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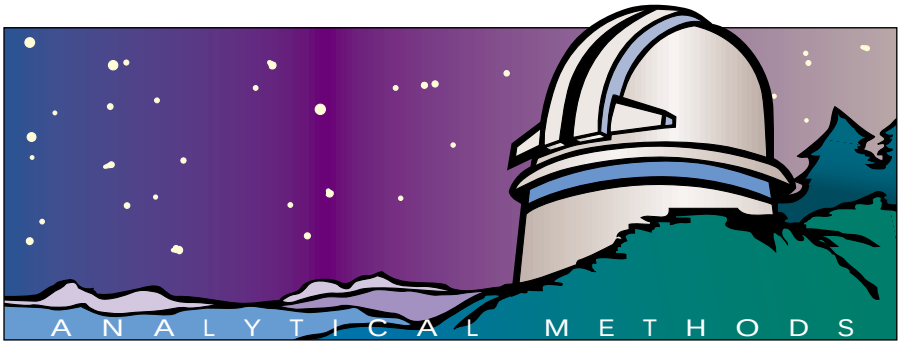

Determining Limits of Detection and Quantitation

Ira Krull and Michael Swartz

In this month's column, we address determining the limits of detection and quantitation in more detail in response to readers' questions about past columns.

Previous "Validation Viewpoint" columns have briefly discussed the determination of the limits of detection (LOD) and quantitation (LOQ) (1–3). However, based upon reader inquiries, the determination of these limits still is an area of great concern. In chapter 1225, the *U.S. Pharmacopeia (USP)* defines limit of detection as the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated (4). A limit of detection test specifies whether or not an analyte is above or below a certain value. The *USP* defines the limit of quantitation as the lowest concentration of an analyte in a sample that can be determined (quantitated) with acceptable precision and accuracy under the stated operational conditions of the method. Like the limit of detection, the limit of quantitation also is expressed as a concentration.

These limits commonly are determined from signal-to-noise ratios (*S/N*). In the case of limit of detection, analysts can use an *S/N* of 2:1 or 3:1, and they can use an *S/N* of 10:1 for limit of quantitation. The 10:1 *S/N* is a rule of thumb because actual limit of quantitation determinations must account for the method objectives of accuracy, precision, and the desired quantitative level.

Typically the signal is measured from baseline to peak apex and divided by the peak-to-peak noise, which is determined from a blank injection. It is important — in this case and in all other cases discussed in this column — that the noise is measured in the blank during the same elution window as the peak of interest.

On the surface, this measurement seems straightforward. Baselines, however, rarely are well behaved, especially at trace levels. Therefore, analysts face another decision: how to measure the noise. Is noise more correctly measured by accounting for the random spikes, by averaging out the random spikes, or by accounting for all baseline disturbances? As early as 1984, researchers proposed changes to the *S/N* convention to clarify limit determination and facilitate comparisons between methods (5). Based on responses to previous "Validation Viewpoint" columns, it has become apparent that the pharmaceutical industry is moving away from the *S/N* convention for limit of detection and quantitation determinations. A new convention is coming into common usage as a result of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q2B Methodology guideline on analytical method validation (6).

THE NEW CONVENTION

The ICH Q2B guideline on validation methodology lists two options in addition to the *S/N* method of determining limits of detection and quantitation: visual noninstrumental methods and limit calculation.

The calculation is based on the standard deviation of the response (σ) and the slope of the calibration curve (*S*) at levels approaching the limits according to equations 1 and 2:

$$LOD = 3.3(\sigma/S) \quad [1]$$

$$LOQ = 10(\sigma/S) \quad [2]$$

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of *y* intercepts of regression lines. The ICH calculation method can reduce the bias that sometimes occurs when determining the *S/N*. This bias can result because of differences in opinion about how to determine and measure the noise.

Equations 1 and 2 are derived from the International Union of Pure and Applied Chemistry (IUPAC) model for spectrochemical analysis adopted in 1975 (7). Although the American Chemical Society (ACS) Subcommittee on Environmental Analysis reaffirmed this model in 1980 (8), it never had been applied specifically to chromatography or other analytical techniques until the ICH guidelines were implemented. In the original IUPAC model, the factor of three in equation 1 was derived from a confidence level, depending upon the probability distribution of the blank signal and the accuracy of the standard deviation. Standard deviation was calculated from 20 or more measurements of the spectroscopic blank signal. Because performing 20 blank injections is somewhat impractical, some researchers have suggested alternatives. The determination of σ was defined in one instance as one-fifth the peak-to-peak noise across a region at least 10 peak widths wide in the region that normally would contain the analyte peak (5). This determination has evolved to the ICH methodology using the standard deviation of the blank, the residual standard deviation of the regression line, or the standard deviation of *y* intercepts of regression lines (6). Spreadsheet software such as Microsoft Excel (Redmond, Washington) can calculate σ in this manner.

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To grasp a complete understanding of the calculations involved, however, consult any good statistics book (9). Essentially the statistical function $S(y/x)$ is calculated by equation 3:

$$S(y/x) = (A/B)^{1/2} \quad [3]$$

where A is the sum of squares of the y residuals and B represents the degrees of freedom. y residuals are calculated from the original x values of the line and the regression equation. Degrees of freedom are equal to $n - 2$ for linear regression. The standard deviation of the y intercept then can be calculated according to equation 4:

$$\sigma = S(y/x)[C^2/nD^2]^{1/2} \quad [4]$$

where C is the sum of x values and D is the sum of the x residuals.

EXAMPLE ICH PROTOCOL

Let's look at an example protocol for measuring the limits of detection and quantitation by the ICH method (10). The limits of detection and quantitation are generated from the linearity data across a range approaching the limits. Standards are prepared from the reporting level to 120% of the control (specification) limit. Workers prepare five standard concentrations spanning the range from dilutions of a stock standard solution. The standards are prepared in this way to minimize errors associated with weighing. Analysts then make two or more replicate injections of each standard and perform regression analysis on the data. Excel software and its linear regression functions can calculate the slope and standard error of the y intercept from the linearity data, according to equations 3 and 4. These results then can be used with equation 1 or 2, as appropriate.

Calculated limits of detection and quantitation have yielded results consistent with those obtained using the standard *S/N* approach (10). In some cases the ICH calibration curve approach yields limits of detection and quantitation that are significantly lower than those obtained by the traditional approach. This situation can occur when the electronic data system has difficulty in consistently identifying the start and end of chromatographic peaks for the lower concentration standards. This situation arises when the standard deviation of the y intercept (based on all the standards) is different than expected because of a bias in the noise for the more-concentrated standards. The key is to look at the standard deviation of the lowest standard injections and compare it with the standard deviation of the highest standard. If noise is constant throughout the range, the standard deviations should be equivalent. If this is not the case, analysts should use the *S/N* determination. Experience has indicated that the two approaches tend to diverge only when the range is large (that is, when the difference between the reporting level and the specification limit is large).

SOME GENERAL CONCLUSIONS

For active assays (*USP* category 1), limits of detection and quantitation are superfluous measurements that do not pertain to the validation of the method for the intended purpose. Generally limits of detection and quantitation are determined only for impurities (*USP* category 2–related substances and residual solvents). One exception to this situation is that analysts may want to examine the limit of quantitation for methods used in evaluating dissolution profiles.

No single method of determining the limits of detection and quantitation is specified in any requirement or guideline. The actual method used by an analyst simply should be documented, and an appropriate number of samples should be analyzed at the limit to validate the level.

Remember that the determination of a limit of quantitation is a compromise between the concentration level and method precision at the limit. Although an *S/N* of 10 or less than 10% RSD are good values as a rule of thumb, users must consider a method's intended use and the required precision. Individual analysts must set the specification because *USP* lists no requirements or guidance for these parameters.

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Sharper peaks yield a higher *S/N*, resulting in lower limits of detection and quantitation (1). Column efficiency therefore can affect these measurements, and analysts should account for both the type and age of the column when determining the limits of detection and quantitation. These parameters usually are determined over the course of time as experience with the method grows, and they are taken into consideration when determining intermediate precision and reproducibility (ruggedness).

Detector response also can affect the ability to measure these limits. For example, the UV detector lamps used in high performance liquid chromatography can lose intensity over time and affect the measurement of limits of detection and quantitation. This phenomenon will become less of a problem because lamp optimization routines in modern detectors compensate for losses in lamp intensity. Other types of detectors, such as those used in gas chromatography, must be properly maintained to provide comparable results over time. Again, these differences should be examined and compensated during method development. When evaluating the method for intermediate precision and reproducibility (ruggedness), analysts should consider instrument differences. When a validated method is transferred, the receiving laboratory should re-evaluate the limits on its analytical systems.

Finally, we want to caution analysts about confusing the limits of detection and quantitation with sensitivity. Sensitivity is defined as the slope of the calibration curve, and as such it usually does not reference the actual limit of detection or quantitation.

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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.

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