## The Use of Internal Standard Calibration with LC/MS/MS Bioanalytical Methods

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is the most commonly used technique for measuring pharmaceutical concentrations in samples such as plasma, blood, urine, and feces. This type of **bioanalytical** method determines the concentration of drug in a sample using an internal standard calibration model. Internal standard calibrations are important when establishing bioanalytical methods to ensure reproducibility and reliability. Since LC/M/SMS uses mass as a means of detection, internal standard methods optimally use an isotopically labeled form of the drug being measured. Synthesizing isotopically labeled chemicals can be expensive and time-consuming. Often a sponsor's sensitivity to time and cost deter them from pursuing isotopically labeled internal standards for LC/MS/MS methods. However, the initial investment in time and cost is more than made up during sample analysis. Many potential problems are completely circumvented and testing is more rapid with significantly lower costs per test and fewer reanalysis.

An internal standard calibration method requires that a known amount of a non-analyte compound (**internal standard**) be added to every sample, calibration standard (**calibrator**) and blank prior to extraction. A calibration curve is constructed for known concentrations of analyte versus the ratio of analyte response to the internal standard response. Since the amount of internal standard added to every calibrator and sample is the same, the concentration in the samples can be calculated from the ratio of analyte to internal standard. One important requirement in using internal standard calibration is that the internal standard and the analyte behave similarly, both during the sample extraction and cleanup steps, and during the LC/MS/MS analysis. Isotopically labeled forms of the analyte usually have 4 to 6 hydrogen atoms replaced with deuterium. This creates a chemical that behaves in all ways identically to the drug being tested for, with the exception that the molecular weight is 4 to 6 atomic mass units (amu) greater. Because the LC/MS/MS is capable of discriminating between two compounds that differ by a few amu, this form of internal standard is ideally suited for LC/MS/MS methods. Other types of analytical instruments that use chemical or spectrometric detection instead of mass cannot use isotopically labeled internal standards.

Bioanalytical methods are used to measure analytes in what are described as "**high matrix**" samples: plasma, blood, urine, feces. These sample types, while containing the analyte at low concentration, also contains a large mix of **endogenous** components, including proteins, salts, minerals, lipids, and other small and large molecules. These are often present in high concentrations and can greatly complicate extraction and analysis. Unlike many analytical methods, bioanalytical methods routinely use calibrators processed in the same manner as the samples. Calibrators are prepared in the same type of sample, known to be free of analyte, and are extracted as the samples are and at the same time. As for any other analytical method, recovery is a required validation element for bioanalytical methods. The complex nature of high matrix samples almost completely eliminates the possibility of 100% recovery of the analyte

from the sample. In fact many bioanalytical methods have absolute recoveries of 70% or less. Bioanalytical methods compensate for low recovery by extracting the calibrators in matching sample type. “Recovery of the analyte need not be 100%, but the extent of the recovery of the analyte and internal standard should be consistent, precise, and reproducible.”[1] Due to the nature of internal standard calibration, it is the reproducibility of the ratio of analyte to the internal standard in samples processed with the method that determines method accuracy, in addition to method precision. Method recovery using internal standard calibration can be independent of absolute analyte recovery as long as the internal standard and analyte recover similarly. The best way to ensure similar recovery is to use an internal standard that is physically and chemically identical to the analyte, as is the case with isotopically labeled internal standards.

The incorporation of an internal standard into every sample compensates for another aspect of the high matrix samples: that each sample can contain very different mixes of the many endogenous components. These differences can cause samples within a set of “similar” samples to extract differently, yielding varying recovery of the analyte of interest. Thus, the few samples used to determine recovery does not necessarily represent method behavior for all samples in any particular set. Endogenous compounds can affect the method behavior of the analyte in a variety of ways. They can actively compete with the analyte for binding sites on an SPE column, change the pH of the extract, change analyte solubility in the extraction solvent, and interact with the analyte making it more or less amenable to extraction. Since each sample can have differing combinations and concentrations of these endogenous compounds, the ability to reproducibly recover the analyte from sample to sample is dramatically reduced. An isotopically labeled internal standard best compensates for sample-to-sample variability.

The addition of internal standard to every calibrator, QC sample, and incurred sample is important for LC/MS/MS methods for a reason besides the difference in extraction from sample to sample. Particularly before the advent of modern atmospheric pressure ionization the response of the mass spectrometer detector could be counted on to fluctuate over the course of the analysis, usually declining with time. By using a calibration model that does not depend upon the absolute response of the analyte one can negate this effect. Since for this method the *ratio* of the analyte response to internal standard response is the important measurement to yield analyte concentration, quantitation is not effected if the overall detector sensitivity fluctuates, as long as the changes in sensitivity are consistent for the analyte and internal standard. This is assured by using an internal standard that is physically and chemically identical to the analyte, as is the case with isotopically labeled internal standards.

Mass spectrometry can only recognize charged compounds, thus the analyte must be ionized in order to be detected. Even for samples that undergo an extensive extraction and cleanup process there are still a wide range of endogenous substances that end up in the final extract and are introduced in the LC system. The LC separates these compounds and the analyte in time, but invariably some have the same retention time

as the analyte. These endogenous substances end up in the ionization chamber of the mass spectrometer, where they can cause greater and lesser degrees of analyte ionization. A change in analyte ionization creates a similar change in detector sensitivity. If the analyte and internal standard elute at the same time, the sensitivity change is compensated for. However, if the analyte and internal standard have even slightly different retention times they can experience very different ionization environments, yielding inaccurately high or low sample analyte concentrations. An isotopically labeled form of the analyte, because it is physically and chemically identical to the analyte, elutes at exactly the same retention time as the analyte ensuring that both experience the same ionization environment as the analyte.

Even when sponsors support the use of an isotopically labeled internal standard, a situation often arises that defeats these good intentions: one or more metabolites are identified and get added to the assay. When a method is to be used to assay more than one analyte an isotopically labeled standard should be made for each analyte. When a one-analyte method is expanded to include additional compounds using only the original internal standard, then the other analytes may be incorrectly quantified as described above. The most notable form of this problem is failed QC sample recovery for the other analytes while the original analyte continues to perform well. Failed QC samples translate to sample reanalysis and thus added time and cost. LC/MS/MS provides a fast and sensitive method for the analysis of bioanalytical samples but can only perform reliably when isotopically labeled internal standards are used for each analyte to be quantitated.

References

1. *Guidance for Industry Bioanalytical Method Validation*, FDA, May 2001