Validation VIEWPOINT

Quantitation in Method Validation

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This month's column examines various methods of quantitation, percent recovery of an analyte as a part of quantitation, and specific approaches to quantitation that must be optimized for complete method validation.

Quantitation means different things to different analysts; therefore, we start this installment of "Validation Viewpoint" with some definitions of quantitation. At times, quantitation has been defined as relative or percent area of individual peaks in a chromatogram, all summing to 100%. Quantitation also has been defined as showing that a substance is present at or above a certain threshold level (l-lo).

In reality, true quantitation relates to an ability to demonstrate exactly how much of a particular analyte is present in a particular sample, It means determining the concentration present in parts per million, micrograms per milliliter, molarity, or other terms that denote the amount of material (mass) in a given amount of the original sample (or volume of solution). Many chromatographic methods for pharmaceutical analysis must demonstrate the quantity of the analytes present per gram of original solid formulation or per volume of the original aqueous solution. Concentration is not area percent or relative area; it is the absolute level or mass per unit volume of an analyte present in a sample matrix.

Analysts cannot validate a method, which depends on demonstrating the accuracy and precision of such measurements, without first being able to accurately and precisely quantitate the level of analytes present in the original samples. Quantitating analytes in samples requires the use of a well-characterized standard of the analyte of interest. Immunoassays, as an alternative to chromatographic assays, also must determine the concentrations present as the mass per unit volume of solution. The very essence of analytical chemistry relies on accurately and precisely quantitating the analytes of interest in the original samples. The U.S. *Pharmacopeia* (*USP*) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) both recognize the unique significance of quantitation in deriving a validated high performance liquid chromatography (HPLC) method (9,10).

Unfortunately, neither the *USP* nor ICH guidelines direct analysts to the best possible method of quantitation. Therefore, chemists must review basic analytical chemistry textbooks that deal with their topic (l-8). The U.S. Food and Drug Administration never will dictate nor even suggest particular quantitation methods for particular samples or HPLC analysis modes; those decisions are the responsibility of individual analysts or their laboratories. Typically analysts must use a trial-and-error approach to determine the best quantitation method for a particular analyte in a particular sample matrix.

Quantitation has no hard and fast rules or guidelines, except that the final method selected must provide the best accuracy and precision possible, the best repeatability, and a high degree of intermediate precision or reproducibility (ruggedness) from analyst to analyst, day to day, and laboratory to laboratory. The method chosen for quantitation also should accomplish these objectives in the shortest time possible with a minimum amount of operator involvement and the smallest amount of sample, resources, and instrument time. The ideal quantitation method in the end will depend on the particular samples that need to be analyzed, the number of samples, the sample matrix complexity, automation possibilities, and availability of sample and standards.

SPECIFIC GOALS OF AN IDEAL QUANTITATIVE METHOD

The ideal quantitative method should possess attributes and advantages such as

- rapid sample throughput with minimum cost, manpower, and instrument requirements;
- high accuracy and precision;
- lack of interference and contamination from matrix and analytical reagents;
- an accounting for percent recovery of analyte;
- an accounting for loss of sample and analyte during sample work-up or analysis; and
- easy routine operation and application by suitably trained technicians.

The real question remains: *Which of the numerous quantitation methods should you select for a particular sample? The* answer often depends on the nature of the sample; that is, whether it is simple with a few peaks or very complex with numerous peaks. Usually, simpler samples (few analytes, simple matrix) can use simpler quantitation methods and external standard calibration plots or even single-point calibrations.

The ability to use single-point, external standard calibration for many samples usually reduces cost, time, and manpower requirements and leads to higher sample throughput. However, analysts can use single-point, external standard calibration only with standard concentrations that are close to the actual concentration of the unknown samples within the linear range of the method. More-complex biological fluid samples that have numerous analytes, difficult-to-remove matrix components, and trace-level concentrations will require more-complex methods of quantitation such as standard additions.

Of course, analysts cannot use any quantitation technique until they demonstrate that the HPLC peak is pure and homogeneous (exhibiting chromatographic selectivity) and that the peak is from the correct analyte. This proof will require some form of sophisticated photodiode-array or mass spectrometry (MS) detection after HPLC separation. Determining peak purity requires measuring the UV and MS spectra throughout each peak of interest

and then using computer software to overlay and compare spectral properties (after normalization or application of sophisticated software algorithms).

Today, software programs routinely perform this task by handling the data, interpreting an impurity profile (peak purity), and creating a purity plot through the chromatographic peak of interest that suggests the absence or presence of impurities in that peak. These software programs can use both photodiode-array and MS data. An assurance of purity by both of these spectral methods before quantitation is even attempted is the ideal approach.

Another software routine — using library files of photodiode-array and MS spectra — then can be used for peak matching to confirm the expected or suspected structure of the peak of interest. There is no sense in performing quantitation until you have proven peak purity and identity otherwise the results could not be correct.

External standard method: In the external standard approach, analysts must generate a calibration plot (see Figure 1) using known concentrations of the standard alone, ideally in the solvent that will be used for actual samples. At least five concentration points should be analyzed three times each ($n = 3$), and, ideally, each concentration should be prepared separately, rather than as dilutions of a single, high concentration solution. This type of analysis will show the true error in preparation of all standard solutions, not just dilution errors.

Additional data should include the precision of each point measurement, the equation of the straight line generated, the y intercept (ideally zero), and the extent of the linear range of the plot. The correlation coefficient of the line, coefficient of linearity or variation (r^2), and linearity (r) values should be described. The calibration plot does not need to be described from the limits of detection to the nonlinear region, but the calibration plot should bracket the expected and actual concentration range found in actual samples. The external standard calculation for determining the concentration of the analyte peak then must be used to derive the concentration for the unknown sample.

$$RF = [\text{std}]/R_{\text{std}} \quad [1]$$

$$[n] = RF \times R_x \quad [2]$$

In these equations, RF refers to response factor, [std] is the standard concentration, R_{std} is the standard response, [x] is the unknown concentration, and R_x is the unknown response. Concentration can be expressed in any of the usual terms, including molarity, normality, and parts per million. The standard response refers to the peak height or area of a known amount of the analyte standard, which ideally is close to the actual concentration in the sample. The unknown response (R_x) refers to the peak height or area of the unknown in the sample.

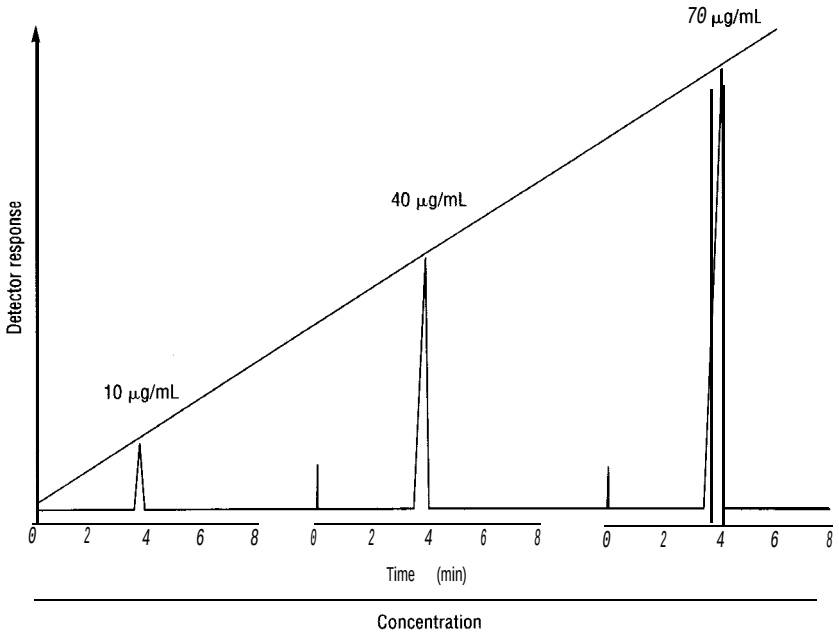

FIGURE 1: A typical external standard calibration plot for three levels of analyte standard injected.

Of course, the use of external standard calibration plots demands knowledge of the analyte's analytical recovery from that particular sample matrix. An external standard calibration plot or single-point external standard usage can become very problematic if the sample matrix changes after a small number of runs or if the samples to be assayed have many different perhaps even unknown — matrix compositions for which the analytical recovery changes from sample to sample.

The only way to learn if these conditions exist is to determine the spiked samples' percent recovery for the various sample types (different sample matrices) and demonstrate their recoveries. If the percent recoveries are very low or if they vary over large values from sample-to-sample, you should pursue another method of quantitation.

Ideally, a calibration plot should go through the origin with a y intercept of 0, but it does not always occur. If the y intercept is significantly greater than 0, it suggests the presence of an interfering or coeluted compound from the blank's background signal. It is possible to quantitate a sample with a non-zero calibration plot accurately, because the reasons for the non-zero y intercept with standards alone also should be present in the samples as well. If the calibration plot's y intercept is negative, it suggests some degree of standard loss during preparation of the injection solutions. This problem should be corrected because the calibration plot error will not carry over to actual samples, and this type of a calibration plot cannot provide accurate quantitation in real samples.

Internal standard method: In the internal standard approach, analysts use a compound that mimics the analyte of interest as much as possible to add to the sample before sample handling, work-up, or preparation. The internal standard should possess chemical, spectral, and chromatographic properties that are similar to those of the analyte, and the standard should be resolved from the analyte of interest. An internal standard must possess a known chemical structure, be available in a high-purity version, and be recoverable from the sample matrix with a percent recovery similar to that of the analyte.

The ideal internal standard is an isotopic analog of the analyte that will be coeluted with the analyte but will be resolvable by MS or an alternative detection method such as radiometric detection. Because photodiode-array UV detection is the most common detection mode in HPLC, isotopic analogs will not suffice. Therefore, users must select an analog of the analyte that has a slightly different structure but similar recovery and chromatographic properties.

The internal standard should have peak shape and symmetry that are similar to those of the analyte. It should be spiked into the sample at a concentration approaching that of the analyte. If you plan to use peak height measurements for the analyte, then you should use the same data with the internal standard to simplify the process.

Figure 2 illustrates a typical internal standard calibration plot, which was generated by maintaining a constant concentration of the internal standard and varying the concentration of the analyte. You should choose at least five analyte concentration points, rather than the

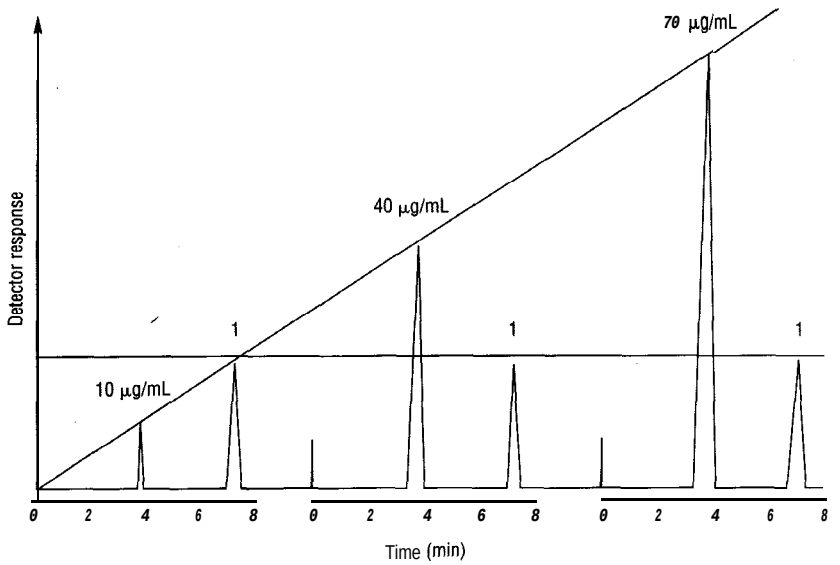

FIGURE 2: A typical internal standard calibration plot with three concentrations of analyte standard and one constant concentration of internal standard derived from three HPLC injections. Peak 1 is the internal standard.

TABLE I: Recoveries of 2-Acetyl-4(5)-(Tetrahydroxybutyl) Imidazole in Caramel Color Samples*

Sample Number	Recovery (%)
VT-1	50.0 ± 5.3
VT-2	64.2 ± 6.0
VT-3	48.5 ± 2.8
VT-4	48.9 ± 1.0
VT-5	65.4 ± 1.8
VT-6	66.5 ± 8.1
VT-8	38.0 ± 2.0
VT-9	61.9 ± 4.5
VT-10	86.4 ± 4.9
VT-I 1	78.0 ± 4.4
VT-I 2	52.0 ± 3.7
VT-13	41.0 ± 6.8

* Recovery represents the original mass of analyte actually obtained from the sample after all sample preparation steps as seen by the detector at the end of the HPLC column. Recovery is given as an average plus or minus a standard deviation for each sample for three separate injections for each sample (n = 3) and one sample work-up.

iree illustrated in Figure 2. The internal standard calculation method differs from the external standard method as follows:

$$RF = (R_{IS}/R_{std})\,[std] \qquad [3]$$

$$[x] = RF(R_x/R_{IS}) \qquad [4]$$

In the equations above, R_{IS} is the response of the internal standard and R_{std} is the response of the analyte standard. Analysts should introduce the same concentration of internal standard as that used for the generation of the internal standard calibration plot. The response can be peak height or area, as long as the variable is used consistently in all calculations. The peak heights and areas for similar internal standard and analyte concentrations should be similar, as should their capacity factors and retention times.

The internal standard approach automatically corrects for percent recovery, so analysts do not need to determine this factor in separate experiments. This attribute of the internal standard method obviates any further need to measure percent recovery or ensure sample integrity for each sample, so long as the percent recovery internal standard-to-standard ratios remain constant from sample matrix to sample matrix. However, users must demonstrate constant recovery ratios for new sample matrices, rather than assuming that they are acceptable.

Errors caused by sample loss from handling or poor injections also are overcome by the internal standard method of quantitation. However, an impure analyte peak will give the wrong quantitative level with this or any other method of quantitation, unless a detector can resolve the impurity from the true analyte peak.

Remember that isotopic dilution techniques, which are used so often in MS, are just a variation of the internal standard method. In isotopic dilution the internal standard is the ideal standard — an isotopic analog such as deuterium, tritium, or a radioisotope.

Standard addition method: As Table I illustrates, caramel samples often differ in their percent recoveries for an impurity (such as 2-acetyl-4[5]-[tetrahydroxybutyl] imidazole) because of the very nature of these samples (7). Because the percent recoveries were variable from sample to sample, though consistently reproducible within any given sample, the only practical method of quantitation was standard addition. This technique accounts for changing percent recovery, matrix effects, loss of sample, and poor injection techniques, but it cannot account or correct for a peak that an analyst falsely assumes to be pure. Again, it is absolutely essential to demonstrate peak purity and identity before attempting any method of quantitation.

In the standard addition method, workers first divide a sample into at least four aliquots of similar volume. The first aliquot is analyzed using an accepted method to generate a peak height for the peak of interest. Then the concentration of this peak is estimated by injecting standards of known concentration of this analyte under the same analytical conditions. Assuming an approximate 50% recovery of the analyte in the sample, an analyst can estimate the concentration that might be present in the original sample. This guess provides only an estimate because it assumes a 100% recovery; however, this assumption is valuable because it provides an initial starting concentration for the analyte standard to be introduced in minimum volumes into the actual sample aliquots (8).

The standard addition method calls for spiking at least three sample aliquots with differing amounts of the standard analyte, as Figure 3 shows. Figure 3 illustrates the use of the standard addition method to determine a particular compound — 2-acetyl-4(5)-(tetrahydroxybutyl) imidazole — in a caramel sample (7). The levels to be spiked usually vary from x/2 to x to 2x, where x represents the estimated concentration level found in the original, external standard estimation study. In any event this amount should bracket the actual concentration. (These figures are not the actual levels spiked in Figure 3. These values are only recommended levels for initial spiking, but they may not provide the most accurate results.)

Of course, analysts must start working with standard additions on a sample that contains no analyte and then spike it at known levels to validate the method of quantitation. Because the actual percent recovery is unknown but assumed to be approximately 50%, some experimentation and flexibility are required in the final spike levels. After some experimentation, those final spike levels should provide acceptable quantitation accuracy and precision, as well as a linear standard addition calibration plot, as shown in Figure 3. If no placebo is available for spiking and no sample is available with zero-incurred original levels of the analyte, then analysts must assume an overall

50% recovery for the real sample, estimate the levels of analyte after recovery (sample work-up and preparation) by a single-point calibration, estimate the external calibration standard injected before and after the actual sample (in replicate), and begin to develop the best possible spiking levels with the starting level of analyte, as above.

Each sample containing the spiked standard then should be assayed and have its peak area or height plotted, as in Figure 3. Each analysis should be performed in triplicate or more, so workers can calculate the standard deviation for the standard addition plot. When all points are plotted using concentration-spiked versus measured-peak area, the plot should form a straight line that intercepts the negative x axis. The zero concentration point on the x axis is the incurred (original) level for the unspiked sample, and the negative x intercept becomes the actual concentration level present in the original sample without any spiking. Robinson (8) describes equations that analysts also can use to derive the original concentration. Often it is simpler and easier to plot the standard addition curve on a computer using statistical software. Several programs that allow users to derive the original concentration without graphing standard addition plots are available (8,11,12).

The standard addition method perhaps is an ideal quantitative approach, except that plots must be generated for each and every sample, which requires an unusual amount of instrument time, effort, energy, and sample quantities. Standard addition probably is the ideal method of quantitation for very complex sample matrices from which recoveries vary from sample to sample and for samples that present problems with sample or analyte loss. The standard addition method will not compensate for an impure peak that is assumed to be pure and to have the correct structure. Therefore, to obtain the best results, analysts must choose a final analytical method that can resolve the analyte peak of interest in the original sample from all other possibly interfering peaks.

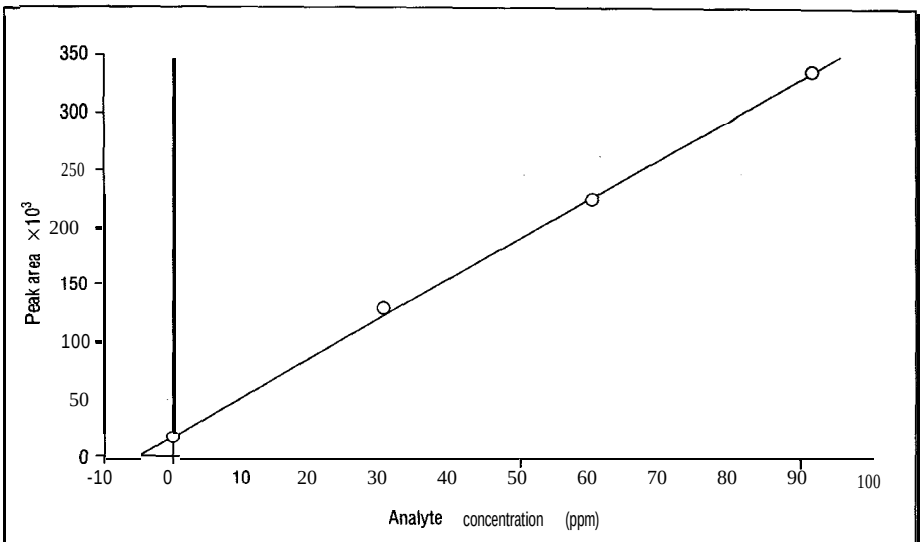

FIGURE 3: A standard addition plot for the analysis of 2-acetyl-4(5)-(tetrahydroxybutyl) imidazole in caramel samples. Three concentrations of 2-acetyl-4(5)-(tetrahydroxybutyl) imidazole were spiked in the original sample. The equation of the straight line plot was $y = 3.473x + 19.65$, and the linearity coefficient (r^2) was 0.999.

CONCLUSIONS

Quantitation is a tricky area, and it must be developed as a part of method validation. Indeed, without accurate and precise quantitation, you cannot have true method validation. Quantitation must be proven, rather than assumed, valid by experimentation using single- or double-blind spiking methods (10). Analysts must use criteria that demonstrate one quantitation method's superiority over another. Analytical chemists must be aware that choosing a method of quantitation depends on factors such as the samples being analyzed, their complexity, the level of available analyte, the total number of samples, the sample-to-sample percent recovery variation, and the time required per sample.

No single method of quantitation works best for any and all samples. The best method for any given situation is derived by experimentation. Of course, if the sample is very simple and has few analytes and possible interferents (for example, a bulk drug sample), then an analyst can start using the simplest methods, namely external standard calibration and single-point calibration. With more-complex samples, such as blood or urine for a metabolism study, workers must assume from the start that a single-point calibration will fail and that they should start with a more rigorous method of quantitation such as standard addition. Chemists also must exercise some judgment when choosing an initial method of quantitation depending on whether their samples have known composition and analyte levels.

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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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