitability of the method for commercial use. This confirmation effort should follow a pre-approved protocol with clear and justifiable acceptance criteria. In the context of the analytical lifecycle, the key components of method validation are as follows:

1. The experimental design of method validation should mimic the qualification design, and acceptance criteria should be linked to the target expectations used in the qualification experiments. In the absence of such rigor, validation experiments become exploratory research and run the risk of undermining the results of the method qualification.
2. Similarly to the qualification (in most cases), the validation acceptance criteria are set based on the type of method and should not differ from target expectations. When qualification target expectations are not met during a qualification study, the rationale for re-evaluation of the acceptance criteria should be proposed in the qualification summary. Setting acceptance criteria for the precision of a method frequently causes confusion, anxiety, and inconsistency in practice. For validation studies, requirements for a reporting interval aligned with the specification for precision studies provide excellent guidance for setting the acceptance criteria for precision and other performance characteristics.
3. Validation acceptance criteria should only include the objective parameters from the qualification to avoid any subjective interpretations, which could impact the outcome of the confirmatory validation studies. For example, frequently during qualification studies, scientists expect that the residual from linear regression does not show any bias (trend). Since the community has not adopted a uniform measure of the bias (which is frequently based on visual evaluation), it is not advisable to include such a requirement in the validation acceptance criteria.

Validation studies should be executed for sample types that will be routinely tested in GMP environments to make decisions about product disposition. This typically includes the following sample types:

- Sample types listed on all release and stability specifications (intermediates, drug substance and drug product);
- Samples associated with process controls and in-process decision points.

5. Performance characteristics

In order to produce a reliable assessment of method performance, all necessary performance characteristics should be evaluated in carefully designed experiments. ICH guideline Q2R1 specifies which performance characteristics should be evaluated for validation. However, interpretation of the table for protein products is not straightforward. This is due in part to the fact that ICH Q2R1, Q6B and the industry used different nomenclature to describe the type of methods. The table below details the performance characteristics that should be assessed during qualification, and subsequently during the confirmatory validation experiments for protein products.

ICH Q2R1 method types	ICH Q6B method types	Industry method types	Performance characteristics					
			Specificity	Linearity	Range	Precision	Accuracy	LOD/LOQ
Testing for impurities	Quantity	Titer	√	√	√	√	√	*
	Purity and impurities	Purity	√	√	√	√	√	√
		Immuno	√	√	√	√	√	√
		DNA	√	√	√	√	√	√
		Peptide map	√			√		√
		Gels	√	√	√	√	√	√
		Process Reagents	√	√	√	√	√	
Assay	Potency	Potency	√	√	√	√	√	√
Identification	Identity	ID	√					

* In some cases may be required by USP <1225>.

Table 1. Performance Characteristics that need to be Evaluated During Qualification/Validation by Method Type

6. Precision

Precision has typically been considered as the most important performance characteristic of the method, because it gives customers/clients of the analytical data direct information on the significance or uncertainly of results. Typically, method precision is established from replicate analyses of the same sample. However, methods for predicting precision have recently been published that allow the assessment of precision based on a single chromatogram (Apostol, Kelner et al. 2012).

Method precision defines the capability of the method expressed in its reporting interval (Holme and Peck 1998). Agut et al. (Agut, Segalini et al. 2006) examined different rules and their application to the reporting interval of results and specifications. The best known and simplest rule to implement is that stated in the AMST standard E-29-02. The rule states that the results of analytical measurements should be rounded to not less than 1/20 of the determined standard deviation (ASTM 2005).

For example, bioassays with a standard deviation of 11.8 should adopt a reporting interval larger than 0.59. However, this 0.59 reporting interval is impractical in day-to-day applications

due to the inability of bioassays to provide precision that would justify such a reporting interval. Therefore, bioassays with a standard deviation of 11.8 would result in a reporting interval of 1. Similarly, an HPLC assay with a standard deviation of 1.3 for the main peak would result in a reporting interval of 0.1. Reporting intervals for impurities (minor peaks) need to be consistent with reporting intervals for the main peak. In general, STD of equal or less than 2 (in units reported by the method) is required to ensure a reporting interval of one decimal place. The argument can be raised that for low level, minor analytes (for example, the dimer in SEC present at 1%), the requirement for STD to be at or below 2% is too generous. This will result in an RSD of 200% for the peak. In such an instance, this would indicate that the minor peak is well below the detection level, because theoretically the RSD at the LOD level should not exceed 33% (Long and Winefordner 1983; Hayashi and Matsuda 1995)

The table below proposes the nearest reporting intervals based on standard deviations obtained during qualification for protein products.

Standard Deviation (in reported units)	Nearest Reporting Interval
≤2.0	0.1
≤20	1

Table 2. Recommended Nearest Reporting Results Based on Standard Deviation

Method precision is closely linked to the concentration of the analyte. The best-known relationship between analyte concentration and RSD is the Horwitz equation (Horwitz 1982; Horwitz and Albert 1997; Horwitz and Albert 1997)

$$RSD = 2^{(1-0.5 \log C)}$$

where, C is the concentration of the analyte in mg/g.

Based on the Horwitz equation, the precision of the measurement, expressed as RSD, doubles for each decrease of analyte concentration of two orders of magnitude.

The Horwitz relationship can provide good guidance for method precision targets during method development and qualification. Intermediate precision obtained during these studies should meet the variability derived from the Horwitz equation for each individual analyte. If, during execution of the qualification experiments, the precision of the measurements exceeds values derived from the Horwitz equation, this may indicate that the assay may need to be redeveloped, or that the technology utilized in the assay may not be fully suitable for the intended application.

Typically, proteins are available for analysis as solutions, with concentrations ranging widely from 1 µg/ml (e.g., a growth factor) to 100 mg/ml or higher (e.g., a monoclonal antibody). In such cases, expectations for the RSD of measurements of the main protein analyte in these solutions, based on the Horwitz relationship (e.g., using protein concentration method), will be 16 and 2.8 %, respectively.

As mentioned earlier, the precision of the method is referred to as uncertainty. The uncertainty of results is a parameter that describes a range within which the measured value is expected to lie (Miller and Miller 2000). Intuitively, we associate this parameter with precision. Therefore, method precision has been viewed as the most important performance characteristic. Typically, method precision has been assessed from replicate analyses of the same sample. The work of Hayashi and Matsuda on FUMAI theory (Hayashi, Rutan et al. 1993; Hayashi and Matsuda 1994; Hayashi and Matsuda 1994; Hayashi and Matsuda 1995; Hayashi, Matsuda et al. 2002; Hayashi, Matsuda et al. 2004) demonstrated that the precision of chromatographic methods can be predicted from noise and the height and width of the signal (peak). However, due to the complexity associated with the required Fourier transformation of chromatograms and the parameterization of the power spectrum called for in implementation of this theoretical construct to the determination of precision, the FUMAI theory approach has not been widely applied.

Apostol et al. (Apostol, Kelner et al. 2012) proposed a new approach to assessing the uncertainty of purity analyses that uses a more holistic approach that is called Uncertainty Based on Current Information (UBCI). The model allows for real-time assessment of all performance characteristics using the results of the specific separation of interest. A fundamental, underlying principle of this approach recognizes that the execution of a purity method is always associated with specific circumstances; therefore, uncertainty about the generated results needs to account for both the operational conditions of the method and the hardware. The authors demonstrated that noise levels, instrument and software settings can be linked directly to all method performance characteristics. Such simplification makes it easy to implement this procedure in a daily operation, and can provide a valuable live assessment of uncertainty instead of extrapolating uncertainty from historical qualification/validation studies.

The UBCI model approximates the maximal uncertainty of the measurement associated with the actual conditions of analysis (test). The obtained precision corresponds to the uncertainty under the most unfavorable conditions, including the highest variability of injection, maximal numeric integration error, expected variability of the peak width, and the most unfavorable contribution of the noise. UBCI shows that the uncertainty of results is not only a function of the method (composition of the mobile phase, gradient, flow rate, temperature), but also is influenced by the hardware associated with the execution of the method (pump pulsation, detector range, status of the lamp, etc.), and the software settings used to acquire the output in the form of chromatograms. Information about these parameters can be extracted from individual chromatograms; therefore, the assessment of method performance characteristics (uncertainty) can be performed real-time, which can be considered as a 'live validation' associated with each individual test result.

It is important to note that historical qualification/validation approaches do not take this fundamental principle into account, such that performance drift may occur over time due to hardware differences and even due to differences in analyst skill levels, such that the

uncertainty of results obtained early in the product lifecycle may not be fully applicable to results obtained later. Application of historical validation data always begs a question about the relevance of these data to the current experimental situation, and sometimes requires investigation, which can delay the approval of results. The UBCI approach, therefore, has the capability of providing not only simplicity, but also a greater level of assessment of the data validity relative to current practices.

7. Accuracy

The determination of accuracy for protein purity methods presents significant challenges. Since it is difficult to establish orthogonal methods for proteins to measure the same quality attribute, it is hard to assess the truthfulness of the accuracy measurements. For example, although SEC-HPLC results can be verified by analytical ultra centrifugation (AUC) techniques, these techniques are based on very different first principles, and may not provide comparable results (Carpenter, Randolph et al. 2010; Svitel, Gabrielson et al. 2011). Therefore, in most cases, the accuracy of purity methods for proteins is inferred when other performance characteristics meet expectations, which is consistent with the principles of ICH Q2R1(ICH 2005).

8. Linearity and range

Linearity and range are typically assessed in a complex experiment demonstrating a linear change of peak area with analyte concentration. Since most of the methods use UV detection, such linearity experiments can be considered as re-confirmation of the Beer-Lambert law for the particular hardware configuration.

9. Specificity

The specificity of analytical methods is typically assessed by examining system interference with the detection and quantification of analytes. Part of this evaluation is the determination of protein recovery from the column (Rossi, Pacholec et al. 1986; Eberlein 1995). The recovery determination requires the knowledge of the extinction coefficient for the protein, which can be calculated from its amino acid composition (Pace, Vajdos et al. 1995) or determined experimentally. It should be noted that the extinction coefficient of a protein may change as a function of pH (Eberlein 1995; Kendrick, Chang et al. 1997). Therefore, direct comparison of the recovery in the neutral pH, size exclusion method with the recovery in an acidic reversed-phase separation may not be valid due to differences in the operating pHs of the methods. The difference may not necessarily reflect the actual recovery, but rather shows pH dependent changes of spectroscopic properties of the protein. With such an approach, the specificity of the method can be assessed in every assay, and reflects dynamically the change in status of consumables (columns and mobile phases) and hardware.

10. LOD and LOQ

Assessment of limit of detection/limit of quantitation (LOD/LOQ) is required for most analytical methods developed to monitor product quality attributes. In the latest version of ICH Q2R1, the terms Detection Limit (LD) and Quantitation Limit (QL) are used instead of LOD and LOQ, respectively. ICH defines LOD (DL) as the minimum level of analyte which can be readily detected, while LOQ (QL) has been defined as the minimum level of analyte which can be quantified with acceptable accuracy and precision. Practical application of LOD is related to the decision about integration of chromatograms, electropherograms or spectra, while LOQ is related to the decision on whether to report the results of tests on official documents, such as the Certificate of Analysis (CoA) for the lot.

ICH Q2R1 suggests three different approaches: visual inspection, signal-to-noise-ratio, or variability of the slope of the calibration curve (statistical approach). Vial and Jardy, and Apostol et al., evaluated different approaches for determining LOD/LOQ and concluded that they generate similar results (Vial and Jardy 1999; Apostol, Miller et al. 2009). It is prudent to verify LOD/LOQ values obtained by different calculations. If those values are not within the same order of magnitude, then the integrity of the source data should be investigated.

The statistical approach is most commonly practiced, and is associated with the use of well known equations:

$$LOD = 3.3 \times SD/S$$
$$LOQ = 10 \times SD/S$$

SD = standard deviation of response

S = slope of calibration curve (sensitivity)

The SD can be easily obtained from linear regression of the data used to create the calibration curves. The most common way to present calibration data for the purpose of linear regression is to graph the expected analyte concentration (spiked, blended) vs. the recorded response (UV, Fl, OD etc). This type of plot is characteristic of analytical methods for which the response is a linear function of the concentration (e.g. UV detection that follows the Beer-Lambert law). In cases where the measured response does not follow a linear dependency with respect to concentration (e.g., multi-parameter fit response of immunoassays), the response should be transformed to a linear format, such as semi-logarithmic plots, so that the equations above can be utilized.

The slope used in these equations is equivalent to instrument sensitivity for the specific analyte, reinforcing the fact that LOD/LOQ are expressed in units of analyte concentration (e.g. mg/ml) or amount (e.g., mg). Since the LOD and LOQ are functions of instrument sensitivity, these values, when defined this way, are not universal properties of the method transferable from instrument to instrument, or from analyte to analyte.

Considering LOD from the perspective of the decision to include or disregard a peak for integration purposes, stresses the importance of signal-to-noise ratio as a key parameter governing peak detection. Defining LOD as 3.3 x noise creates a detection limit, which can serve as a universal property of methods applicable to all analytes and different instruments (because sensitivity factor has been disregarded in this form of the equation). LOD expressed in this format is a dynamic property due to the dependency on the type of instrument, status of the instrument, and quality of the consumables. LOD determined this way will be expressed in units of peak height, e.g. mV or mAU.

The decision about reporting a specific analyte on the CoA is typically linked to specifications. After the decision about integration has been made for all analytes resolved (defined) by the method, the results are recorded in the database (e.g. LIMS). When all analytical tests are completed, the manufacturer creates the CoA by extracting the relevant information from the database. Only a subset of the results, which are defined by specifications, will be listed on the CoA. The specifications will depend on the extent of peak characterization and the clinical significance of the various peaks (Apostol, Schofield et al. 2008). Therefore, the list will change (evolve) with the stage of drug development. In such a context, LOQ should be considered as the analyte specific value expressed in units of protein concentration, a calculation for which instrument sensitivity cannot be disregarded (in contrast to LOD estimation). This indicates that a potential exists for diverse approaches to the practice of determining the LOD and LOQ.

Application of LOD/LOQ to purity methods presents specific challenges that deserve additional consideration. The reporting unit for purity methods is percent (%) purity, a unit that is not compatible with the unit in which LOD or LOQ are typically expressed (units of concentration or amount). The signal created by the analyte may vary with the load, while the relative percentage of the analyte does not change. This creates a situation where the analyte of interest can be hidden within the noise or, alternatively, can be significantly above the noise for the same sample analyzed at two different load levels within the range allowed by the method. This has been addressed by the concept of "dynamic LOQ" by combining statistical and S/N approaches (Apostol, Miller et al. 2009).

$$LOQ = 10 \times \left(S/N\right)^{-1} \times P$$

N = level of peak-to-peak noise
S = peak height for the analyte of interest
P = purity level for the analyte of interest

The above equation expresses LOQ as a function of signal-to-noise ratio and the observed purity of the analyte. Both parameters can change from test-to-test, due to equipment variability and sample purity variability. Therefore this equation should be viewed as the dynamic (live) assessment of LOQ.

11. System suitability

System suitability is intended to demonstrate that all constituents of the analytical system, including hardware, software, consumables, controls, and samples, are functioning as required to assure the integrity of the test results. System suitability testing is an integral part of any analytical method, as specified by ICH Q2R1. However, guidance is vague and reference is often made to Pharmacopeias for additional information. The USP, EP and JP contain guidance for a broad scope of HPLC assays, including assays of the active substance or related substances assays, assays quantified by standards (external or internal) or by normalization procedures, and quantitative or limit tests. While each type of assay is described in the compendia, the specific system suitability parameters to be applied for each type of assay, is not included with the description. Thus, some interpretation is required. The interpretation of how to best meet the requirements of the various compendia while still maintaining operational efficiency is a significant challenge for industry.

Existing guidance for system suitability was developed for pharmaceutical compounds and may not be directly applicable for proteins which, due to their structural complexity and inherent heterogeneity, require additional considerations beyond those typically required for small molecules. For example, appraisal of resolution by measuring the number of theoretical plates (commonly done for small molecules), may not be the best way to assess the system readiness to resolve charge isoforms of a protein on an ion exchange column. This may be due to the relatively poor resolution of protein peaks resulting from inherent product microheterogeneity, when compared to the resolution typically seen with small molecules. However, this methodology (the number of theoretical plates) may be a very good indicator to measure the system performance for size exclusion chromatography (SEC), which does not typically resolve product isoforms resulting from microheterogeneity.

To appropriately establish system suitability, we need to consider both the parameter that will be assessed and the numerical or logical value(s), generally articulated as acceptance criteria, associated with each parameter. System suitability parameters are the operating parameters that are the critical identifiers of an analytical method's performance. System suitability should be demonstrated throughout an assay by the analysis of appropriate controls at appropriate intervals. It is a good practice to establish the system suitability parameters during method development, and to demonstrate during qualification that these parameters adequately evaluate the operational readiness of the system with regard to such factors as resolution, reproducibility, calibration and overall assay performance. Prior to validation, the system suitability parameters and acceptance criteria should be reviewed in order to verify that the previously selected parameters are still meaningful, and to establish limits of those parameters, such that meaningful system suitability for validation is firmly established.

One important issue that merits consideration is that the setting of appropriate system suitability parameters is a major contribution to operational performance in a Quality environment, as measured by metrics such as invalid assay rates. A key concept is that the

purpose of system suitability is to ensure appropriate system performance (including standards and controls), not to try to differentiate individual sample results from historical trends (e.g., determining equivalence of results from run-to-run). In practice, setting system suitability parameters that are inappropriately stringent can result in the rejection of assay results with acceptable precision and accuracy. It is highly advisable to ensure the participation of Quality Engineers and/or other staff members with appropriate statistical expertise when setting system suitability parameters.

12. Method robustness

ICH Q2R1 prescribes that the evaluation of robustness should be considered during the development phase. The robustness studies should demonstrate that the output of an analytical procedure is unaffected by small but deliberate variations in method parameters. Robustness studies are key elements of the analytical method progression and are connected to the corresponding qualification studies.

Method robustness experiments cannot start before the final conditions of the method are established. It is a good practice to identify operational parameters for the method and to divide them in the order of importance into subcategories according to their relative importance, which are exemplified below:

1. "Essential" category: includes method parameters that are critical to the method output and therefore require evaluation;
2. "Less important" category: includes method parameters that are not as critical as those in the "Essential" category, but may still affect the method output. These parameters should be evaluated at the scientist's discretion;
3. "Depends on Method" category: includes parameters that may affect the method output differently for different methods, such that these parameters should be treated differently for each method;
4. "Not useful" category: includes method parameters that are known to have no impact on the method output, such that these parameters need not be evaluated for robustness.

It is highly impractical to evaluate the impact of all possible parameters on the output of the method. Therefore, robustness studies could be limited to the demonstration that the reported assay values are not affected by small variations of "essential" operational parameters. It is a good practice to prospectively establish a general design (outline) for such studies. Typically, in these types of studies a reference standard and/or other appropriate samples are analyzed at the nominal load. The studies may be carried out using the one-factor-at-a-time approach or a Design of Experiment (DOE) approach. The selection of assay parameters can vary according to the method type and capabilities of the factorial design, if applicable. It is essential to study the impact of all essential factors, and it is important to establish prospectively "target expectations" for acceptable changes in the output, to ensure that these robustness studies do not repeat the development work. The maximum allowable

change in the output of the analytical method can be linked to the target expectations for the precision of the method, which are derived from the Horwitz equation (Horwitz 1982; Horwitz and Albert 1997; Horwitz and Albert 1997). Recently a number of software packages have become available to assist with the design and data analysis (Turpin, Lukulay et al. 2009; Jones and Sall 2011; Karmarkar, Garber et al. 2011).

13. Challenges associated with validated methods

Remediation of validated analytical methods is typically triggered by the need to improve existing methods used for disposition of commercial products. The improvement may be required due to an unacceptable rate of method failures in the GMP environment, lengthy run times, obsolete instruments or consumables, the changing regulatory environment for specifications or stability testing, or for other business reasons.

We anticipate that technological advances will continue to drive analytical methods toward increasing throughput. In this context, it appears that many release methods are destined for change as soon as the product has been approved for commercial use (Apostol and Kelner 2008; Apostol and Kelner 2008). This is due to the fact that it takes more than 10 years to commercialize a biotechnology drug, resulting in significant aging of the methods developed at the conception of the project. Therefore, the industry and regulators will need to continuously adjust strategies to address the issue of old vs. new methods, particularly with respect to how these advances impact product specifications (Apostol, Schofield et al. 2008). Frequently, old methods have to be replaced by methods using newer technologies, creating a significant challenge for the industry in providing demonstration of method equivalency and a corresponding level of validation for the methods.

14. Concluding remarks

When we consider the critical role that analytical method development, qualification and validation play in the biopharmaceutical industry, the importance of a well designed strategy for the myriad analytical activities involved in the development and commercial production of biotechnology products becomes evident.

The method qualification activities provide a strong scientific foundation during which the performance characteristics of the method can be assessed relative to pre-established target expectations. This strong scientific foundation is key to long-term high performance in a Quality environment, following the method validation, which serves as a critical pivotal point in the product development lifecycle. As noted previously, the method validation often serves as the point at which the Quality organization assumes full ownership of analytical activities. If done properly, these activities contribute to operational excellence, as evidenced by low method failure rates, a key expectation that must be met to guarantee organizational success. Without the strong scientific foundation provided by successful method development and qualification, it is unlikely that operational excellence in the

Quality environment can be achieved. As analytical technologies continue to evolve, both the biotechnology industry and the regulatory authorities will need to continuously develop concepts and strategies to address how new technologies impact the way in which the Quality by Design principles inherent in the analytical lifecycle approach are applied to the development of biopharmaceutical products. The basic concepts are described in ICH guidelines Q8, Q9 and Q10 (ICH 2005; ICH 2008).

The ICH Q2 guideline requires that an analytical method be validated for commercial pharmaceutical and bio-pharmaceutical applications. Frequently, validation is done only once in the method's lifetime. This is particularly of concern when the future testing is performed on an instrument with different technical characteristics, in different geographic locations within the company and/or at contract laboratories around the world, using different consumables, different analysts, etc. This concern is exacerbated by the requirement for modern pharmaceutical and biopharmaceutical companies to seek regulatory approval in multiple jurisdictions, where the instrumentation, consumables, and scientific staff experience at the testing location may be very different than that present in the place where the drug was developed. These considerations raise questions about the value of the current format of the validation studies conducted by the industry. Moreover, it is not clear how the validation data obtained using existing methodologies should or even could be used toward the assessment of the uncertainty of the future results, given the many factors that contribute to the uncertainty.

Perhaps the time is right for the industry to consider the use of a combination of sound science and reasonable risk assessment to change the current practice of the retrospective use of method validation to the new paradigm of live validation of purity methods based on the current information embedded in the chromatogram. Laboratories that work in a GMP environment are required to produce extensive documentation to show that the methods are suitable. Pharmaceutical and biopharmaceutical companies thoroughly adhere to these requirements, inundating industry with an avalanche of validation work that has questionable value toward the future assessment of uncertainty. The predication of uncertainty provides an alternative that has the potential to reduce the work required to demonstrate method suitability and, in turn, provide greater assurance of the validity of the results from the specific analysis in real time.

The establishment of qualification target expectations can be considered as a form of Quality by Design (QbD), since this methodology establishes quality expectations for the method in advance of the completion of method development. Also, the analytical lifecycle described here covers all aspects of method progression, starting with method development, the establishment of system suitability parameters, and qualification and robustness activities, culminating in method validation, which confirms that the method is of suitable quality for testing in Quality laboratories. The entire analytical lifecycle framework can be considered as a QbD process, consistent with evolving regulatory expectations for pharmaceutical and biopharmaceutical process and product development.

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