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Internal standard (IS) variation case studies: Emerging from three common IS challenges

The use of internal standards (IS) is essential for developing and applying liquid chromatography-tandem mass spectrometric (LC-MS/MS) quantitative bioanalytical methods. As bioanalytical knowledge rapidly advances and regulatory requirements continue to harmonize globally, attention has shifted to focus on IS of chromatographic methods.

IS in LC-MS/MS methods plays a crucial role in normalizing the variation of analyte responses from the entire experimental conditions and instrumental conditions. With typical LC-MS/MS assays, laboratories can adequately monitor internal standard variation (ISV) across an analytical run and identify abnormal response(s) as common practice for modern regulated bioanalytical laboratories. However, appropriate methodologies or criteria to assess the ISV are still ambiguous and differ widely among laboratories.

Due diligence amongst ambiguity

Industry experts have discussed the establishment of feasible acceptance criteria for ISV at length during international conferences and various other occasions, but there is still no definitive common consent. In fact, descriptions of IS response variations are limited in most regulatory guidance for bioanalytical method validation and its applications[1-3]. Meanwhile, governing health authorities have not defined assessment procedures or acceptance criteria.

Scientists must ensure the accuracy of the quantitation results. At a minimum, they should thoroughly document the monitoring of ISV and proactively establish the criteria to trigger samples' reassay when presented with an abnormal IS response. In 2019, the U.S. FDA published guidance on this topic reflecting its current thinking on ISV: "Evaluation of Internal Standard Responses During Chromatographic Bioanalysis: Questions and Answers."[4] Even though clear statistical acceptance criteria is not included in this guidance, this publication provides recommendations on frequently observed scenarios and is the first step toward harmonized practice on monitoring of IS responses in the regulated bioanalysis community.

Handling variations with IS

Incorporating the IS is fundamental to determine the reliability of the target analyte's concentration data, and there has been a trend pushing to integrate stringent criteria for IS responses in the study samples. This evolution has resulted in considerable complications moving forward from conventional protein precipitation, liquid-liquid extraction, solid-phase extraction, to the incorporation of hydrolysis and/or derivatization steps, and immunocapture hybrid LC-MS/MS techniques. To resolve these issues, scientists use IS in a bioanalytical method to correct any potential variations originating from recovery differences propagated along with sample pre-treatment procedures. The responsibility of closely monitoring IS response variation falls on the scientists using IS, which becomes increasingly difficult for an analog compound to the analyte in tandem with the experimental setup's complexity.

The more complicated and lengthy the experimental setup, the more significant the sample-to-sample variations. An IS's presence serves to normalize potential variations for the entire sample preparation process and identify any mishandling of samples or instrument malfunction to produce reliable quantitative bioanalytical results.

Incorporating IS is crucial to develop, validate and apply an acceptable LC-MS/MS bioanalytical method, both for regulated and non-regulated studies. Scientists also utilize IS to correct any instrumental conditions variations during an analytical run, especially for analysis with automated steps. Modern laboratories commonly employ such analysis to monitor any instrumental malfunction or failure that produces abnormal data. Although recent advancements in the ultra/ultra-high-performance liquid chromatography (U/UHPLC) systems have already demonstrated minimal instrumental variations, errors or operational faults require monitoring of the IS response. Minor issues such as air bubble traps in the liquid line, leakages or blockages can all interrupt accurate data collection.

Additionally, since the observed biological matrices come from healthy individuals or diseased states, IS can help isolate any abnormal responses from a particular matrix or subject. Especially when a mass spectrometer is the detection technique, such responses alter the ionization efficiency and might affect the validity of results. In addition, ISV is a reliable indicator of analytical quality regarding the chromatographic peak shape and analyte/IS retention times.

Applying the above considerations can be complex to understand and apply to a specific molecule. Still, companies that can grasp a few common root causes of ISVs is a beneficial starting point for any bioanalytical method development team. The following case studies outline real examples originated from sample matrices, analyte characteristics and sample pre-treatment of LC-MS/MS bioanalytical methods.

Case 1: Scattering of internal standard responses

A bioanalytical method employing liquid-liquid extraction (LLE), stable isotope labeled-internal standard (SIL-IS), automated liquid handling and modern LC-MS/MS instrumentation should be considered one of the mature methodologies with the merit of simplicity. In reality, when such a method is unexpectedly surprised with significant ISV, the root cause may be fundamental.

Figure 1 shows the IS responses of the samples from a simple and straight forward LLE method in a 96-well plate format, observed from an accuracy and precision run during method validation. The IS responses reported evenly and distributed within a narrow range throughout. The sample analysis frequency was once a year to accommodate a Phase III clinical trial, typically lasting for several years.

When this method was re-applied, the scientists observed severe scattering of IS responses, as shown in Figure 2. Bioanalysts then reanalyzed the entire batch of sample extracts on a different instrument using exactly the same method conditions. The result reproduced an identical variation pattern. This result immediately ruled out the possibility of instrumental error and narrowed the possible causes down to the matrices or sample pre-treatment procedure. Using the IS variation to observe calibration standards, quality control samples and study samples, bioanalysts indicated that analyte concentration and matrices were not the contributing factors as the batch acceptance criteria were well matched whereas such ISV was not limited to study samples. No approach thus far could establish a relationship between the IS response and these two factors.

Finally, the laboratory identified the root cause – the shaker speed setting for the liquid-liquid extraction step. In the bioanalytical methodology, only shaking speed set at "high" was documented, which is a subjective description that varied according to individual scientists' practical experience. In addition, no equipment model or brand name was mentioned, which added another layer of uncertainty on the true speed. This variability produces descriptive traps that fail to deliver expected results. This case is an excellent example of the fact that in-depth and detailed instructions and documentation are required to capture all experimental steps to ensure a method's reproducibility and performance throughout its life cycle.

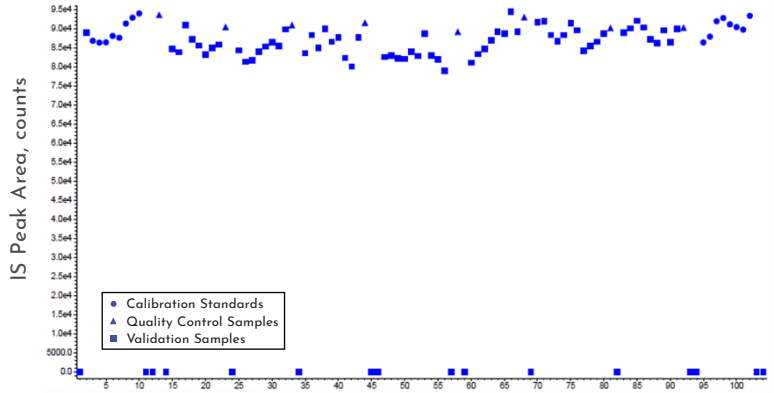

Fig. 1: Typical IS responses observed during method validation

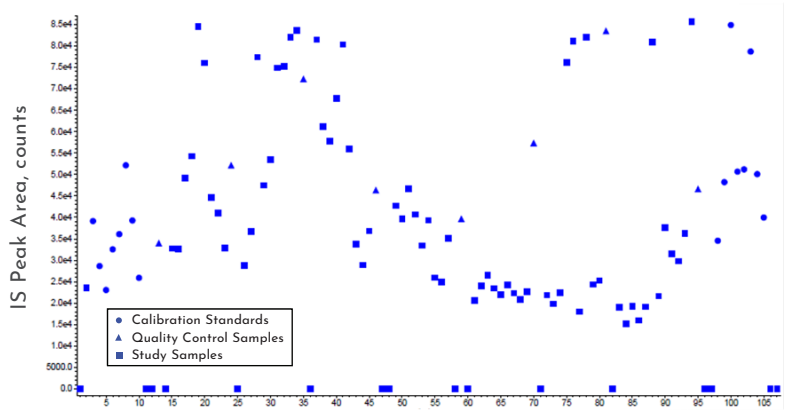

Fig. 2: Scattering IS responses observed during sample analysis

Case 2: Ionization efficiency competition

Figure 3 illustrates the relationship between IS response and analyte concentration. The method that generated this data was a typical protein precipitation employing SIL-IS.

From the calibration standards, it is evident that the IS responses dropped as the analyte concentration increased. This phenomenon was reproduced from the analysis of six sets of quality-control samples sequentially injected with increasing concentration. In the middle of the injection sequence, two additional sets of quality-control samples, prepared from two different individual matrices, also revealed similar observations.

Since this method used SIL-IS, the unlabeled analyte's coelution competed significantly with the SIL-IS under a limited ionization efficiency. Such IS response trends due to ion suppression are common and sometimes unavoidable. This often happens or becomes apparent when the span of the calibration range is too high or the analyte/IS response is too strong. To resolve or minimize the spread, scientists must carefully adjust the IS concentration, achieving a balanced instrumental response.

In some cases, the fine adjustment of mass spectrometer ionization parameters, such as voltage or probe position, might be viable solutions to this phenomenon.

Case 3: The characteristic property of the analyte

Scientists observed a standout case of ISV during the method development of a Quaternary Ammonium

Drug (QAD). QADs are an inherently charged entity, and in this case, the molecular weight was over 1200 Da, where protonation during mass spectral ionization leads to the formation of a doubly charged precursor ion.

Figure 4 depicts the full scan mass spectrum. This bioanalytical method utilized SIL-IS, which means the IS itself also exhibited two charge states upon ionization. As such, the laboratory could select both singly- and doubly-charged precursor ions for the multiple reaction monitoring (MRM) mode for quantitative analysis.

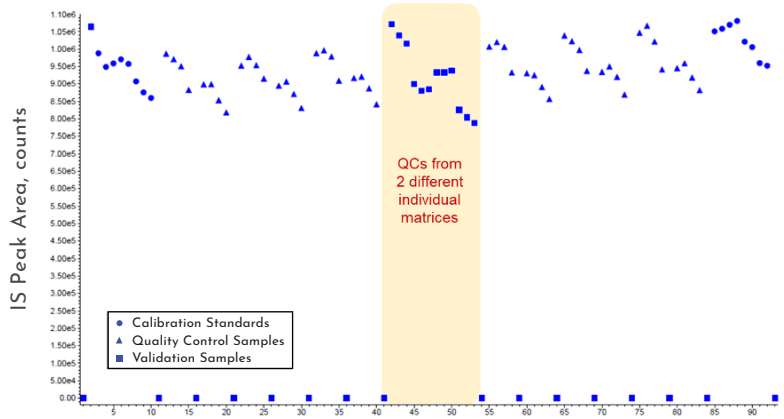

Fig. 3: Changes of IS response along with concentrations.

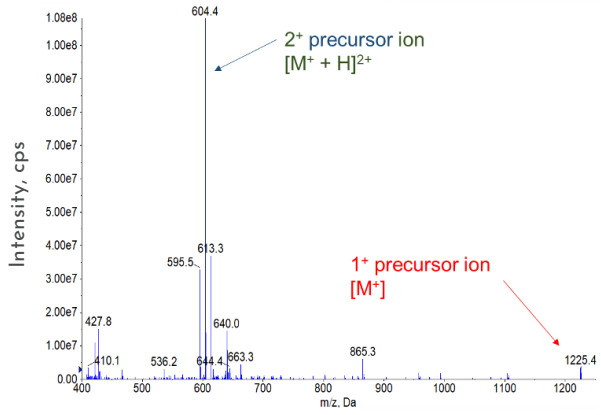

Fig. 4: Full Scan MS of a Quaternary Ammonium Drug (QAD)

Scientists injected two sets of calibration standards in the early method development stage in the order of low to high concentrations (Figure 5). Then, they observed that the IS responses of the singly-charged transition increased with the growth of the calibrators' concentration. Alternatively, the doubly-charged transition exhibited the opposite. In this case, the IS trend was fairly complicated as ion suppression should have been involved due to increased analyte concentration. But in the meantime, the protonation efficiency of the IS seemed to be impacted simultaneously.

In addition, this phenomenon was even more noticeable after surpassing 250 times (the fifth calibrator) the LLOQ (the first calibrator with the lowest concentration). As the calibration range could not be condensed, the solutions focused on optimizing ionization and protonation.

Figure 6, an accuracy and precision run, illustrates the IS response variation after optimization.

Scientists monitored two MRM transitions per each charge state (i.e., 2+ for the upper section and 1+ for the lower section of the IS plot). The results revealed acceptable and consistent IS responses for each transition with variation independent of analyte concentration. As suggested in the second case study, careful adjustment of IS concentration could have contributed to the improvement. Meanwhile, another key measure was the use of optimized mobile phases to properly control or stabilize the extent of protonation of QAD during MS ionization. The optimization of mobile phases and an understanding of electrospray ionization techniques are critical to achieving stable and consistent IS responses. Understanding the analyte's physiochemical property, the ionization technique, and instrumental parameters, including mobile phases, set an excellent example for the value of the scientist's experience and knowledge in finding the solution to develop a reliable and robust analytical method.

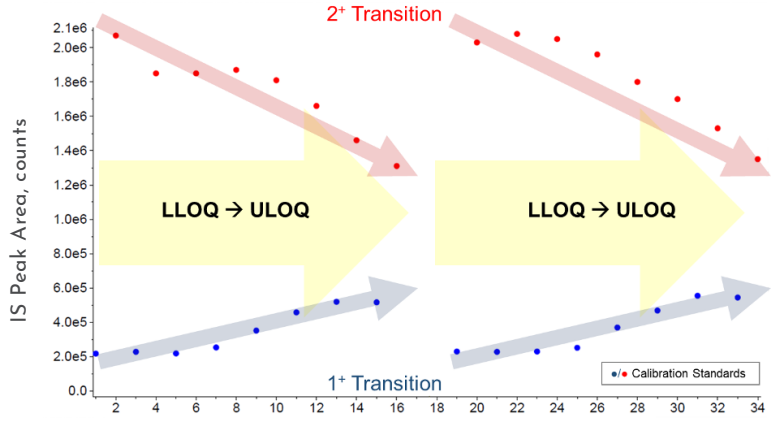

Fig. 5: Interactive trending of IS responses between the 1+ and 2+ charge state of the QAD precursor ions along with the change of concentration.

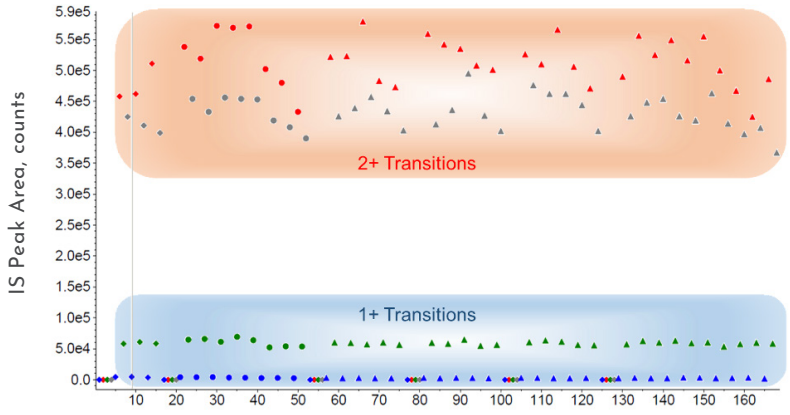

Fig. 6: Optimized IS responses for the QAD from the analysis of an accuracy and precision run

Cultivating reliable data

Incorporating IS in bioanalytical methodology is critical to ensure the reliability of the quantitation results obtained from the method. The publication of U.S. FDA Q &A regarding ISV serves as an excellent remedial to the currently available regulatory guidance for bioanalytical method validation and its application. This guidance also emphasizes the value in applying ISs and monitoring the IS response variation during bioanalysis.

Although no concrete assessment method or acceptance criteria is available from current regulatory guidance, a few takeaways are clear. First, each laboratory is responsible for properly defining IS variability acceptance criteria.

And secondly, repeat sample analysis rules and procedures and the subsequent decision rationale on reporting values within in-house Standard Operation Procedures or bioanalytical protocol. These in-house established guidelines help evaluate the potential impact on the data's reliability and constitute the unambiguous reassay or investigation plan to identify the root cause of such variation. In short, laboratories and drug developers alike must prioritize thorough documentation, methodology design and fundamental knowledge of the analytical technique applied in routine bioanalytical work.

References

1. EMEA Guideline on Bioanalytical Method Validation, effective 1 February 2012.
2. US FDA Guidance for Industry, Bioanalytical Method Validation, May 2018.
3. ICH Harmonised Guideline, Bioanalytical Method Validation, February 2019, Draft.
4. US FDA Guidance for Industry, Evaluation of Internal Standard Responses During Chromatographic Bioanalysis: Questions and Answers, September 2019.

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