The 4th Calibration and Validation Group Workshop on Recent Issues in Regulated Bioanalysis, a 2-day full immersion workshop, was organized by the Calibration and Validation Group. Contract research organizations, pharmaceutical companies and regulatory agencies came together to discuss several ‘hot’ topics concerning bioanalytical issues and regulatory challenges and to reach a consensus among panelists and attendees on many points regarding method validation of small and large molecules.

The 4th Annual Workshop on Recent Issues in Regulated Bioanalysis and Global Harmonization of Bioanalytical Guidance was hosted in Montreal, Quebec, Canada on 22 and 23 April 2010. This workshop was organized by the Calibration and Validation Group (CVG) and chaired by Fabio Garofolo. CVG is a Canadian-based, nonprofit scientific organization that partners with industrial, academic and regulatory bodies to provide an educational forum for the discussion of calibration and validation practices within the pharmaceutical community [1].

This year the workshop was expanded to include, for the first time, a section on large molecules. As in previous years [2,3], the discussions resulted in consensus on many of the issues raised regarding the bioanalysis of both small and large molecules.

Although consensus has always been a goal of the workshop, it was taken a step further this year, when the main theme surrounding this workshop was the global harmonization of bioanalytical guidance. Though workshops of this type have always attempted to harmonize approaches within the industry, release of the draft European Medicines Agency (EMA) guideline on Validation of Bioanalytical Methods [4], and the news at last year’s CVG workshop that the US FDA was revising their guidance [3] brought harmonization to the forefront of the industry’s attention. The needs of the companies governed by these guidelines were thoroughly discussed by the professionals and the representatives of multiple regulatory agencies in attendance in order to determine how best to proceed on a truly global scale.

**Attendance**
A total of 281 delegates from North America, Latin America, Europe, Africa and Asia attended this workshop representing 165 companies working in the field of regulated bioanalysis. Scientists, managers, directors and executives from contract research organizations (CROs), pharmaceutical companies and regulatory agencies were represented.

**Goals & objectives**
The goal of the 4th 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 discussion included issues on small and large molecules.

Several ‘hot’ topics were addressed during the presentations and panel discussions:

- Regulatory updates from the FDA, EMA, Health Canada Therapeutic Product Directorate (TPD) and UK Medicines and Healthcare products Regulatory Agency (MHRA);
- Review of ANVISA Resolution RE no. 899, 23 May 2003 [5], pertaining to method validation and sample analysis;
- CRO’s certification by ANVISA, in order to perform bioequivalence (BE) studies for submission in Brazil;
- Is global harmonization of bioanalytical method validation and sample analysis possible? What is the best/fastest way to achieve it? International Conference on Harmonization (ICH) Guidelines or Organisation for Economic Co-operation and Development (OECD) Guidelines?

Affiliations continued overleaf.
(OECD) Guidelines or a Bioanalytical World Congress with the active participation of both industry and regulatory agencies?

- Lipemic and hemolyzed plasma samples: is it allowed to just define these samples as ‘not reportable’ values or is further method development needed to analyze them?
- Statistical challenge to the current validation criteria: how well does presudy validation predict the quality of individual incurred sample results?

**Urine and tissue analysis: tissue analysis is not controlled until the sample is homogenized and urine analysis is not controlled during sample collection and sampling. What is your approach?**

- Multi-analyte assays, repeat analysis and failed runs: if repeating for failure on one analyte, do we need to regress and report repeat analyte concentrations that passed previously? At what point does an investigation for an ‘assignable’ cause for failed runs end, and the outcome can be deemed ‘non-assignable’?

- Preparation of calibration standards: what is the best technique to use between preparing calibration standards in bulk versus preparing them fresh? Is it possible to agree upon a standard uniform approach?

- Critical reagent stability and assignment of expiration dates for large molecule methods: when conjugates from reference material are produced, how do you establish the new expiration dates?

- Sample handling from receipt to disposal: regulatory agencies constantly target sample handling during inspections: collection, storage, chain of custody in a laboratory information management system (LIMS) system, and sample labeling. What are the many issues in sample handling, monitoring and tracking and how do we fix them? How can we improve sample tracking to better meet the requirements of the regulatory agencies?

- Challenges in endogenous analyte assays (e.g., vitamins, hormones and coenzymes): what are the implications for regulated biomarker bioanalytical methods? What are the regulatory agency perspectives? Are bioanalytical method validation guidelines good enough for endogenous analytes?

- **Carryover** criteria: is it feasible to perform a sample-by-sample assessment of potential impact if carryover greater than 20% of the LLOQ is suspected? What does this measurement estimate? Are new carryover criteria (i.e., 5% possible contribution from previous injection) accepted by regulatory agencies? What are industry standards after Crystal City III and the use of nonrandomized sequences?

**Global harmonization of bioanalytical regulations**

Beginning with the first Crystal City conference and subsequent report [6], the FDA has been at the forefront of providing guidance on bioanalytical method validation. This guidance comes in the form of an official FDA guidance document released in 2001 [7], as well as subsequent conference reports [8,9] which outline the Agency’s current thinking on various issues. A recent example of this is the workshop report discussing the current expectations regarding incurred sample reproducibility (ISR) [9].

However, technology has moved forward since the release of the official FDA guidance document, and conference reports were never intended to represent official Agency policy. Brian Booth of the FDA updated the attendees on the guidance revision process, stating that a draft is expected to be released in 2011. In addition to the recommendations already outlined at the 2006 Crystal City conference [8] and the ISR workshop [9], the content will also be updated in order to expand the section on ligand-binding assays (LBAs), with recommendations expected to be similar to the LBA white paper [10]. Acceptance criteria for LBAs are anticipated to be that 75% of standards must be within 20% of their nominal concentrations (25% at the LLOQ), and the total error cannot exceed 30% (40% at the LLOQ). The guidance is also expected to discuss the number of QC samples required to span the dynamic range of the standard curve, and when partial revalidation is required if additional QC levels are added to sample analysis runs. Furthermore, the stability section is likely to stipulate that evaluations be performed at the actual temperature that the samples are being stored at, implying that stability proven at -20°C does not prove stability at -70°C. Furthermore, stability data will be required for LBA reagents.

Some other topics under consideration are the need and extent of validation for biomarkers, microsampling, endogenous compounds and diagnostic kits.
The attendees’ comments regarding this guideline strongly suggested that any necessary evaluations should be scientifically driven, not just blanket requirements. In response, the Agency reiterated that the prevailing thinking was to avoid being too prescriptive.

To complement the FDA guideline, the EMA released its own draft guideline [4]. This guidance was created based on a need outlined in a concept paper [11] containing recommendations for a guideline for the validation of bioanalytical methods, which had so far been lacking for the European community.

Jan Welink, representing the EMA, presented an introduction and status report on this guidance document. He explained that the EMA organized a drafting group headed by a rapporteur (The Netherlands) and a co-rapporteur (France), whose roles were to write the document with input from the other member states. After circulating the document for internal comments for approximately 1 year, a draft was released for public consultation in December 2009 (the deadline for public comments was 31 May 2010). Welink then presented a summary of the various sections of the guidance, stating that in some cases the drafting group had already recognized the need to adjust certain sections (e.g., LBAs). In addition, some requirements such as not allowing pharmacokinetic (PK) repeats will be clarified as applying only to BE studies.

Peter van Amsterdam presented the European Bioanalysis Forum’s (EBF) perspective on the EMA draft guideline, and some of the comments that they submitted during the public comment period, gathered from its members. The main issues the EBF presented were matrix effect, study reports, legal basis, ISR, definitions of complete/partial/cross validation, stability and accuracy. For example, EMA specifies that hemolyzed and lipemic lots of matrix must be tested as part of the matrix effect evaluation. The EBF proposed that the test is not always relevant, depending on the molecule and expected use of the method, and therefore should be an option. Furthermore, the EBF recommended that the EMA use the EBF published approach to ISR evaluations [12].

The differences between the EMA and FDA guidance documents have been previously discussed [13] amid growing concern from international companies wishing to submit studies to both the USA and European countries. When one adds the ANVISA guidelines [5,14,15] into the mix, as presented during the workshop by ANVISA representative Arthur Leonardo Lopes de Silva, or the fact that more and more studies are being conducted in India and China, the issues can get even more complex. Harmonization would resolve all these complex issues.

Surendra Bansal presented an introduction to harmonization which touched on many of these points. He pointed out that currently only the FDA [6–8] and EMA [4] provide comprehensive guidance for bioanalysis; other guidances were either limited abbreviated instructions [5,14,16–18], or references to other guidelines [16,19]. He further discussed that besides bioanalytical guidance, other regulations/guidances such as Good Laboratory Practices (GLPs), Good Clinical Practices (GCPs) and guidance on analytical instrument qualification have a profound impact on how bioanalysis is conducted and would require global harmonization. He concluded with the point that bioanalytical science is universal, work is global, but regulatory guidance is national.

In response to these challenges, three editorials [20–22] and one open letter [23] have already been published strongly recommending harmonization. Due to the globalization of the drug market, it is in everybody’s interest to harmonize the requirements. Besides the different associations backing this recommendation (American Association of Pharmaceutical Scientists [AAPS], Applied Pharmaceutical Analysis [APA], CVG, EBF), Jan Welink also addressed this topic in his presentation, stating that harmonization is desirable, but will be challenging when the needs of all the different agencies are taken into account. Similarly, Viswanathan mentioned during the FDA presentation that not only is harmonization necessary where different countries’ guidelines would be similar in content, but also globalization of the guidance, where one document would be used by all countries. Eric Ormsby of the TPD recognized the same need, not only facilitating submissions for the industry, but also from an agency point of view. He likened it to the creation of the Common Technical Document created by the ICH, which has facilitated review for all agencies. Finally, da Silva of ANVISA stated that his agency also supports this idea. The idea was unanimously endorsed by panelists and attendees during the panel discussions.

Therefore, a consensus was reached at the workshop. Ideally, a nonprescriptive, science-based document that describes the rationale for its elements should be developed for the

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**Key Term**

**Matrix effect:** Suppression or enhancement of ionization of analytes by the presence of matrix components in the biological samples.
global community to provide guidance to ensure the development and use of accurate bioanalytical methods that generate reliable data. It will be important to look at the global picture when drafting the guidance; however, it must be accepted by all countries for the initiative to ultimately be successful. Many roadblocks are anticipated (e.g., variable scientific experience, politics and language interpretations), but harmonization is a crucial step for the pharmaceutical, biotechnology and CRO industries.

This rallying call will outline a plan to create an all inclusive Global Bioanalytical Consortium (GBC) consisting of scientific associations with worldwide influence (AAPS, APA, CVG, EBF, among others) and industry to merge the FDA, EMA, ANVISA, Health Canada and all the other guidances in order to create one, unified document that can be presented to the regulators in various countries. The expectation is that the document will be influential enough that all agencies will want to adopt it, and therefore harmonize their inspection approaches.

Regulatory agency presentations & updates
In addition to the remarks presented by representatives of the FDA, EMA, TPD and ANVISA, discussed above, representatives from the MHRA and TPD also presented additional topics important to their respective agencies.

- TPD regulatory harmonization
Eric Ormsby (Manager, Office of Science, Health Canada: Therapeutic Product Directorate) discussed not only harmonization of the validation guidance, but the importance that is placed on international companies following the ICH GCP regulations. These regulations are mandatory for clinical trials run in Canada, however the TPD reviews submissions from other countries as well, some of which do not adhere to the ICH GCPs. This creates challenges for the agency when reviewing these studies. Furthermore, Canada is a signatory of the OECD, meaning that Health Canada must recognize and implement their Council Acts. One of these is the OECD GLP principles. Therefore, in 2009, Health Canada developed a GLP policy in the form of a guidance document [24]. This policy designates the Standards Council of Canada (SCC) as the country’s GLP Monitoring Authority, and is responsible for auditing and accrediting GLP facilities located in Canada. Nonclinical studies performed at these facilities will be eligible to be submitted for review in other OECD countries with the certainty that GLP principles were adhered to. Finally, Ormsby briefly discussed harmonization of the BE guidances. While he did say that some aspects could be harmonized (e.g., analysis of data with sound statistical methods and programs, study design to protect Type I error at 5%), there would be some portions that would need to remain country specific (e.g., reference products and BE standards).

- MHRA update
Jason Wakelin-Smith (GCP Inspector, UK MHRA) updated the attendees on some of the guidelines relevant to BE investigations/inspections performed by the MHRA. The first is the European Bioequivalence Guideline [25], which was adopted by the Committee for Medicinal Products for Human Use (CHMP) in January 2010 and became effective in August 2010. This guidance outlines the Committee’s position (i.e., those involved in the assessment of European BE applications) regarding adherence to GLP, GMP and GCP in BE investigations. For example, GMP regulations are applied for labeling and unequivocal identification of product administration. Also, although the GLP regulations technically do not apply to BE studies, the guidance indicates that their spirit should be adopted; further clarity for MHRA expectations in this aspect is provided in a local guidance available on the MHRA website [26]. Other guidelines existing within the MHRA describe Phase I accreditation [27] and the procedure for responding to inspection reports [28]. One final guidance document presented was the MHRA guidance on the maintenance of regulatory compliance in laboratories that perform the analysis or evaluation of clinical trial samples [26]. This document contains 23 sections related to the planning, conduct, reporting and archiving of clinical laboratory studies from the point of contract set-up to the reporting of the results. It outlines the fundamentals of good practice directed by GCP for the laboratory, with a focus on both the patient and the data. However, the take-home message from this presentation is that it is necessary to understand the GCPs and how they apply to the quality system in place. A common non-compliance issue during MHRA inspections is that a claim of GLP compliance is made where a GCP compliance claim is expected.
The second topic addressed by Wakelin-Smith was the MHRA approaches to inspections. They have two types of inspections: routine (risk-based) and triggered. Risk-based inspections are proportionate relative to compliance risks; those that demonstrate the lowest level of compliance will be inspected more frequently. This approach includes elements of self-assessment and self-monitoring. Routine and triggered inspections are appropriate particularly to the BE setting; routine inspections are proposed for those organizations who submit data most commonly for license applications. Whilst this does not preclude a triggered inspection (i.e., an inspection ‘for cause’ based on particular questions or concerns relating to the submitted data), it is anticipated that a routine program may reduce inspection burden for organizations by making system-based compliance information available within and between European Regulators and those with whom agreements are in place. Factors that are considered for routine systems inspections are: the frequency the facility is named on submissions, the number and nature of the questions that may arise in current or previous submissions, the quality of the data and responses provided to the agency, and previous inspection history.

**Biosimilars, biologics & large molecules**

- **Biosimilars & the guidance documents that support them**

Representatives of both the MHRA and Health Canada’s Biologics and Genetic Therapies Directorate (BGTD) presented an introduction to the approaches each agency is taking to inspections. Louise Mawer (Senior GCP and GLP Inspector, UK MHRA) defined a biosimilar, from European Council Directives [29], as a medicine similar to a biological medicine already on the market, having different starting materials and/or production pathways but the same activity (though the mechanism of action may differ) and same dose as the marketed reference product.

There are unique considerations when working with biosimilars: there cannot be any substitution of product without the consultation of a physician or the patient being made aware; and changes in the product, process or purity can have a major impact from a clinical point of view.

The European Directives and guidance state that these products must be essentially similar to the marketed reference product and the active constituent must have a well-established use that is effective and has a known safety profile. The submission process is essentially the same as for small molecules, however immunogenicity and comparability tests are always required and there may be a need to provide additional preclinical and clinical data depending on the comparability test results. If the therapeutic use of the two medicines differs, then extra data must be provided. Mawer indicated that EU guidelines exist [30] to clarify expectations regarding the immunogenicity tests required, as well as different quality issues that are important to the process (e.g., manufacturing process, comparability exercises and choice of reference product). Some analytical considerations she presented included ensuring sufficient method sensitivity, the statistical assessment of results and ensuring that the methods used for comparability tests are adequate for determining any potential differences between the two products.

Patrick Bedford (Senior Policy Analyst, Office of Policy and International Collaboration, Health Canada: Biologics and Genetic Therapies Directorate) expanded on this topic, from Health Canada’s perspective. Guidelines released in 2010 state that the demonstration of similarity between the test product and the reference biologic drug can be the basis for accepting a reduced clinical data package (as is the case with the European submissions) [31]. Some of the drivers for the submission of these products in Canada are that an increasing number of biologic drug patents are expiring, their review is scientifically feasible with advancing technologies and the public demand is increasing for affordable alternatives to innovator biologic drugs. The underlying principles of the Canadian guidance document include the sponsor being responsible for providing the necessary evidence of safety, quality and efficacy to support all aspects of an application for authorization. The basis for accepting a reduced nonclinical and clinical data package is supported by existing regulations, and hinges on demonstrated similarity between the product under consideration and the reference biologic drug. Once a Notice of Compliance is issued for a subsequent entry biologic it is considered to be a new biologic drug, and is regulated accordingly.

A key point to note across both presentations is that biosimilars are not considered generic molecules.
**Key Term**

**Calibration curve:** A set of several calibration standards of various concentrations. A calibration standard is matrix that has been fortified with a known quantity of analyte(s).

- **Large molecules: ligand-binding versus chromatographic assays**
  
  Joseph C Marini (Associate Director, Centocor Research and Development, Johnson & Johnson) presented the attendees with an overview of LBAs, their differences from chromatographic assays and the challenges involved during method development and validation. LBAs are used to detect and measure a macromolecular interaction between a ligand and a binding molecule. The standard curves used in these methods are typically nonlinear and relatively limited in dynamic range. The imprecision is greater than for chromatographic assays and reagents are unique to each product tested. There are several advantages to LBAs, including high sensitivity due to the high affinity of the binding agents, high specificity and the samples can be assayed without separation from the matrix. However, there are some disadvantages to these assays as well. They are time consuming to develop and validate (6–12 months), reagents are difficult to produce and scale-up, and because lot-to-lot variations can severely affect assay performance, reagent use needs to be closely monitored. Different LBA platforms exist, and Marini showed some examples (ELISA, DELFIA and Gyrolab™). One point to consider when using LBAs is that there is no internal standard. Furthermore, there is one standard curve and one set of QC samples per plate, and consideration should be given to organizing samples so that there are complete subject sets per plate, and hence, per batch. Finally, additional sample dilutions are routinely required; robotic sample dilution is one option for dealing with this issue.

  Conversely, Steve Lowes (Senior Vice President – Scientific, Advion BioServices, Inc.) discussed the application of LC–MS technology to large molecule biomarker assays and considerations that should be given to defending the data in a regulated bioanalysis environment. He pointed to the advantages of LC–MS including that it is a well-established technology amenable to a wide range of analytes, a solid regulatory framework is in place to support the use of LC–MS, it afforded high selectivity, and the inherent multiplexing advantage of chromatography. The challenges and disadvantages of LC–MS were then presented including large molecular weights of protein biomarkers, which are typically heterogeneous, as well as a frequent lack of available qualified reference standards. One approach is to use a combination of analyte digestion of a diagnostic peptide combined with immunoaffinity capture of the protein and/or released peptide.

  Biomarkers are endogenous compounds, and this presents the most profound difference from the basis around which regulated bioanalysis guidance is written. Consideration must be given to this fact when deciding on the approach used to analyze these compounds. The standard addition approach to handling endogenous presence of analyte was the first option discussed, although it was highlighted that this relies on calibration curve extrapolation to determine endogenous concentration. Bioanalytical regulatory guidance currently discourages extrapolation of calibration curves and as such this approach cannot be advised. Using a surrogate matrix (e.g., charcoal-stripped plasma or synthetic matrix and phosphate-buffered saline) is a common approach. This approach requires that parallelism experiments are added to the method validation to ensure that the surrogate matrix behaves as the study sample matrix and Lowes discussed this. A final option would be to use a surrogate reference standard when preparing calibrator and QC samples. This approach, unique to mass spectrometry detection, uses stable-isotope-labeled isotopologues. While elegant in principle, method validation experiments must demonstrate equivalence between the surrogate standard and the native iso fraction analyte of interest.

  No matter which approach is chosen, a fit-for-purpose LC–MS biomarker assay validation strategy should be considered. The validation categories proposed by Lowes were screening, qualified and validated, each with their own pros and cons. Screening assays are the most economic and quickest to develop, however, they do not meet regulatory standards and the relative quantitation data provided may be difficult to interpret. Validated assays emulate what is done today with a definitive LC–MS validation of a xenobiotic bioanalytical assay. However, a validated assay is not always needed to address the objective of an in vivo study and without well-qualified analytical standards may not even be possible. Therefore, Lowes described the qualified assay designed to accommodate the needs of the study and the constraints that may apply. In each case, a specific, customized validation plan would be established outlining the tests needed to meet a fit-for-purpose outcome. Lowes concluded his presentation stating
that justification of the validation limitations is necessary and scientific defense of the approach taken is paramount.

**Method design, development, validation & routine analysis: beyond the guidance**

- **Method design: laboratory & scientific affairs partnership**

Marc Lefebvre (VP Scientific & Regulatory Affairs, Algorithme Pharma) provided attendees with insight into the challenges faced by CROs when developing a bioanalytical method in relation to scientific and/or regulatory requirements. He reinforced that communication between the scientific affairs group and the method development group is essential for ensuring that an appropriate bioanalytical method is developed. BE requirements have been updated by several agencies since 2003 (FDA), with the most recent by the TPD and EMA (2009–2010). These updated requirements can be based on four types of data. The first, based on a comparison of serum/plasma/blood PK data, is applicable to dosage forms intended to deliver the active moiety to the bloodstream for systemic distribution. The second, based on the comparison of urine PK data, is mainly used when the drug cannot be quantified in other matrices, and is excreted mainly in urine. The third, based on the comparison of pharmacodynamic measurements, is applicable when there is no method to assay for the active moiety, but there is a method to assay for an acute pharmacological affect. Finally, the fourth type, based on the comparison of clinical endpoints, is not as accurate, sensitive or reproducible as the other three types, but may be applicable when assessing dosage forms intended to deliver the active moiety locally.

Less than a decade ago, technology was not available to detect very low plasma concentrations of some active moieties. Therefore, many PK profiles of parent drugs could not be adequately determined, and BE/bioavailability relied, in many cases, on the main metabolite concentrations. An example of this is valacyclovir, which shows peak concentrations of approximately 50–100 ng/ml, but that is rapidly eliminated from plasma within a few hours after dosing. In contrast, its main active metabolite, acyclovir, shows C_{max} concentrations that are 30-times greater. However, with more sensitive technology, the BE/bioavailability requirements have since been appropriately updated.

As a consequence of these adaptations, the development and validation of bioanalytical methods has become more complicated. Furthermore, with the commercialization of more powerful drugs, doses are lower and PK profiles are more difficult to determine. Ranges are typically set by determining the C_{max} and covering approximately four to five half-lives in order to ensure that all subjects are monitored accurately; whether they are normal, fast or slow metabolizers. It is also necessary to take into consideration the challenges raised by long half-life drugs compared with short half-life drugs, endogenous compounds, enterohepatic recycling, first-in-human trials, special populations, food effect studies and metabolites. In all cases, successful method design and development can only be achieved with strong lines of communication between all stakeholders.

- **Bioanalytical risk & method quality**

When developing and validating a bioanalytical method, it is important to become aware of the bioanalytical risk (which changes as the bioanalyst’s knowledge grows) and the acceptable bioanalytical risk tolerance. Mohammed Jemal (Senior Research Fellow, Bristol-Myers Squibb) discussed this topic by presenting examples where awareness of certain risks was not very high even 10 years ago. His first example, awareness of the impact of phospholipids on quantitation, is only just becoming a requirement for method development/validation. It is now necessary to ensure that phospholipids are either separated chromatographically from the analyte of interest, or else that the extraction method is selective enough to remove them from the sample altogether. Another example Jemal provided is the impact of metabolites on quantitation. It is possible that a metabolite is isobaric with the parent drug or that it produces the isobaric species via in-source conversion. In such cases, chromatographic separation is essential. It is also possible that back-conversion of the metabolite to the parent compound occurs during the multiple steps involved in sample analysis, including sample handling and extraction. Jemal highly recommended using pooled incurred samples during method development to ensure that metabolites do not impact analyte quantification. In this way, the impact of even previously unknown metabolites can be assessed. It is important to realize that inherent method quality is built during method development. The subsequent method validation
only augments sound method development. It should be noted that the use of incurred samples for method development is different from the current practice of ISR. Jemal stressed that most of the nonoperational ISR failures could be eliminated by using incurred samples during method development.

Bioanalytical risk tolerance is a function of the intended outcome of the study for which the method is going to be used. This is in line with regulatory expectations. For example, the Crystal City III conference report [8] indicates a flexible, tiered approach to method validation for metabolites, so that methods become ‘fit-for-purpose’. Thus, some of these methods may not be fully ‘validated’, fulfilling the normally prescribed sets of validation procedures and the normally adopted fixed acceptance criteria. Instead, the performance of the methods may be assessed by conducting fit-for-purpose tests appropriate for the intended clinical or preclinical study with the requisite acceptance criteria. Such ‘qualified’ methods, if preceded by sound method development, may even be better than validated methods not preceded by sound method development.

Qualified methods are recommended for fulfilling the Metabolites in Safety Testing (MIST) Guidance until after human multiple ascending dose studies. Metabolite analysis using a validated method with fixed acceptance criteria can then be implemented for those metabolites deemed important as per the MIST Guidance.

- Dried blood spot use in regulated bioanalysis

Christopher Evans (US Head, Bioanalytical Science and Development, GlaxoSmithKline [GSK]) presented his company’s advancements in the area of dried blood spot (DBS) technology. This technique, which has been around for approximately 40 years, originated as a technique for blood collection and detection of phenylketonuria in newborns, and is currently widely applied for the detection of a variety of disorders in newborns. Although GSK had found some applications for this method as early as 6 years ago, they determined that many of their advantages were not very useful at the drug discovery stage. However, approximately 4 years ago, research into this technology for use in routine drug development and analysis was initiated. The pressures of the industry currently require companies to deliver high-quality PK data in a short period of time and to reduce, refine and replace the use of animals in drug development. More specifically, using plasma in bioanalytical methods requires the collection of large volumes of blood, which is difficult to obtain when using rodents, the typical subjects of preclinical studies used in drug development. Based on ethical considerations, it is often necessary to do composite sampling instead of serial sampling to obtain the volumes required, where terminal exsanguination of the animals is necessary. When specialized rodent models are employed, this is a wasteful and costly procedure. Furthermore, regulatory agencies are requiring more pediatric PK and juvenile toxicokinetic (TK) studies. The primary advantage of DBS is the reduction in collection volume, which alleviates concerns for serial sampling of rodents, pediatric studies and improved recruitment due to simplified sample collection. Furthermore, shipping and storage can be done at ambient conditions, since stability concerns in the typical liquid matrices are no longer relevant. This is important for studies performed in developing countries where freezers and dry ice for transportation are difficult to obtain.

Dried blood spot technology uses either untreated cards or cards treated with additives to lyse cells and denature proteins. Approximately 15 µl of blood is spotted onto the card in triplicate. The cards are dried and then shipped or stored at ambient temperature in a sealable bag containing desiccant. Over 150 validated methods for more than 90 compounds have been developed at GSK. Processing the DBS involves punching a disc in the spot to obtain a sample then soaking it in solvent for extraction. Processed samples are then injected onto LC–MS/MS systems for quantitation.

One of the conclusions was that this technology potentially allows for greater metabolite stability. Furthermore, when DBS results were compared with the results of in vivo samples assayed using traditional bioanalytical methods, an excellent correlation was achieved between both methods. Validation requirements remain essentially the same, with the exception that no freeze–thaw stability evaluation is required, dilution of samples is more difficult to perform and matrix effects can be observed based on card treatments. Sensitivity using this technology can be limited with existing technology, in that it cannot typically be used for pg/ml ranges.

The advantages of this technique currently lie primarily with the clinical portion of a study. However, there is potential for future work to
be done to improve cards and treatments, to apply this technology to biopharmaceutical/biomarkers and to enable direct analysis of the cards, without prior treatment with a solvent, thereby streamlining the procedure. DBS represents a viable alternative to traditional sampling methods. It is robust enough to support validated methodologies to internationally accepted criteria, but it is still in its infancy and is likely to change as more research emerges.

Interpreting routine sample analysis results

Eric Woolf (Senior Director, Merck Research Laboratories) discussed the scientific analysis of routine sample analysis data and the importance of looking beyond simply the batch acceptance criteria. His main point is that although guidance documents and SOPs exist providing batch acceptance criteria, and all data are generated under an appropriate quality system, these do not necessarily ensure good science. Guidance documents serve as a baseline and may not recommend all the possible data necessary to ensure that a method is robust. By way of example, Woolf provided two additional parameters that may be reviewed, but that are not addressed by guidance document acceptance criteria: internal standard response variation and standard curve slope variation.

Batch criteria as outlined in the guidance documents assume that study samples will perform identically to calibrator and QC samples, and that the internal standard will correct for any variability between matrix sources. However, since subject samples may contain more or different endogenous compounds than the typical matrix used to prepare the calibrators and QC samples, there may be room for doubt, and the internal standard can provide insight into what is actually going on with the study samples. An example was presented of a study run where all batch criteria were met. Based on these results, one could assume that there are no analytical issues with study samples. However, when the internal standard responses of samples in this run are observed more closely, it becomes apparent that the internal standard responses of calibrators and QC samples are two- to three-times higher than the responses in study samples. In this case, an investigation is recommended to reinforce the validity of the data and confirm that the internal standard does compensate for matrix effects. One possibility is to spike predose samples with the analyte to determine if the back-calculated concentration is correct. Another is to dilute study samples with control matrix to the point where internal standard variation is eliminated, to demonstrate that the results of the diluted samples match with those of the undiluted samples. To ensure that reliable and good quality data is always generated, consider adding internal standard response limits to an SOP: identify minimum and maximum internal standard responses from acceptable calibrator and QC samples. Study samples with internal standard responses less than 50% or greater than 150% should be reassayed.

A second parameter to regularly observe is calibration curve slope variation. Woolf recommended analyzing the slopes of five sets of calibration standards prepared with different matrix lots during method validation. A low coefficient of variation (<4%) indicates no relative matrix effects, however variations higher than this can indicate changes in precision of 15–20% at each point. Furthermore, tracking slope values during routine sample analysis may provide insight into operator or instrument issues. The example provided demonstrated a significant change in the slope beginning and ending on specific dates. All batches met acceptance criteria, however, when asked to provide more information during an FDA inspection, it was discovered that a new lot of internal standard working solution had been prepared immediately prior to obtaining the results. Although there was no direct impact to study sample results since the same working solution was added to all samples, the FDA representative still cited that the analysts failed to correctly follow the analytical method by incorrectly preparing the solution. If the investigation had been performed during the study, this point may have been identified and the samples reanalyzed. It is difficult to identify significant changes in slope values from day to day (can be up to twice), and they may vary from instrument to instrument due to slight differences in tuning/resolution parameters, however, it is often possible to identify very large differences, which may indicate a problem.

Woolf concluded that the typical acceptance criteria, while important, are only the minimum requirements for proving reliable data. The examples provided demonstrated that even with these criteria, a good scientific review of the data can provide insight to otherwise undetectable potential quality issues.
Value of incurred samples during method validation

With the introduction of ISR [9], the limitations of using only spiked quality control samples in order to validate bioanalytical methods are becoming apparent. Only when ISR evaluations meet the acceptance requirements are we truly able to confirm that a method is reproducible.

Robert Massé (Vice President, Bioanalytical Division, Anapharm) highlighted the essential differences between spiked samples and incurred samples. First, the screening process for matrix used to spike samples is generally loose, whereas the screening of subjects is very rigid. Spiked samples are often prepared using multiple, pooled lots of matrix, diminishing any individual matrix characteristics that could be seen in subject samples (e.g., pH). Incurred samples typically have more endogenous compounds associated with the drug, therefore increasing the possibility of interference with quantification. Finally, the amount of anti-coagulant in the samples can vary, depending on how much blood is collected. Massé followed this discussion by presenting several case studies of failed ISR evaluations, their investigation and resolution. The first case where ISR directly led to insight into an analytical issue occurred when only 42% of samples for an Atorvastatin metabolite met acceptance criteria. The sample processing temperature and pH were questioned, and it was determined that there was an increase in conversion of the metabolite’s associated lactone form. Since this metabolite is not present in QC samples, this effect was not seen during method validation. A second case involved a small synthetic peptide. Stability tests run during validation showed a negative, but acceptable trend. However, due to the nature of the incurred sample matrix, its instability was more pronounced. A final example was a case where 40% of incurred samples failed the ISR test, mostly occurring at the low end of the curve. It was determined that the IS response of the ISR batch was half that of the other batches (assumed to be due to a sample processing error), thereby changing the slope of the curve and affecting the low end concentrations. It is evident that the limited number of batches during validation do not catch all the nuances of a method that is assumed to be robust. Although study data was not affected in this case, it was determined that IS response is critical to the reproducibility of the method.

Mario Rocci (Executive Vice President, ICON Development Solutions) used ISR failure case studies to highlight ICON’s approach for investigating ISR failures. The first case presented was for a method used to quantitate a parent compound and its metabolite. This method had been in use for 5 years prior to the ISR requirements. The method was very robust, with little or no need for reanalysis. ISR test results had a substantial negative bias for both the parent and metabolite. Investigation began by retesting the stability of the analytes in QC samples, which confirmed that the problem was with the incurred samples. During the next clinical study, additional duplicate aliquots of samples were provided in order to evaluate the incurred sample reproducibility of the method unconfounded by the freeze–thawing of samples. When ISR was evaluated between previously unthawed duplicate samples, the agreement in results was excellent, pointing to a freeze–thaw stability issue with incurred samples that was not reflected in the QC samples. In order to help support previous use of the method, the incurred samples were used to determine the limits and conditions where acceptable results could be obtained.

The second case involved a newly validated method that appeared robust. Two studies were analyzed using the method, with acceptable ISR results for both parent and metabolite. During the third study, ISR results for the parent failed (metabolite results were acceptable), with unacceptable ISR results occurring for samples obtained late in the elimination phase. When the designs of the three studies were compared, it was noted that the administered dose for the third study was twice that of the dose administered in the first two studies. Using this information, it was hypothesized that an unmeasured metabolite could be converting back to the parent during the thawing of the samples. Since the putative metabolite concentrations were likely to be lower in the first two studies, this conversion might not impact the assay of the parent compound for these studies.

The method was adjusted so that sample thawing was performed within a limited time in an ice bath and the method was used with success. However, two more studies arrived from separate clinics and one of the ISR evaluations failed. This created concern about the study where the ISR results ‘passed’ since the conversion of the putative metabolite back to the parent may have conceivably occurred at the clinic. This concern highlighted the need to critically
evaluate ISR results in the context of the analytical work, since passing ISR results in this case would not necessarily indicate that the results were valid. Further evaluation established the need to flash-freeze the samples after aliquotting to prevent degradation. This case highlighted the need to perform ‘vein to vessel’ stability assessments for certain compounds. Without the use of the incurred samples, neither of these stability issues could have been identified.

A last approach for using incurred samples during validation was presented by Joleen White (Senior Research Investigator, Bristol-Myers Squibb). White used these samples to assess the impact of ligand-binding method changes and cross-validation. Changes to a previously validated method are sometimes necessary, and it is recommended that a risk-based approach be taken to determine the likelihood of a difference in the results due to the change. Some changes have a lower risk of impacting the final concentrations (e.g., changing instruments vs changing critical reagents). The higher the risk, the more important it is to use incurred samples to determine the impact. For instance, when clinical efficacy was observed at lower doses than anticipated, it was necessary to lower the assay LLOQ. The approach chosen was to change the instrumentation and detection label. Incurred samples from several studies were re-assayed with the new method and results compared with the original analysis. It was discovered that samples from older studies had a net negative bias relative to the original results. The hypothesis was that the new instrumentation was more sensitive to long-term stability issues. Going forward, a new method will be developed to attempt to mitigate these issues, and the subsequent cross-validation will also include incurred samples. Another case occurred where it was necessary to broaden the quantitative range in order to reduce the dilution factor used for study samples. The capture antibody was changed and the format changed from sandwich ELISA to competitive ELISA. Cross-validation using pooled incurred samples as well as QC samples met criteria. However, PK results from large studies obtained using the new assay no longer matched historical results obtained with the old method. When the incurred samples from the large study were re-assayed with the old method, there was poor correlation with results that had been obtained using the new method. For the new assay under development, the cross-validation will include full PK profiles already evaluated with the original method.

All of these case studies presented serve to highlight that knowledge of method validation is evolving as new technologies and guidelines are being introduced.

Panel discussions & consensus points
The final half-day of the workshop involved discussion on topics proposed by the attendees and CVG members outlined in the ‘Goals and objectives’ section of this paper. What follows is a summary of those discussions, as well as any consensus reached by the workshop attendees. The first topic of discussion revolved around harmonization of the bioanalytical method validation guidance documents, previously discussed in this article. Furthermore, the question regarding biomarker assays meeting regulatory guidance was addressed during the discussion of Lowes’ presentation. Consensus was achieved regarding the use of a tiered validation approach for biomarkers.

- Lipemic & hemolyzed plasma samples
For many years, only ANVISA addressed the testing of hemolyzed and lipemic samples [5,14]. Recently, however, the EMA has included performing matrix effect on these plasma types as part of their draft guidance [4] and the FDA has started focusing their attention on the subject during inspections as well. Previous industry conferences included discussion on the need to incorporate hemolysis and lipemic testing as part of method validation [3,4]; however, since no guidance includes information on how to perform these evaluations, these tests have not been adopted at large. Some questions that have been raised include the maximum hemolysis and lipemic levels that should be tested, and how to identify affected study samples. Identification of hemolysis may seem straightforward with the use of a color chart, but evaluating the color of affected samples is very subjective, especially when nearing the upper limit of hemolysis being tested in the facility. As for potential lipemic samples, visual identification is not always possible and no chart exists in case samples are visually identifiable. This led to the inevitable question of how to treat samples exceeding the tested levels, and whether these tests have even been performed during method validation.

During the discussion, it was evident that a clear majority of the attendees and panelists were not disputing the usefulness of investigating the impact of hemolysis or lipids on the method. For example, the physiological properties of the
compound should be taken into consideration, particularly the partitioning constant when it is available. If a compound is expected to bind to red blood cells, then additional testing should be done (an example was provided by an attendee regarding a malaria drug where this was a problem). Furthermore, lipids can cause a matrix effect if not taken into account. If extraction procedures remove the lipids or if they are chromatographically separated, then this test should not be required. If a method is well developed then these factors would have been taken into account and the method would have been adjusted, until any impact from these factors was negated. One way to do this is to use a stable-labeled internal standard; ANVISA noted that they require these tests because they do not oblige the use of stable-labeled internal standards.

In general, it was felt that good science should prevail; any anomalous results should be investigated taking the state of the plasma into account. However, there was no consensus reached about whether to include these tests as part of method validation or not. Since agency trends demonstrate that these evaluations are requested, it is advised that the potential impact of hemolyzed or lipemic plasma is investigated either in method development or method validation.

One additional question on this topic was asked during the discussion. Hemolysed or lipemic samples may need to be diluted during the assay due to issues such as limited sample volume and concentration above the upper limit of the range. One question that was asked was whether the blank plasma used to dilute these samples needed to ‘match’ the samples (i.e., hemolyzed or lipemic). It was decided that if the ruggedness of the method was demonstrated along with tolerance to hemolyzed or lipemic content, then the type of plasma used should not matter.

### Statistical challenge to the current validation criteria

The FDA Bioanalytical Method Validation guidance [7] and subsequent Crystal City conference [8], as well as the draft EMA guidance document [4] recommend the 4–6–20 rule as a general, easy-to-apply criteria for method validation and routine sample analysis batch acceptance, although both acknowledge that a statistical approach can be used. Boulanger et al. disagree with using this methodology, questioning how well the prestudy validation will predict the quality of individual incurred samples [33]. They argue that the 4–6–20 rule focuses on the performance of procedures instead of putting the focus on the quality of future results. Additionally, they claim that the rule is defined based on technological capabilities instead of evaluating the ‘fitness-for-use’ of a method. They suggest using a statistical approach when determining the acceptance criteria to apply to a method; their conclusion recommends using the β-expectation tolerance interval.

The consensus reached by all was that both methods are valid and it was desired that future iterations of guidance documents not be too prescriptive by recommending one approach over the other. The robustness of a method will not be truly determined until it has been used to assay incurred samples, no matter which approach is used. It was also pointed out that when the statistical analysis approach is discussed, the worst case scenario is used to evaluate the worth of the 4–6–20 rule, and that the results of most runs are much better than the limits of acceptability, leading to aggregate assay performance well below the 4–6–20 across all accepted run QCs.

### Urine & tissues analysis

Urine sample collection is typically not a controlled process. Many samples are often pooled within a certain timeframe to create one collective sample. Tissue analysis requires that the tissue samples be truly homogenized, which is potentially difficult to achieve. Because of these factors, the approach taken to validate urine and tissue methods cannot be the same as the approach taken with more typical biological matrices.

The attendees all agreed that these types of methods are atypical and should not be regulated in the same way as, for example, plasma methods. They should be fit-for-purpose, such that the end-point of the study can be answered with ‘qualified’ methods; validating these methods is not necessary and often not possible. For example, acquiring stability data is not possible for tissue methods (spiked tissue samples are not representative of incurred samples). Therefore, these methods cannot be considered validated since stability data would be missing. They could, however, be considered qualified and held to the highest standard that is achievable. Furthermore, urine methods should be analyzed on a case-by-case basis. It is not common practice anymore to require urine results as primary data. However, if urine results are the primary end-point, then the collection process needs to be strictly controlled and the method fully validated.
Multi-analyte assays, repeat analysis & failed runs
The FDA Bioanalytical Method Validation guidance [7] states very clearly that “samples involving multiple analytes should not be rejected based on the data from one analyte failing the acceptance criteria.” Therefore, for multi-analyte studies, repeating for only one analyte raises the question of what to do with the previously passing analyte(s) data. One proposed procedure was to analyze samples for all analytes, but quantitatively process only the data for the analyte that is needed. The extra data would not be processed or reported.

The attendees all agreed with the proposal; only the originally accepted data needs to be reported. However, two additional options were proposed and deemed acceptable alternatives. The first would be to use the available repeated data as part of the ISR test. Another was to cross-validate the method analyzing all analytes versus methods assessing each analyte individually. Therefore, repeat samples can be accurately repeated using the appropriate single analyte method.

An additional question on the topic of repeat analysis was put forward by an audience member. They had cases where the FDA had issued notifications requiring extrapolated data for samples originally above the upper limit of quantitation (ULQ) or below the lower limit of quantitation (LOQ) that had been analyzed with dilution or data reported as analytically unacceptable. There was some question as to the rationale for asking for a value that has been identified as unreliable based on an observed/documented analytical issue. The consensus reached was that these values should not be reported.

Preparation of calibration standards
There are two options when preparing calibration standards: prepare in bulk and use within the determined stability timeframe, or prepare freshly for each analytical batch. Both approaches have their own advantages and disadvantages. Preparing bulk standards is very efficient, and allows the standards to be tested for acceptability prior to study sample analysis. On the other hand, bulk preparations require increased storage space, and long-term stability data is required to cover their use. Fewer stability issues occur when preparing fresh calibration curves and no storage space is required, but there is a higher risk of intra-assay mispreparation issues and this method is resource intensive.

Overall, it was determined that it was not necessary to decide on a common approach, since both were allowed according to the Crystal City III conference report [8]. It was specified that freshly prepared calibration curves were necessary for stability evaluations, and many attendees used fresh preparations for all validation batches. Only a small percentage of attendees always used freshly prepared standard curves. Bulk preparations were perfectly adequate as long as long-term stability data existed to cover their use.

LBA critical reagent stability
LBAs are for the most part indirect assays, typically relying upon the use of secondary reagents to reveal the presence/amount of the analyte of interest. The expectation is that these secondary reagents (e.g., antibodies and antibody conjugates) have expiration dates assigned in order to determine their ability to be used over the long term although the tests that can be used to assign an expiration date will vary according to the nature of the specific reagents.

The panelists all agreed that the published article by Rup and O’Hara [34] represented the industry standard on this topic. Consensus was that a re-test date is first assigned based on previous history with the material or similar materials, however, assay performance should be continually monitored in order to determine the ultimate expiration date. It is recommended that processes and criteria be set up a priori during method development and validation for monitoring ongoing assay performance to expose any stability issues. The initial re-test date should be set at the shortest interval of component parts if it is a composite reagent.

Consensus was reached on several other questions raised while discussing this topic. The most important point made was that although LBA methodology is different, the scientific needs are still essentially the same as those for small molecules with possibly widened criteria.

A question was raised about having acceptance criteria for anchor points. The conclusion was that such acceptance criteria, if applied, must look at aggregate deviations of the calibrators within the analytical range, and must demonstrate that the presence of the anchor point has an effect on batch acceptance. It was clear, though, that anchor points should never be rejected in order to force the acceptance of the QC samples.

Another attendee asked whether a selectivity sample could be excluded from the selectivity evaluation. It was concluded that selectivity samples, similar to validation QC samples,
should be excluded only when a documented analytical cause warrants their removal.

Finally, the number of ISR samples required for LBA studies was raised, and if the same dilution factor should be used as was used for the original analysis. The number of samples required is the same as for small molecules: 5–10% of the total number of study samples. Furthermore, if parallelism experiments have been done successfully, then the dilution factor used should not impact the results obtained. However, in practice, using the same dilution factor reduces the number of variables between the two analyses.

- Sample handling

Study sample chain of custody is always an important point during regulatory inspections. There is an increased focus in the industry on cold-chain management, especially regarding the shipping of medications. Furthermore, sample storage units require continuous monitoring in order to ensure sample integrity over the duration of the study.

Some potential issues included validation and CFR part 21 compliance of LIMS, development of a sample handling stability program, practical considerations associated with inventory of the samples and finally, samples shipment on dry ice (representing -80°C), even though they may be stored long term at -20°C, potentially requiring stability evaluations at both temperatures. A majority of the attendees performed stability evaluations at both -20°C and -80°C, however, this was not unanimous. Furthermore, it may be necessary to perform the stability directly on dry ice, since the presence of CO₂ may have an effect on pH-sensitive compounds.

The workshop consensus was that a well-controlled procedure must be established, with good documentation and a defined chain of custody. When developing the procedure, one must consider limitations about storage specifications in different parts of the world. It is essential to have good communication between the clinic and lab facilities so that samples are handled properly from the beginning of the study. One critical issue raised by an attendee was to make sure the sites are aware that they should change gloves between handling dose solutions and sample collection tubes to avoid contamination.

- Carryover

Carryover is an important issue to address during method development, and the extent of carryover must be determined. If carryover is inevitable, a nonrandomized sequence is typically used. There is no guidance document that provides criteria for estimating potential sample-by-sample carryover. It was agreed upon during the discussion that carryover must be addressed during method development and sample analysis by creating a strategic approach to minimize it. Most of those in attendance injected a blank or zero sample after an ULQ sample or multiple ULQ samples. In general, it was important to use scientific rationale to determine the impact of any carryover and to be transparent in reporting it. Furthermore, it is important to note that a method previously determined to be free of carryover tested using ULQ samples may demonstrate carryover caused by samples that fall above the upper limit of quantitation.

- Stability requirements for co-administration studies

One attendee brought an additional question to the workshop addressing the stability requirements for co-administration studies. This references a recent FDA 483 issued for not proving freeze–thaw and long-term stability in the presence of a co-administered compound. Since this appears to be a new approach to the established validation requirements, the issue was discussed during the workshop to determine if this was a widespread trend by the agency, or if any of the attendees had seen any scientific evidence for concern. A poll of the audience indicated that no one could provide an example where analyte stability was affected by co-administered compounds, thus bringing into question the scientific rationale for such stability assessment requirements. However, one person came forward stating that they had been asked for this type of data before, and had supplied it with no issues observed.

Conclusion

All the speakers present at the 4th annual Workshop on Recent Issues in Regulated Bioanalysis 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 points:

- Global harmonization of bioanalytical guidance is an important and necessary step in facilitating global submissions of studies supporting the release of new drugs;
An all-inclusive Global Bioanalytical Consortium should be created with representation from scientific associations and industry to discuss harmonization of bioanalytical regulatory guidance and ideally propose unified guidance language that can be presented to regional agencies;

Method robustness will not be confirmed during prestudy validation by either the 4–6–20 rule or statistical evaluation until incurred samples are tested during sample analysis. Either methodology could be used;

For matrices or analytes that fall outside of the traditional bioanalytical methods (e.g., tissues, urine, biomarkers), a tiered approach to method validation should be used. The attendees recommend that the following categories be used to denote the level of validation achieved: screening, qualified and validated. All levels of validation should be scientifically justified a priori based on the intended use of the data;

For multi-analyte methods, there is no need to regress repeat data of any analyte that previously passed the original analysis;

The use of either bulk or fresh preparations of calibration standards can be used during sample analysis, with proper supporting stability data, if necessary;

The retest date of critical reagents is first assigned based on previous history with the material, and stability is continually monitored in order to determine the final expiration date;

Anchor points should have predefined criteria for removal based on calibrators in the quantitative range, and should not be removed in an effort to pass the QC samples;

Between 5 and 10% of ISR samples should be assayed for LBAs. The same dilution factor as original analysis is not required as long as supporting parallelism data exists, but is usually selected in practice;

LBA selectivity samples should not be excluded without analytical cause;

A well-documented, controlled sample handling procedure must exist;

Carryover must be investigated during method development and minimized.

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