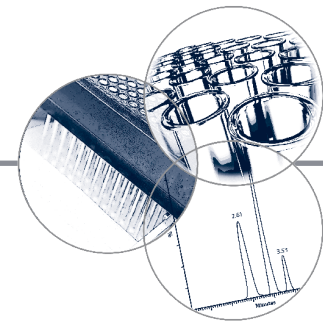

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How to manage having no incurred sample reanalysis evaluation failures

"…our determination to develop a robust, scientifically sound and high-quality method, as well as our employees' professionalism and our quality system, keep us away from incurred sample reanalysis failures."

Keywords: acyl glucuronides ■ advanced method development ■ bioanalytical guidelines ■ ISR ■ ISR criteria ■ ISR failure ■ unstable metabolites ■ US FDA ■ White Paper in bioanalysis

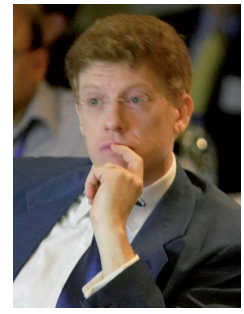
According to the 1992 Health Protection Branch Canadian Guidance, randomly selected 15% incurred samples were required to be reanalyzed as part of bioavailability and bioequivalence studies [1]. However, perhaps due to a lack of acceptance criteria, the Therapeutic Products Directorate of Health Canada revoked this requirement in 2003 and incurred sample reanalysis (ISR) was laid aside for approximately 3 years. In May 2006, at the third AAPS/US FDA Bioanalytical Workshop (Crystal City III) [2], the US FDA stated that the evaluation of ISR needs to be performed as part of both pre-clinical and clinical studies. Consequently, ISR became a highly discussed topic of multiple bioanalytical conferences, such as the AAPS Bioanalytical Workshop on ISR in 2008 [3] and the 2008 and 2009 CVG Workshops on Recent Issues in Regulated Bioanalysis [4–6]. The EMA has also included this requirement in their draft Guidance for Validation of Bioanalytical Methods [101].

Since the introduction of the ISR evaluation as a requirement, the treatment of a failure of this evaluation has become an international issue. Therefore, bioanalytical scientists are using their best scientific knowledge and common resources to avoid ISR failure during bioavailability and bioequivalence studies. Commonly known causes of failure are the occurrence of analyte and/or labile metabolite instability such as from conjugates and lactones, and matrix effects. In order to avoid ISR failure, precautions must be taken in the early stages of method development. Furthermore, although this evaluation is not meant to evaluate the analytical quality of a study, it is also important to have a solid GxP compliance program to ensure that all staff are properly trained and all written procedures are clear and followed, so as to eliminate this potential cause for failure.

During the last year, many ISR failure investigations, presented in conferences and/or published in scientific journals, demonstrated that the root cause of the failure was due to drug instability [4–7]. In order to mitigate this possibility in our laboratory, at the beginning of each method development a literature search for any known instability of the drug in solution or in matrix is performed so that the scientist will be able to avoid unstable conditions from the beginning of development.

Adjustment of the sample handling procedures may need to be performed in order to stabilize the analyte in solution and/or matrix. Common types of adjustments may include either performing sample extraction at 4°C, adding a preservative to the matrix samples or storing the solution or matrix samples at stabilization temperature. For example, simvastatin, as with other lactone compounds, is well known for being unstable due to hydrolysis conversion to its hydroxy acid form. Therefore, the sample extraction and sample storage temperatures were set at 4 and -80°C, respectively, preventing the opening of the lactone ring, which could possibly result in variability of back-calculated concentrations and, consequently, ISR failure.

Furthermore, as part of the extensive literature search, our scientists focus on the existence of all potentially problematic metabolites (labile or otherwise). Indeed, labile metabolites are known to cause ISR failure, however, there is also a possibility of interference due to nonlabile metabolites that are isobaric or have an isotopic contribution at the analyte and/or internal standard molecular weight [8,9]. Therefore, the presence of labile or other known metabolites is noted as well as their expected concentrations in the subject samples.



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Incurred sample reanalysis failure for the *p*-hydroxy-atorvastatin quantification was recently described, whereas the ISR for *o*-hydroxy-atorvastatin and atorvastatin did meet the acceptance criteria [10]. After investigation, it was found that the back-conversion of the *p*-hydroxy-atorvastatin lactone to its *p*-hydroxy-atorvastatin acid form was the cause of the ISR failure. Moreover, another ISR failure was demonstrated to be caused by the short-term instability of a conjugated metabolite, which converted back to the parent drug [6].

In order to perform stability evaluations that best represent the incurred samples, our scientists include the labile metabolite(s) in quality control (QC) samples used for all the evaluations; short-term, long-term, freeze–thaw and processed reconstituted stability as well as in whole blood stability for bioanalytical methods. Based on these stability results, the sample handling procedure may have to be refined to avoid back conversion of the labile metabolite(s) to the analyte of interest [8,9]. For example, the possible presence of rheinacyl glucuronide, which was found to be present in urine samples after a dose of diacerein [11], justified the inclusion of rheinacyl glucuronide in the stability samples during method development of the quantification of rhein in human plasma. Results demonstrated the occurrence of the back conversion of rheinacyl glucuronide to rhein in storage conditions determined to be optimal for rhein. It became necessary to evaluate multiple stabilization additives and storage conditions to achieve the stability of rhein acyl glucuronide in plasma as well. Finally, a storage temperature of -80°C was found to be optimal for the stability of rheinacyl glucuronide in plasma and the clinical site informed of the change prior to sample collection, eliminating the chance of ISR failure.

In the case of the quantification in plasma of tramadol and its metabolite, *O*-desmethyl-tramadol, there is a nonquantified, isobaric metabolite to *O*-desmethyl-tramadol: *N*-desmethyl-tramadol. The presence of *N*-desmethyl-tramadol may interfere with *O*-desmethyl-tramadol quantification, and consequently may cause variation in the calculated concentrations. Based on this information collected as part of the literature search, the selectivity of the mass transition of *O*-desmethyl-tramadol was optimized in order to avoid detecting *N*-desmethyl-tramadol. Moreover, the chromatographic conditions were selected to achieve chromatographic separation of *N*-desmethyl-tramadol and *O*-desmethyl-tramadol, making sure that our method is free of *N*-desmethyl-tramadol interference and accurate for *O*-desmethyl-tramadol quatification. In addition, for *O*-desmethyl-tramadol quantification, the concentration of *O*-desmethyl-tramadol glucuronide was found to be larger than expected, which gave us the opportunity to successfully perform reliable stability evaluations in the presence of the labile metabolite during method validation and demonstrate that there is no degradation of the *O*-desmethyl-tramadol glucuronide using our method assay conditions.

Since matrix effect is also a major cause of ISR failure, it is critical to avoid it for assurance of an acceptable ISR [12]. Our view is that there can never be too much investigation into the impact of the matrix on a bioanalytical method. Therefore, to evaluate the robustness of our methods, the method development group evaluates matrix effect over ten lots of matrices (male and female), as well as one hemolyzed matrix (7.5%) and one lipemic matrix, for plasma quantification. Furthermore, for these matrix lots, possible late peaks are monitored over five-times the method run-time and the ionization enhancement and/or suppression profile monitored by postcolumn infusion [13]. Chromatographic run-time may be adjusted or a flush gradient program may be added due to the presence of a late peak to avoid variability in analyte peak intensity. In addition, our scientists ensure that the peak of interest elutes far from areas where there is suppression and/or enhancement of the signal due to matrix. We have observed matrix effects coming from hemolyzed plasma in some of our human plasma method developments, for example, lamotrigine and morphine quantification. For these cases, the use of a stable-labeled internal standard was found to be an efficient way to avoid any effect on the analyte back-calculated concentrations.

Furthermore, thorough training, a GxP compliant environment and good communication during the method transfer step from method development to validation are also key for acceptable ISR evaluations. It was observed that good laboratory practice minimized the analytical errors that directly have an impact on ISR results [14,15].

Another interesting case was the discovery of clopidogrel transesterification. Owing to well-trained staff, the presence of an additional peak

noticed during the chromatogram review of the initial batches led to investigation. The cause was found to be an on-column conversion of clopidogrel acyl glucuronide, which occurs via a transesterification reaction mediated by methanol present in the mobile phase [16]. Therefore, chromatographic conditions were changed, using acetonitrile instead of methanol in the mobile phase. This fast response to a small discrepancy gave us the opportunity to quickly correct the method and consequently avoid ISR failure during samples analysis.

Training is part of a GxP compliant environment, but equally important are well written, clear procedures. In order to be able to reproduce the analyte concentrations, one must be able to accurately reproduce the steps used to acquire them. Therefore, our methods are written by the developing scientists and reviewed by several future users to make sure that all the information contained in the documents is complete and unambiguous. Then, a pre-study meeting is held with all participants to review the method and further clarify important points.

In our facility, the transfer of the developed method to the validation group is an important step. Therefore, at the end of method development a stressing procedure is applied in order to test and stress the newly developed bioanalytical method. This procedure is tougher and more rigorous than the typical validation requirements and is carried out to ensure that the method will easily and successfully perform during both the validation phase and sample analysis. Moreover, to ensure success, three batches are extracted concurrently by the scientist and the validation analyst to make sure that the critical points of the method are well known and will not cause unwanted issues.

In conclusion, our determination to develop a robust, scientifically sound and high-quality method, as well as our employees' professionalism and our quality system, keep us away from ISR failures. Continuing to avoid ISR failures is one of our main objectives, motivating us to constantly innovate scientifically and technologically.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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