



# 2009 White Paper on Recent Issues in Regulated Bioanalysis from The 3rd Calibration and Validation Group Workshop

The 3rd Calibration and Validation Group Workshop on Recent Issues in Regulated Bioanalysis was organized by the Calibration and Validation Group as a 1.5-day full immersion workshop for contract research organizations, pharmaceutical companies and regulatory agencies to discuss several 'hot' topics concerning bioanalytical issues and regulatory challenges. A consensus was reached among panelists and attendees on many points regarding method validation of small molecules.

The Calibration and Validation Group (CVG) is a Canadian-based, nonprofit scientific organization that partners with industrial, academic and regulatory bodies to provide education and forums for discussion on calibration and validation practices within the pharmaceutical community [1]. On 16th and 17th April 2009, the CVG held its 3rd Workshop on Recent Issues in Regulated Bioanalysis. The 3rd Workshop continued the discussion on the topics published in the White Paper of the 2nd Workshop [2], as well as other recent issues in the bioanalytical field.

The bioanalytical industry is governed by enacted laws and regulations issued under those laws by regulatory agencies of many different countries. These regulatory agencies often provide suggestions of practices that they will generally accept under these laws and regulations by issuing guidance documents. These guidance documents are often written in general terms and agencies leave the specifics of execution up to the industry. Furthermore, due to the fast-paced evolution of technology and regulatory requirements, the guidance documents are often not updated with enough regularity to reflect the current bioanalytical climate. Agencies use forums and workshops to disseminate new, and clarify old, expectations. These venues also allow members of the bioanalytical community to come together and attempt to harmonize their approaches to the guidances.

## Attendance

A total of 142 attendees from USA, Canada, Europe, Mexico and Asia were present at this workshop. These represented scientists,

managers, directors and executives from contract research organizations (CROs), pharmaceutical companies and regulatory agencies.

## Goals & objectives

The goal of the 3rd Workshop was to provide a forum to reunite, exchange knowledge and share ideas on bioanalytical issues and regulatory challenges faced by the bioanalytical community. The focus was primarily on small molecule. Several hot topics were addressed during the presentations and panel discussions:

- Regulatory updates from the US FDA, Health Canada Therapeutic Product Directorate (TPD) and UK Medicines and Healthcare Products Regulatory Agency (MHRA);
- New instruments and techniques. Can these new instruments and techniques really improve quality in regulated bioanalysis? What were the experiences of those present?
- The worldwide acetonitrile (ACN) shortage: what are the strategies for addressing this shortage?
- Manually modified chromatograms: following the release of the EMEA Draft Guidance for Bioequivalence [9], how are manually modified chromatograms reported? What are the procedures regarding manually modified chromatograms? Do users have a cutoff criterion that allows for a modification (e.g., 5% difference in response)?
- Hemolysis testing: what evaluations are required? At what level of hemolysis? How do users determine the level of hemolysis in incurred samples?

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**INCURRED SAMPLE**

Biological study sample taken during a clinical or preclinical study

**OVER-THE-COUNTER MEDICATIONS**

Drugs sold legally without a prescription from a health professional

**ANTICOAGULANT**

Agent used to prevent the formation of blood clots

- Metabolite testing: what evaluations do users perform when there are unstable metabolites? Do analysts perform these tests during method validation?
- Specificity in the presence of **over-the-counter medications** (OTCs): what are the procedures regarding the testing of OTCs? Do laboratories also test for contraceptives and their metabolites? Which ones?
- Use of variable injection volumes: do users test the impact of variable injection volumes? Do laboratories perform the test during validation?
- **Anticoagulant** counter-ions: are additional validation tests needed when changing anti-coagulant counter-ions? What validation parameters should be evaluated? Have any cases of true counter-ion impact been encountered?
- Stability in whole blood: what is the maximum processing time of clinical samples in a typical laboratory? Is this based on blood stability? How do laboratories perform blood stability?
- Investigating acceptable results: when data assay acceptance criteria are met, but a potentially questionable result is encountered, is an investigation needed?
- Incurred sample reanalysis (ISR): a follow-up after implementation by the industry;
- Workshop presentation summaries.

**What's new from TPD**

Eric Ormsby (Office of Science, Health Canada, TPD) announced that Health Canada is developing and discussing several proposed guidance documents. It was expected that they would be posted for external consultation in May 2009, however, as of December 2009, the guidance documents had still not been made available; they are expected in early 2010.

The Guidance for Bioequivalence currently exists in two parts: A (oral dosage formulations) and B (modified release formulations) [3]. These will be revised in order to combine them and to clarify some positions. New approaches for collecting and analyzing bioequivalence data will also be added. An additional guidance will also be prepared to address 'Report C' drugs, which include highly toxic drugs, drugs with nonlinear kinetics and combination drug products, amongst others. Overall, these guidance documents are set to replace ten currently existing documents.

In the Guidance for Bioequivalence, several modifications were discussed and a sampling will be presented. For example, currently, the guidance states that add-ons are permitted with the passing of consistency tests. However, no error guidelines were included. Add-ons will still be permitted, however, a Bonferroni adjustment will be introduced to keep the error rate during statistical analysis at 5%.

Furthermore, study design approaches are being reconsidered to allow for sequential and adaptive designs. The sequential design would be used when variation and expected mean differences are uncertain. This design would set a total number of subjects, N, but would allow stopping the trial at  $n < N$  if bioequivalence results were determinable. This is clearly an advantage with regards to time and costs associated with the study. The adaptive design is similar to the sequential design; however, the stopping rules would be based on the cumulative results collected.

On the subject of statistical analysis, it is proposed that mixed effects models be used, since they allow for all types of crossover designs and handle missing values.

Extreme values will be addressed. The current parametric approach, which is not tolerant to outliers, allows the removal of 5% of subjects with justification. One option would be to construct a nonparametric approach in order to reduce the influence of outlier subjects on bioequivalence. Another option would be to re-test the anomalous subjects with both formulations. If the new results are consistent with the proposed model, then the original results could be removed. Health Canada will maintain their position regarding ISR (i.e., it will not be required at this time. The requirement for steady-state studies for modified release formulations was questioned. Since steady-state studies do not appear to give any better comparison of the two formulations, it will be proposed that the requirement be removed.

The guidance documents are unclear regarding the use of metabolites to determine bioequivalence. The proposal is that since it is the formulated drug being absorbed, then this is the drug that should be assayed. Only if this is not possible would a metabolite, preferably the primary metabolite, be permitted, as long as this is justified and stated in the protocol.

The second guidance addressed was the Guidance for Inhaled Corticosteroid Products [4]. The proposed recommendation is to use eosinophil as the primary outcome measure.

Secondary measurements would include FEV1 and asthma symptoms. Furthermore, both test and reference products should have at least a 50% increase over the placebo. Finally, using a 90% confidence interval, the test versus reference results should be between 80–125%. In the case of steroid nasal products, the proposed guidance includes the possibility of requesting a waiver from safety and efficacy data if the ingredients in solution are identical to the Canadian Reference Product (CRP). The design would have 3 arms: CRP, test and placebo. Daily nasal symptom scores would be measured and there should be a statistical difference between the active products and the placebo. Furthermore, the mean changes from baseline should be compared for the test and the reference, using a 90% confidence interval. Results should be between 80–125%.

### A few recent FDA updates

Brian P Booth (Deputy Director, Office of Clinical Pharmacology, Center for Drug Evaluation and Research, US FDA) announced that a draft revision to the Bioanalytical Method Validation guidance (May 2001) [5] will start in the Fall of 2009. Several topics will be addressed as part of this revision, beginning with the acceptance criteria for ligand-binding assays. The proposal is to allow 20–25% deviations, for a total error of  $\pm 30\%$  ( $\pm 40\%$  LLOQ). Furthermore, 75% of the standards would need to be  $\pm 20\%$  ( $\pm 25\%$  at LLOQ). The QC acceptance would follow the 4/6/20 rule.

For small molecules, QC samples would need to span the dynamic range, where additional QC levels would be added if required. Carryover/contamination and **matrix effect** requirements will be addressed specifically. The new expectations with regard to ISR will be included, as well as those regarding the reporting of rejected runs, QC results and re-assayed samples. A tiered approach to ‘metabolites in safety testing’ (MIST) will be addressed [6,7]. The stability tests should mimic actual sample storage conditions and stability at  $-20^{\circ}\text{C}$  does not represent stability at  $-70^{\circ}\text{C}$ . Finally, the guidance regarding system suitability will be amended to what was presented as part of the Crystal City III White Paper [7].

There remain some topics with no consensus. It is still unclear what level of validation is needed for changes in anticoagulants or changes from one sex to the other per species in pre-clinical studies. The agency will also begin to look at the validation of assays for biomarkers.

Brian Booth revisited the FDA recommendations regarding ISR originally presented at the 2008 Boston Society for Advanced Therapeutics conference and outlined in the recently published White Paper from the 2008 American Association of Pharmaceutical Scientists (AAPS) ISR Workshop [8]. Some recent issues observed during audits included the re-assay of too few subjects, procedures with a cap on the number of samples re-assayed (5–10% were not necessarily included), criteria allowing at least 20% difference and instances where some replicates noted differences of approximately 30% with no subsequent investigation.

The Guidance for Industry: Safety Testing of Drug Metabolites was finalized in February 2008 [6]. The FDA/AAPS Workshop report from Crystal City 2006 proposed that a tiered approach be taken in validating assays for unique human metabolites that account for 10% or more of the parent AUC [7]. However, currently no detailed description is available. A possible approach may be to use limited validation of metabolites prior to human studies. The next step might be to identify unique human metabolites in first-in-man studies, which would require a toxicological evaluation. This step might necessitate a higher level of validation. If the outcome of the toxicological studies necessitates characterization of the metabolites in human, then the highest level of assay validation would be needed.

The FDA also updated the ICH S9 Guidance in 2009, which is of importance because it clarifies that the need for additional safety studies for the metabolite of a cytotoxic drug would generally not be needed [10]. The reason is that the toxicity of the parent and the metabolite would have been assessed in totality in the first-in-human studies.

### Bioanalytical ISR failure & scientific investigation into out-of-specification results

- Steve Lowes (Advion BioServices, NY, USA) used an example of a failed ISR evaluation to present one approach to failure investigations, their execution and the documentation of the results. The example involved an LC–MS/MS bioanalytical method applied to samples from two pharmacokinetic (PK) studies in separate pre-clinical species. The ISR experiment for one species passed predetermined reproducibility acceptance criteria but failed for the second

#### MATRIX EFFECT

Suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples

**PK REPEATS**

Samples designated by a pharmacokinetic scientist as needing to be repeated for poor pharmacokinetic fit

species. Results from the failed ISR experiment showed greater than 60% of PK repeats in excess of  $\pm 20\%$  from the original measured concentration. The study was put on hold and an investigation was initiated.

In the approach described, investigations are initiated when there is an unexpected result with no assignable cause. This may include sequential failed runs, high failure rate, a result not expected for a given sample (e.g., > LLOQ for a predose sample), evidence of carryover and so on.

The first phase of the investigation involves the initial evaluation of the data. The documentation was reviewed with a focus on calculation errors, sample preparation mistakes and instrument problems. At this point it is proposed that the project leader makes one of three determinations:

- Assignable cause found;
- Probable cause found;
- No assignable cause found.

The first scenario triggers a corrective action, approval from management, repeats as required and any training required to prevent recurrence. The second scenario allows for one single test to verify the hypothesis. Following the results of that test, one of the two other scenarios is triggered. Allowing a single evaluation experiment is a practical response to ensuring that a likely reason for the problem is tested but minimizes extrapolation to extensive troubleshooting. The third scenario triggers the second phase of the procedure – a full investigation.

In the ISR failure example described, the project leader chose the second scenario. A hypothesis involving inhomogeneous sampling of the study samples was tested by repeating the ISR samples in duplicate. Since results were not conclusive, a full investigation (third scenario) was triggered. At this point, representatives from other science and operations groups (e.g., quality assurance and scientific management) were introduced to the investigation process. All meeting minutes and communications were kept with the investigation documentation. An investigation plan was established, with sufficient flexibility to accommodate the progression of the investigation experiments.

The example given concluded that the ISR irreproducibility correlated to analyte instability. Further investigation suggested the

presence of species-specific, unstable, Phase II metabolites capable of degradation to the parent compound under freeze–thaw conditions.

Steve Lowes went on to describe the reporting and conclusion of the investigation using the ISR example. All investigation documentation and results were consolidated into one location to facilitate complete reconstruction of the investigation process. Assuming a root cause is found, a corrective and preventive action plan should be created to correct the problem and identify any deficiencies to the quality systems or personnel training programs. Finally, the impact of the cause on previous analytical results should be evaluated. If no root cause is found, it must be demonstrated that the method is reliable and that the unexpected results have no negative impact on the study data.

Steve received a question regarding the generation of additional ISR data and how that data was reported. He emphasized that although there is a general reluctance to generating replicate data, in some cases it is necessary in order to focus the investigation in the right direction.

One comment from an attendee highlighted that this investigation demonstrated the limitation of performing ISR experiments. Long-term stability and freeze–thaw tests are not required on incurred samples and the ISR evaluation as currently implemented does not take into account limitations due to these potential stability issues. Steve acknowledged the point; however he noted that the investigation was necessary to differentiate the stability issue from a potential assay reproducibility problem.

#### **Method specificity: a systematic approach for concomitant medications**

Xia Yin (Scientific Leader, Bioanalytical Development, Apotex Inc., ON, Canada) began by explaining that ideally, concomitant medications should typically not be used so as not to interfere with the bioanalytical methods in bioequivalence studies. Concomitant medications may be used in other studies where specifically detailed in an approved study protocol. However, Canadian [3], FDA [5] and European [9] requirements all dictate that methods should be selective even when these medications are present. Therefore, it is necessary to decide which medications to consider, how to ensure that the method is specific in their presence and what to do when they are present.

When evaluating which medications to include in the specificity evaluation, one must consider the situations where these compounds are used. They may be intermittently permitted within the study (e.g., acetaminophen in case of headache), specifically required by the protocol (e.g., co-administered compounds), expected in patients included within the study, oral contraceptives if female volunteers are permitted and other compounds that might be present but forgotten about during volunteer disclosure (e.g., caffeine, antihistamines and nicotine). When available, it is important to know the details of what was taken when, including: compound(s), formulation and dosage strength.

Several likely candidates to include in the specificity procedure are acetaminophen, oral contraceptives, nicotine and cotinine. Other candidates should be included if a risk assessment determines that the frequency of exposure is high or that the elimination is long, to reduce the impact if they were taken when not in the clinically controlled environment (e.g., prior to housing or during the wash-out phase).

Method development should take the physicochemical properties of the compounds into account when designing the method. Some approaches to eliminating unwanted interference from concomitant medications are to exclude them through extraction (pH selection and solubility), separate them chromatographically or exclude them through detection (masses different than the analytes of interest are not detected in MS/MS). One approach is to prepare a cocktail with all compounds and analyze it with MS/MS detection using all applicable mass transitions. This allows for the identification of where all potential interferences elute in relation to the analytes of interest. If they do potentially interfere during detection, then include those compounds in the extraction procedure to identify if they are extracted and to ensure that they do not affect quantitation.

In conclusion, it is recommended to plan for concomitant medications in advance. Contact the clinical facility to identify potential compounds that could be used. Furthermore, for some compounds it might also be necessary to consider their metabolites during analysis. It is also recommended to monitor trends of OTCs available in other countries to determine if they need to be addressed.

Attendees noted that this process was easier when healthy volunteers are used, since the use of concomitant medications is better controlled.

However, for studies including patient populations, this procedure becomes impractical, since each could be on a variety of medications. One approach in these cases would be to analyze pre-dose samples to detect any interferences.

### Challenges in the bioanalysis of endogenous drugs

Rupinder Phull (Director, Pharmacokinetics & Bioequivalence, Barr Laboratories, Inc., NJ, USA) introduced the topic of endogenous compounds, the challenges of developing such a method and accurately and reliably measuring the endogenous baseline levels, especially at low concentration levels (pg/ml). The current requirement for bioequivalence studies is to meet the 90% confidence interval criteria for both baseline corrected and uncorrected data.

The FDA provides no formal guidance on developing methods for endogenous compounds, however, the Bioanalytical Method Validation Guidance (May 2001) [5] states that “whenever possible, the same biological matrix as the matrix in the intended samples should be used for validation purposes.” Furthermore, if “stripped or altered matrix is used for preparation of study calibration standards and/or QC samples, stability evaluations still must be conducted in samples prepared in unaltered matrix.”

The challenges caused by these requirements are largely dependent on the class of compounds due to their physicochemical and solubility properties. Another challenge may be the availability of stripped matrix or matrix with sufficiently low baseline levels to complete the entire method validation and study sample analysis.

When selecting a suitable matrix for method validation and use during sample analysis, take into account the target assay range of the compound. One typical approach is to use unaltered or altered matrix for the calibrants and unaltered matrix for the QC samples. This approach would then satisfy the requirement regarding performing stability evaluations in unaltered matrix.

Unaltered matrix may be used when there is a large anticipated difference between baseline levels and levels in the study samples. It would be necessary to screen multiple donors in order to find sufficient amounts of matrix with low endogenous levels, to complete method development, validation and sample analysis. When those lots are detected, they may be used to prepare calibrants and QC samples.

There are two typical types of altered matrix: charcoal-stripped and solvent-treated matrix. Charcoal-stripped matrix can have batch-to-batch variability and trace amounts of the charcoal residue may interfere with recovery at low concentrations, which also affects precision and accuracy at the LLOQ. Solvent-treated matrix may also have batch-to-batch variability and may contain different solvents, which can result in different extraction efficiencies from altered versus unaltered matrices.

When unaltered matrix contains endogenous levels below or approaching the LLOQ, typical validation procedures are fairly straightforward. However, when endogenous levels approach mid-range levels, the question of how to prepare low concentration QC samples must be addressed and presents a practical challenge.

One approach is the standard addition method. The endogenous level is accurately determined by measuring six replicates of unaltered matrix against a stripped curve and assigning a value to the lot of matrix. The value is then subtracted from the desired concentration and the matrix is spiked with the difference in the concentration level.

Another approach is the over-spike method. In this case, the target concentration is spiked in addition to the endogenous level already present. All calibrants and QC samples must contain the same endogenous level and the curve will have positive intercepts.

An additional challenge to endogenous compound methods is that the use of stable-labelled isotopes as an internal standard does not always compensate for the difference in recovery between altered and unaltered matrices.

Other factors to consider include selecting the matrix to use for sample dilutions and whether the selected matrix can impact recovery. Furthermore, the data reported after baseline adjustment can result in negative values and how to treat the negative values that occur before the absorption phase and those in the elimination phase needs to be addressed in the study protocol.

### Labile metabolites and their potential impact on bioanalytical data reliability: typical case stories

Robert Massé (VP, Bioanalytical Division, Anapharm, QC, Canada) began his presentation by outlining some classes of drugs that pose a bioanalytical challenge. Some examples of these are hydroxyl carboxylic acid compounds, such as atorvastatin and simvastatin, which can

rearrange into lactones, poly-unsaturated compounds, such as acitretin and retinol, light- and temperature-sensitive drugs, such as isotretinoin and acitretin, and drugs with glucuronides, such as enalapril and perindopril, which can back-convert into the parent compound. One specific example of a challenging compound is perindopril, a member of the angiotensin-converting enzyme inhibitor family of drugs. During *in vivo* metabolism of this compound, the ester function is cleaved to form perindoprilat and both compounds can then undergo glucuronidation of the carboxylic acid moieties. The four resulting compounds will be present in different relative amounts during analysis, depending on the conditions of the method. Therefore, it is essential to take the structures and metabolic features of the compounds of interest into account when developing a method. Also, this implies that blood-collection procedures must be thoroughly assessed to ensure that the actual analyte concentrations at any time-point of the PK profile are not biased by such Phase II metabolite back-conversion phenomenon. An additional stability issue to be aware of during method development is the possibility that light-sensitive compounds may also demonstrate a time/temperature dependency. An example was provided where stability evaluations of a light-sensitive drug were performed over a 9–622 day period at -20°C. Results demonstrate that after approximately 110 days, there is significant degradation; however, when stability was evaluated at -80°C for up to 584 days, samples were still stable.

All of these issues need to be assessed when dealing with ISR failure. For example, an unstable Phase II metabolite could convert to the parent during sample storage. If this process is uncontrolled, perhaps by acidification/stabilization during clinical processing, reproducibility may be poor. Another technique being developed, which may help with stabilization during the clinical processing step, is collection using **dried blood spots** (note that this technique will be discussed further at next year's CVG workshop). However, although this technique might be useful from a practical point of view, research is still necessary to determine whether bioequivalence could be determined using this technique. It may also be necessary to prove reproducibility independent of stability, by assaying and re-assaying samples in consecutive runs.

Metabolite back-conversion could equally occur during sample processing, where, if nonreproducibility is demonstrated, it might be

#### DRIED BLOOD SPOTS

Technique where a drop of blood is collected onto filter paper, dried, then subjected to analysis

necessary to adjust extraction pH or to control the phenomenon by chromatographic separation. The presence of high metabolite concentrations, even if not directly detected, could cause suppression or enhancement of the analyte of interest. This phenomenon can be observed across timepoints and its relative intensity should vary with dose levels. Poor control of the separation of these compounds from the analyte could result in nonreproducible results.

Although there is no official guidance on ISR yet available from the regulatory agencies, there are strong arguments against including accuracy testing as part of the requirements. Proving accuracy is difficult to impossible. One possible method would be a standard addition technique, where samples are analyzed, then spiked and reanalyzed. Another option would be to analyze samples using two fundamentally different techniques (LC-MS/MS vs LC-UV). Both options are expensive, time- and resource-consuming and can be inconclusive. However, the use of stable-labeled isotope internal standards should help improve ISR variability, particularly in cases where matrix effects and ion suppression may have an effect on instrument response or when the disease state of the study participants may alter the biological content of the test matrix with respect to that of healthy subjects.

### **Challenges with method development, qualification & transfer: a CRO's approach to addressing the challenges**

Mario Rocci Jr (Executive VP, Prevalere Life Sciences, LLC, NY, USA) presented an overview of the relationship factors that are responsible for a successful client-CRO relationship. The success of a CRO depends on scientific expertise, operational efficiency, turnaround times, customer service, regulatory compliance and cost. However, the relationship between the CRO and the client is lucrative only if there is a shared sense of values and purpose, the client expectations are met, there is effective communication and each has the ability to effectively and efficiently resolve tough problems that arise during the partnership. In order to achieve this, it is important that all parties state expectations up front.

In order to clarify expectations of all parties *a priori*, as well as facilitate client and FDA inspections, it is recommended to create an individual project protocol, which would serve as a bridge between the client's and CRO's standard operating procedures (SOPs), allowing

for a customized, formalized document that indicates which of the CRO's SOPs are used or superseded.

One way to achieve operational efficiency in a CRO is to allow for fluid movement of personnel between method development and sample production groups. This accommodates the peaks and valleys that occur in method development and sample analysis work and allows for more varied work for the staff involved, thereby allowing them to improve their scientific development. More importantly, it also allows for the transition of scientific expertise across all parts of the project cycle.

Another way to be more efficient is to allow for real-time processes. By redefining the process to allow for real-time data flow, the data is scientifically and QA reviewed as it is generated, resulting in more efficient handling of QA observations and a decrease in report release turnaround times.

There are different types of method development projects. The first is *de novo*, where a method is developed 'from scratch'. The second is adapting a method from one matrix to another. One final type is adapting a method developed and used at least once at another facility for current use. This last type requires method transfer and qualification/validation.

Statistics were presented which showed that average method development and validation times decrease by 5 days when a deuterated standard is used, resulting in cost savings of thousands of dollars. Another way to increase the efficiency of method transfer and qualification is to accumulate as much information about the method prior to starting, in order to learn where the weak/stress points of the assay are. For particularly difficult methods, it is often cost effective to send an analyst to the client site, if possible, in order to gain experience with the method prior to transfer.

### **Considerations for the assessment of processed sample stability in bioanalytical methods**

Patrick Vallano (Bioanalytical Laboratory, Mylan Pharmaceuticals, WV, USA) presented this topic in an attempt to clarify a general confusion in the industry in regards to the requirements for processed sample stability. The following terminology was reviewed:

- Processed sample stability: generic term that refers to the stability of the sample as a function of time under a defined set of conditions after extraction is completed;

**AUTOSAMPLER STABILITY**

Stability of processed samples over time when stored in the autosampler during batch injection

- Post-preparative stability: term used in the Bio-analytical Method Validation guidance [5] for the stability of processed samples demonstrated using freshly extracted calibration samples;
- Processed sample integrity: typically refers to a length of time over which a complete batch of samples may be stored prior to injection. This is not usually verified using a freshly extracted curve;
- **Autosampler stability**: stability experiment where processed samples are stored in the autosampler;
- Reinjection reproducibility: typically a full batch of samples that is reinjected.

Patrick presented the argument that processed sample stability is not a true stability. Since the internal standard is present and could presumably degrade at the same rate as the analyte, the ratios and, therefore, back-calculated concentrations, would remain constant and stability criteria would be met despite the degradation. Following the release of the Bioanalytical Method Validation guidance [5], the expectation of the FDA was that QC sample stability was verified against freshly extracted standards. However, this position was reversed in the Crystal City III White Paper, which stated that the evaluation should be performed as needed and that freshly extracted standards were not required if performing autosampler reinjection reproducibility.

Currently, the industry appears to be struggling to decide the correct experiments to perform during method validation. Therefore, it is necessary to analyze the scientific factors that could affect quantitation and to take into consideration their effects on a sealed versus punctured sample container. Appropriate validation experiments are dependent upon the way samples are treated in each particular laboratory.

The first factor is that there may be a change in the relative responses of the analyte and internal standard (i.e., analyte and internal standard response changes at different rates). This could be due to degradation, adsorption, precipitation or the appearance of additive or multiplicative interferences. The effect is a change in response ratio and an error in back-calculated concentrations. However, the magnitude of the error in study samples depends on changes in relative response and how samples will be run in the production environment. For example, if it takes 120 h for the relative change in ratios to cause a

30% error to the back-calculated concentrations, but samples are run within a 5 h period (at which point, the error is only 1.5%), then there is no impact on the final results.

The second factor is that there may be a decrease in the response of the analyte and the internal standard (i.e., analyte and internal standard response changes at a similar rate). This could be due to degradation or the appearance of multiplicative interferences. The effect is a decrease in signal-to-noise (S/N) ratios, with a potential impact on the precision and accuracy at the LLOQ. For example, if the S/N ratio of the LLOQ is typically 10 and there is a 60% decrease of analyte response over 120 h, the precision and accuracy of the LLOQ may be affected and the regulated S/N ratio of 5 will not be met. However, if it is possible to analyze batches within 100 h and still obtain acceptable sensitivity, precision and accuracy at the LLOQ, then there is no impact on the final results.

Validation experiments should reflect how study samples will be treated and may be addressed using different validation evaluations. One situation was presented as a length-of-batch stability. This evaluation determines that samples are stable for at least the batch run time (samples injected at the end of the batch are as stable as those injected at the beginning of the batch). In this case, changes in response ratios will have a direct impact on the determined concentrations and freshly extracted standards should be used. Furthermore, if the total number of samples in a typical batch will exceed the maximum number of samples allowed on the autosampler, then the storage conditions of the extra samples must also be evaluated.

The second situation that was presented was 'processed sample integrity'. In this case, the length of time a batch can be stored prior to injection is evaluated and freshly extracted standards are not required. The rationale is that the degradation of all samples occurs at an equal rate. Therefore, it becomes necessary to simply verify sensitivity, precision, accuracy and lack of interference in the blank samples. It is recommended that a precision and accuracy run be used to assess this evaluation.

One final situation typical to the production environment is reinjected samples. Behavior of punctured versus sealed samples might not be the same, due to changes in solvent evaporation and oxidation caused by air exposure, for example. Therefore, the validity of reinjecting

previously injected (i.e., punctured) samples must be proven, taking into account the allowable procedures defined by management (e.g., full batch reinjection versus partial batch reinjection).

### **ISR, MIST & the EMEA**

Peter van Amsterdam (Head of Global Bioanalytics, Solvay Pharmaceuticals, Weesp, Netherlands) represented the European Bioanalysis Forum (EBF). This group was created following a meeting of bioanalytical scientists from approximately ten European pharmaceutical companies and CROs to discuss incurred sample reproducibility. The meeting was such a success that the focus was widened to a broad array of bioanalytical topics within pharmaceutical research and development. The EBF currently has 27 member pharmaceutical companies.

Following that initial meeting, the EBF continued to have internal discussions and surveys on many topics and has become a strong voice in global bioanalysis. Recently, they prepared a full paper, including recommendations for the bioanalytical community regarding incurred sample reproducibility [10]. Discussions centered around which samples were reanalyzed for the evaluation, how many samples were required, were individual samples or pooled samples allowed, when was the evaluation performed, what was the acceptance criteria and what was the reported result.

The final EBF recommendations regarding ISR are that ISR should be considered as part of method validation, although process checks are also valuable. It should be performed once per species or in case of a major method change, for preclinical studies and, for clinical studies, at least during first-in-human and patient-population studies, for special populations and for bioequivalence studies. The criteria for small molecules should be within 20% of the original value and within 30% for large molecules. Although not explicitly discussed, it was taken for granted that failures should be investigated. The number of samples should be left to the scientific judgment of the company and they do not recommend a fixed percentage. Finally, the only recommendation with regard to reporting the data from this evaluation was that it should be in the analytical report for bioequivalence studies. Following the release of the FDA, Guidance for Industry on Safety Testing of Drug Metabolites [6], members of the EBF answered some questionnaires in order to

share their practices on MIST implementation. One item that was raised was the terminology adopted by the guideline. EBF recommended that better definitions of method types be provided, based on the degree of validation and the purpose of use. Some suggestions were:

- Screening method: this would not usually be generated by bioanalysis departments and they have no acceptance criteria. Data would be qualitative and used for early decision making;
- Qualified method: method with appropriate level of scientific validation (accuracy, precision and stability) to allow documented and reproducible judgment based upon the concentrations of the metabolites;
- Validated method: method validated in accordance with the FDA Bioanalytical Method Validation guidance, supplemented with the recommendations from the recently published conference report.

The Concept Paper/Recommendations on the need for a Committee for Medicinal Products for Human Use Guideline on the Validation of Bioanalytical Methods [11] was reviewed by the EBF. Members agreed to send consolidated feedback to the EMEA regarding this concept paper, however, further discussions took place at the next EBF open meeting in December 2009, where several regulatory agencies were invited to present their points of view.

### **A bioanalytical view of GLP modernization**

Douglas Fast (Director, Bioanalytical Research Pharmacokinetics, Dynamics and Metabolism, Pfizer, CT, USA) presented a short history of GLP, discussed how some practices and techniques have changed since their inception and proposed some updates.

The GLP regulations contain high-level concepts, written very generically. However, over the last 30 years of use, professional organizations have helped shape the practical implementation of the regulations through the Crystal City conferences and numerous workshops.

Many things have changed within the industry since the regulations were first written. Detection instrumentation and practices have developed dramatically (e.g., MS vs UV and LC-MS/MS vs GC-MS), there are new HPLC column diameters and packing materials, computers are now required as more than just number crunchers, liquid handling has evolved

to include multiwell-plates, robots and pipettes, documentation has become electronic and global and the nature of the analytes tested has changed. Furthermore, there has been a change of approach to quality systems, where GLP and good manufacturing practice (GMP) requirements are beginning to converge, formal quality programs (Total Quality Management, International Organization for Standardization) are being implemented and global harmonization is desired.

However, what has not changed is the need to assure reliable data for regulatory agencies and, ultimately, the users of the medications. There is still a need to provide this data as quickly as possible, but at the same time, reduce costs from a business point of view. Finally, there is still a need for scientific advancement.

Keeping these ideas in mind, Douglas proposed some general ideas for the FDA to consider while they undertake the modernization of the GLP regulations. First, the regulations should not include recommendations. These should be limited to guidance documents or workshop reports. Modernization suggestions should not only come from the agency, but from consultation with industry as well. Furthermore, there should be a bias towards statistically-determined measures of quality (e.g., confidence intervals rather than acceptance limits, statistical criteria for regression models and weighting, for example). It was recommended that regulators be willing to accept standards-based, risk-evaluated systems for quality rather than depending on a quality assurance unit review of individual studies. A consolidation of nonclinical and clinical bioanalytical practices is suggested, as well as outlining whether studies conducted under regulations of other jurisdictions would be accepted as equivalent to those done under 21 CFR Part 58 [102].

Some specific suggestions were also presented. The role of Principal Investigator should be defined, as well as their role in multisite studies. The requirements for electronic records should be specifically referred to. The use of databases rather than sheets for the master schedule should be addressed, as well as possible maintenance of the master schedule by another department other than the QA unit. For test articles (including metabolites), the regulations should allow characterization testing appropriate to the stage of development. Wording should be clarified to allow data generated during a nonclinical study to be entered into electronic laboratory notebooks. Finally, the method of correcting or adding to contributing scientist reports needs to be addressed.

### Bioequivalence studies: a good clinical practice inspector's perspective

Louise Mawer (Senior Good Clinical Practice Inspector, UK MHRA, London, UK) presented an overview of the auditing process adopted by the UK MHRA. The most recent revision of the EMEA Guideline on the Investigation of Bioequivalence [12] is expected to be finalized by the end of 2009.

Louise outlined the types of inspections performed by the agency. These are routine good clinical practice (GCP) systems inspections, routine study-specific inspections, some voluntary overseas inspections, triggered inspections (within and outside the UK) and clinical laboratory inspections. The inspection process includes, from a clinical point of view, subjects (and their rights, including consent and confidentiality), investigational medicinal products and samples. From a laboratory point of view, points to be inspected would be sample chain of custody, analysis methods, results management, reports, facilities and equipment.

Some inspectional issues faced by inspectors are often related to local language, culture and travel. Attempting to ask questions, obtain information and occasionally provide bad news in another language can often lead to miscommunication and frustration on the part of the audited body as well as the auditor. Furthermore, the scales of compliance of foreign submissions can vary and the auditor is faced answering the questions of whether documentation omissions are compliant or not compliant.

To assist UK clinical laboratories in their understanding of the application of clinical trial regulations and GCP principles, Louise concluded her presentation with an overview of guidance for clinical laboratories pending publication on the MHRA website [13].

### When & how much do anomalous results need to be investigated?

Fabio Garofolo (VP Bioanalytical Services, Algorithmic Pharma, QC, Canada) presented five case studies, in order to stimulate discussions regarding the need to investigate acceptable, yet anomalous results. There is currently no guidance document available for bioanalytical requirements regarding investigating irregular results, although current GMP guidances can be adapted to the cause.

The Bioanalytical Method Validation guidance provides acceptance criteria for method validation and batch acceptance [5]. Furthermore,

the expectation is that if an irregular or anomalous result is obtained, an investigation should be initiated. However, results can meet acceptance criteria and yet, a hidden problem is suspected. A scientist is then faced with a decision of providing acceptable results versus higher quality results. Practical considerations when making this decision include the inability of a company to investigate every small deviation, which would require too much time and resources. Additionally, from a CRO perspective, sponsors are divided on the issue. Some prefer that every small issue be investigated. Others take the position that problems that do not affect batch criteria have no probable impact on a study. A balance must be struck.

The first case outlined a contamination issue that was isolated to one particular autosampler. The needle and loop capillary were worn out and, after replacing these pieces, the contamination disappeared. Four batches were analyzed using this piece of equipment, although only one batch failed to meet the batch acceptance criteria. The attendees agreed that all batches, even the acceptable ones, should have been repeated following the corrective action, in order to rule out any sporadic contamination that may have been undetected during the initial analysis.

The second case demonstrated a matrix effect in four out of 32 subjects. Internal standard responses of these subject samples were 55–75% of the response of standards and QC samples despite the matrix effect evaluation being acceptable during method validation. Therefore, to determine the impact of the phenomenon, predose samples of the applicable subjects were spiked with known quantities of analyte and extracted. Since the precision and accuracy of the back-calculated concentrations met acceptance criteria, no further investigation was warranted.

The third case involved results of the LLOQ standard that were near the limit of acceptability for precision and accuracy (116%). Furthermore, there was a high batch-rejection rate due to unacceptable deviations at the lower end of the **calibration curve**. The investigation uncovered that the amount of solvent added during the spiking process needed to be identical in all calibrants, even if it was always well within the 2% allowable limit. This procedure was adopted, new standards were prepared and precision and accuracy was greatly improved (106% at the LLOQ). More importantly, batch success rate was improved to 85%.

The fourth case demonstrated chromatographic anomalies. When using the MS detectors, electronic spikes were present in the chromatograms. These spikes were not limited to the retention times of interest, nor were they method-specific. Most of the occurrences were outside the retention time window and in the rare case where a peak of interest was affected, the sample was simply repeated. It was decided to open an investigation even though the impact of the spikes was minimal, since overall efficiency was reduced. The root cause was a miscommunication between the manufacturer and the technical support group regarding the latest recommendation for capillary adjustment.

The final case occurred during sample analysis, where there were unexpectedly low concentrations for the metabolite. However, PK outliers for the metabolite were not allowed and so there were no PK indicators provided to the lab to confirm this observation. Since the method employed only one internal standard in the quantification of the analyte and the metabolite, an additional stable-labeled internal standard was added to the method to better monitor the metabolite. Despite successfully validating the method using this second internal standard, it was noted during sample analysis that both the stable-labeled internal standard and the associated metabolite would periodically disappear. The root cause was multivariable and involved specific subject matrices being incompatible with specific lots of hexane over time. Therefore, despite batch acceptance and no outside input to confirm potential analytical issues, the investigation did uncover a method issue.

In conclusion, just because results are acceptable does not mean there may not be a problem. Quality and good science are a must.

### Panel discussions & consensus points

The second day of the workshop involved discussion on the various topics addressed in the presentations and proposed by the attendees and CVG members (listed in the 'Goals and objectives' section of this paper). Outlined below is a summary of those discussions, as well as any consensus reached by the workshop attendees.

#### ■ New instruments & techniques to improve data quality

The audience and the panel discussed several different technologies for improving data quality and efficiency of method development, method validation and biological sample analysis. Several

#### CALIBRATION CURVE

Set of several calibration standards of various concentrations. A calibration standard is matrix that has been fortified with a known quantity of analytes

techniques were discussed during the workshop, particularly small-particle columns, fused-core particle high-resolution columns, high-field asymmetric waveform ion mobility spectrometry and increased mass-resolution MS/MS.

The audience experience with small particles (sub-2- $\mu\text{m}$  particles) and fused-core high-resolution columns was extensive and very positive. It was confirmed that these chromatographic technologies were often already in daily use in the bioanalytical laboratory. It was mentioned that one of limitations of small-particle columns is that the tubing connections need to be optimal in order to minimize peak broadening due to dead volumes. Furthermore, although run times were significantly shortened using these columns, the practical limitation of the analysis time became the cycle time of the autosampler and wash cycle. This could be circumvented, using some autosampler models, due to a feature where a high-pressure valve allows injector washing to be concurrent with detection of the previously injected sample.

According to the audience, high-field asymmetric waveform ion mobility spectrometry has been used to reduce background noise, separate isobaric ions and eliminate problems of in-source fragmentation. High resolution has also been used with success by some workshop attendees (0.2–0.3 full width at half maximum) in order to decrease the noise through increased selectivity.

#### ■ Worldwide ACN shortage

Acetonitrile is a byproduct of acrylonitrile manufacturing for the automotive industry and others with large volume needs for manufactured plastic. There are no facilities dedicated to the production of ACN. Therefore, due to the recent economic downturn, there has been a global disruption in the supply of ACN.

Elution strength, low chemical reactivity, low viscosity and good miscibility with water have made this solvent a popular choice for LC mobile phases and biological sample preparation. It is also the preferred organic solvent for LC–MS/MS due to a lower background signal than methanol. Analytical departments are reliant on large volumes of ACN and have been required to find solutions to reduce their use of this solvent.

According to the audience, the most common way to address the shortage has been to move towards low flow rate chromatography, substitution with methanol, minimize waste, solvent

substitute for the needle wash and check alternate suppliers to ensure equivalent quality in order to have additional supplier options.

The option of recycling the solvent was being researched and indications are that this is a possible option using commercial solvent recycling units or other distillation systems. Care must always be taken to avoid lot-to-lot contaminants and residual impurity determinations and certification should be considered for use in supporting regulated work. Finally, for departments that do not require highly pure ACN, using a lower grade of the solvent should be considered.

#### ■ Manually integrated chromatograms

The EMEA issued a draft revision of the Guidance for Bioequivalence [12]. Within this guidance are expectations with respect to manually integrated chromatograms. It is proposed that the specific chromatograms that have not been automatically integrated must be listed, the change justified and the value before and after the modification must be reported. According to the audience, it is preferable to use a single integration method for the whole batch and, when possible, the whole study. However, although the integration algorithms have greatly improved with time, they are still not perfect and the scientific judgment of the user is still required. There may be cases where one or two chromatograms are not correctly integrated using the predetermined method, however, these should remain a very small percentage of the overall number of chromatograms. If the modification is judged scientifically necessary, it must be properly documented (person executing the modification and the justification) and approved by management. Although discussed, no cut-off criterion was recommended, since the decision should be based on scientific necessity. Furthermore, analysts are typically blinded to the randomization scheme. Therefore, the decision to perform a manual integration is based on the chromatogram and not on the final outcome of the study. Currently, none of the panelists are reporting manually integrated chromatograms in the final reports; however, results from manually integrated or modified chromatograms may be reported.

One attendee provided first-hand experience of chromatogram-integration challenges from a FDA audit. In the case cited, the integration method parameters for a specific analytical run were modified three to four times to achieve consistent and acceptable peak baselines across

the entire run. All changes were recorded in the audit trail. However, the analyst did not document a specific reason for each of the integration modifications. Even though the integration method was set while the analyst was blinded to the final concentrations, the inspector determined that the procedure was lacking sufficient and specific detail to justify the reiteration of integration parameters. Most attendees found this citation to be extreme and unnecessary. When setting the method parameters, one must be able to start somewhere and trial and error is often the case until the whole run is optimized. However, in a case where only one batch needs major adjustments and all other batches do not, it may not be unreasonable for an auditor to require additional explanations.

#### ■ Metabolite testing during method validation

The FDA Bioanalytical Method Validation guidance [5] includes the expectation that selectivity of the method is demonstrated. Typically during method development, consideration should be given to the presence of metabolites based on prior work and an extensive literature search. All the metabolites that can impact analysis are cause for concern, including those with the same mass as the analyte of interest and those that are unstable or have a possibility of intra-assay conversion to the analyte of interest. Tests may need to be performed during development, perhaps using incurred samples, to make sure that there is no co-elution that can impact quantitation.

The responsibility of conducting appropriate method development and method validation lies with the analyst. Appropriate documentation of strategy that is inclusive of known metabolites should be maintained for regulatory agency review. Potential assay problems from a metabolite (e.g., stability of a lactone) should be evaluated with method validation experiments that include metabolites (where available) to demonstrate transparency and control. It is difficult to standardize the required tests, because different metabolites can present unique analytical challenges.

One limitation faced by bioanalytical scientists is that the reference standards of the metabolites are not always available. In this case, it is suggested that as soon as incurred samples are available the practical impact of *in vivo* metabolites on the method should be scanned and the impact on the method assessed.

#### ■ Specificity in the presence of OTCs

The specificity requirements discussed above apply equally to OTCs. Since these compounds can be extensively used by the public and their use is not controlled at all times during the study (e.g., prior to initial dosing or during the wash-out period, when subjects are not housed within the clinical facility), regulatory agencies and sponsors sometimes require proof that the presence of OTCs does not impact bioanalytical method quantitation. The same applies to patient populations who often take several different medications.

One challenge of specificity testing in the presence of these compounds is that they can have many metabolites (e.g., aspirin results in at least 12 possible metabolites). These are not necessarily all adequately described in the literature;  $C_{\max}$  values are often unavailable for nonactive but present metabolites. Furthermore, reference standards are typically unavailable for the metabolites.

All these issues make it nearly impossible to perform a meaningful specificity evaluation in the presence of these compounds. Therefore, there was no consensus on whether this evaluation was performed or not and to what extent the metabolites were tested. Most OTC metabolites are not tested unless they are known to be the primary active component and present in high concentrations (e.g., salicylic acid). Some attendees questioned the need for this test given the selectivity of MS/MS.

One suggestion to circumvent this potential issue was to use the predose samples to detect any impact or to use the placebo-arm samples to represent populations with possible OTCs. One limitation of this approach is that these samples are typically only checked for interference and not for suppression or enhancement of the signal for the analyte of interest.

#### ■ Investigating acceptable results

All regulatory agencies provide guidance documents that include acceptance criteria to apply to method validation and batch acceptance. The criteria most often used in the industry are outlined in the FDA Bioanalytical Method Validation guidance [5]. However, although it is important to set limits of acceptability, good science must prevail. Therefore, although results may meet pre-established acceptance criteria, they must be reviewed with a scientific eye to ensure that there are no anomalies that could indicate a hidden problem. There should be a SOP in place that outlines when and how to perform any investigations and it should include not only out of specifications, but any anomalous results or trends in the data.

**ION SUPPRESSION OR ENHANCEMENT**

Analyte response suppression or enhancement caused by the co-elution of other species with the analyte of interest (i.e., metabolites, endogenous compounds, etc.)

Attendees indicated that, in general, most have a SOP such as this in place. One challenge that most face is determining when to stop an investigation. Since human nature is to digress when the first one or two specific hypotheses are rejected, it is important to remain focused on the issue at hand. Time and resources are often limited and if no root cause is found, then higher management needs to get involved to ensure that the ending of the investigation was valid. An equally important factor is the knowledge of when to initiate an investigation. Not all out-of-specification observations require an in-depth investigation; scientific judgment should be used.

There are many options to documenting and reporting investigations. There was no consensus from the attendees on how and where investigations were reported, however, depending on the severity of the issue, the final bioanalytical report or a separate investigation report were the popular options.

■ **Anticoagulant counterions**

Anticoagulants come in different salt forms (EDTA, K<sub>2</sub>, K<sub>3</sub>, Na, heparin and Li). Either one or many different anticoagulant salt forms may be used over the course of validation or sample analysis. There are contradictory results in the literature on the possible impact of anticoagulant counter ions on bioanalytical data [14,15].

Some regulatory agencies (e.g., Health Canada TPD) have started requesting proof that the method is not impacted when different counter ions are used.

A consensus was reached that a check should be performed to ensure that the anticoagulant and counterion described in the study protocol is consistent with the bioanalytical method validation experiments. If not, additional tests should be performed. Since no guidelines have been issued, there was no consensus on which tests are required to provide proof, however, it was agreed that at a minimum, a precision and accuracy batch is required. Some attendees performed precision and accuracy using six different lots of the different counter-ion anticoagulants. Others only performed one precision and accuracy run and the freeze–thaw or long-term stability evaluation. Still others redid all stability evaluations.

■ **Variable injection volumes**

Elevated injection volumes can impact quantitation using LC–MS/MS by saturating the detector, generating unacceptable chromatography (overload, split peaks, poor resolution),

causing ion suppression or enhancement or increasing the chances of column blockage over time. The audience's consensus was that the use of variable injection volumes should be investigated during method development and tested during validation. Validating a range of volumes for use was considered an acceptable procedure. Linearity, precision, accuracy and matrix effect were suggested as suitable tests of method ruggedness and tolerance of variable injection volume.

■ **Hemolysis testing during method validation**

Hemolysis describes the rupture of red blood cells and the release of hemoglobin into the surrounding plasma. Hemolysis constitutes a special case of matrix effect, since certain compounds may behave differently in the presence of red blood cells. Bioanalytical methods are optimized for specific matrices and some argue that hemolyzed plasma is a different matrix than plasma, resulting in potential differences in quantitation, stability and chromatography. Consequently, high numbers of hemolyzed samples may indicate a potential training issue with those who perform the blood collection.

The only agency currently including hemolysis testing in method validation as part of their guidance documents is Agência Nacional de Vigilância Sanitária (National Health Surveillance Agency Brazil) [16]. No other guidances exist requiring this information or outlining the requirements for testing.

No consensus was achieved regarding where and how to perform this testing. Some do it as part of method development only, others only do it if hemolyzed samples arrive for testing, but most attendees seemed to do a tolerance test as part of method validation. It was recommended to perform such a test at least during method development if it is not being carried out in validation. Most perform the test using simulated hemolyzed plasma (2–5% of lysed red blood cells added to plasma), however, it is difficult to determine the level of hemolysis in incurred samples, so this value may be over- or under-evaluated.

■ **Blood stability testing during method validation**

The FDA Method Validation guidance states that drug stability should be proven during sample collection and handling, where the drug is still in blood form [5]. Since analytical methods

are typically designed for plasma samples, there is a challenge to performing this test in a representative fashion. Typically, an aliquot of samples containing the drug is spun down immediately to obtain plasma at time zero and another aliquot is spun down at a later time (e.g., after 2 h), to obtain the stability sample. This does not provide a true stability, but a 'sample-collection process stability.' The results are qualitative, since the partition between blood cells and plasma is often unknown. In order to provide a true stability result, two methods for each analyte would need to be developed: one in plasma and one in blood, and this is not a practical solution.

It is recommended to perform the sample collection-process stability during method validation, taking care to use the minimum amount of solvent possible during sample preparation in order to avoid hemolyzing the blood (verify the possibility of using a saline solution). Following the results of this experiment, it is important to transmit the method details to the clinic so that they can adjust their collection method if required.

#### ■ Incurred sample reanalysis

An update on the application of ISR was requested from attendees. There was still no consensus on the number of samples assayed, with some basing the decision on the size of the study (5% for large studies and 10% for small studies), and others selecting a specific percentage in all cases (5, 10 or 10% up to 1000 samples and then 5% of the remainder). Typically, the number of samples reanalyzed fell between 5 and 10%.

There was also no consensus if this evaluation was performed only for studies destined for FDA submission. This appeared to be sponsor-dependant. However, Brian Booth, the FDA representative, made it clear that in the case where there is an FDA submission ISR results would likely be expected in the submission.

Finally, ISR failures experienced by the attendees so far seem to indicate that the problems encountered are not due to method reproducibility (the original purpose of the evaluation), but to matrix effects or stability issues.

#### Conclusion

Several hot topics were discussed during the 3rd Workshop on Recent Issues in Regulated Bioanalysis. All the speakers were industry experts and regulatory representatives and formed the panel that was given the mandate to achieve an initial consensus on several key points, which was then presented for further discussion with attendees.

Consensus was achieved for the following:

- Manually integrated chromatograms should occur infrequently since it is important to correctly set the integration method optimally for all samples in a run. However, good scientific judgment must be used in the cases where a change is required and they must be documented with management approval. Manually modified chromatograms are not generally reported in the final report but they may be reported if desired;
- The impact of the presence of metabolites on quantitation should be determined as much as possible during method development and if an issue with a specific metabolite is uncovered, then the impact should also be assessed during method validation in order to minimize the chances of a problem with incurred samples;
- A SOP must exist outlining the procedure for investigations. Investigations should not only be initiated in the case of out of specifications, but also if there are any anomalous results or unexpected trends in the data;
- The anticoagulant used in the study protocol should be consistent with the validation, otherwise additional testing should be done;
- The effect of hemolysis should, at a minimum, be investigated during method development;
- Blood stability testing is a collection process stability experiment and should be performed during method validation;
- The number of samples re-assayed for the ISR evaluation should be between 5% and 10%, based on the attendees' experiences.

No consensus was reached on the following:

- The specific tests required during method validation for metabolite testing, anti-coagulant counter-ion testing, hemolysis testing and blood stability testing;
- The need to perform specificity in the presence of OTCs and the ability to make it a meaningful evaluation when metabolites of these compounds are taken into consideration;
- Where to report the results of investigations;
- The need and method to perform tests to validate a range of injection volumes during method validation.

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