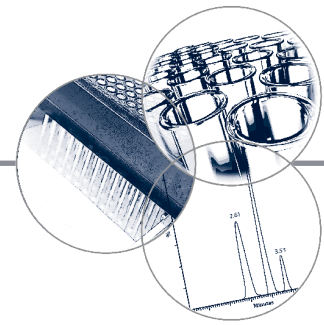
White Paper



2012 White Paper on Recent Issues in Bioanalysis and Alignment of Multiple Guidelines

Over 400 professionals representing pharmaceutical companies, CROs, and multiple regulatory agencies participated in the 6th Workshop on Recent Issues in Bioanalysis (WRIB). Like the previous sessions, this event was in the format of a practical, focused, highly interactive and informative workshop aiming for high-quality, improved regulatory compliance and scientific excellence. Numerous 'hot' topics in bioanalysis of both small and large molecules were shared and discussed, leading to consensus and recommendations among panelists and attendees representing the bioanalytical community. The major outcome of this year's workshop was the noticeable alignment of multiple bioanalytical guidance/guidelines from different regulatory agencies. This represents a concrete step forward in the global harmonization of bioanalytical activities. The present 2012 White Paper acts as a practical and useful reference document that provides key information and solutions on several topics and issues in the constantly evolving world of bioanalysis.

The 6th Workshop on Recent Issues in Bioanalysis (WRIB), 'Where Regulators and the Industry Convene', was hosted in San Antonio, TX, USA on 26–29 March 2012. It was the first time this event had taken place in the USA, as all previous workshops were held in Montreal, Canada. The 6th WRIB continued to be an open forum for discussing, sharing perspectives and providing potential solutions for the most recent issues in bioanalysis. The contributing chairs included Binodh DeSilva (Executive Director, Bioanalytical Sciences-Biologics, Bristol-Myers Squibb, USA), Fabio Garofolo (Vice President, Bioanalytical Services, Algorithme Pharma Inc., Canada), Gabriella Szekely-Klepser (Vice President, Drug Safety Evaluation, Allergan, USA), Russell Weiner (Executive Director Clinical Development Lab., Merck Research Laboratories, USA), and Mario Rocci (President, ICON Development Solutions, USA). Attendance at this year's meeting grew over previous years with global representation from over 400 professionals representing more than 200 companies, including pharmaceutical companies, CROs and regulatory agencies working in the bioanalysis field. The contributing regulatory agency representatives included Brian Booth (US FDA), Sam Haidar (FDA), Eric Ormsby (Health Canada TPD), Jan Welink (Dutch Medicines Evaluation Board), Olivier Le Blaye (French ANSM, formerly AFSSAPS), João Tavares Neto (Brazil ANVISA) and Toshinari Mitsuoka (Japan MHLW).

It was announced at the 6th WRIB that while the Calibration and Validation Group [3] has successfully handled the previous editions of the workshop, the future WRIB meetings will be organized by the newly formed Canadian Forum for Analytical and Bioanalytical Sciences, a non-profit scientific association fully dedicated to and focused on bioanalysis [101]. This new organization was created to better respond to the challenges of global expansion of bioanalytical sciences in the past several years. It will continue in the footsteps of the Calibration and Validation Group by carrying on the tradition and format of the WRIB meetings [4–7,102].

Discussion topics

A considerable number of topics were addressed during the speakers' presentations and panel discussions. The present White Paper focuses on the discussions, consensus and resulting recommendations on 16 recent issues ('hot' topics) in bioanalysis, as well as the topics of interest addressed during the presentations.

The 16 hot topics included issues pertaining to both LC–MS methods and **ligand-binding assays** (LBAs), and are listed below.

LC–MS discussion topics:

1 Reference standards play a critical role in bioanalysis. How can the quality of reference

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standard material (RSM) and stable isotope labeled (SIL) internal standards' (IS) isotopic purity be improved?

2 How can electronic common technical documents (eCTDs) be harmonized for regulatory submissions? What are the global standards for electronic submission format? Is automated bioanalytical report writing a realistic goal for global submissions?

3 Should LBA or LC–MS acceptance criteria be followed when large molecules are analyzed by LC–MS? If so, what is the rationale?

4 Assay **specificity** [1]: how do co-administered drugs or drugs present in drug–drug interaction studies influence the selectivity of the assay?

5 Incurred sample stability (ISS): what are the differences between ISS and incurred sample reanalysis (ISR)? Should we consider performing ISS? Should ISS become a regulatory requirement? When do you need to perform ISS?

6 Dried blood spots (DBS): what are the best approaches to storage, drying time and dilution? Is the age of the blood sample a factor to consider? What is the best way to use IS with DBS? Should calibrants and QC samples have the same hematocrit as study samples?

LBA discussion topics:

7 **Hemolysis** and lipemic samples: what are the general approaches to analysis? How do we quantify the degree of hemolysis and lipemic levels? When should these experiments be performed?

8 **Total error** [2]: should the total error be reported in LBA calculations?

9 Dilution QC samples: should dilution QC samples be analyzed routinely in sample analysis runs (in-study)?

10 Automation: what platforms are in use today and what types of data flows/processes are optimal?

11 Can the term GLP be used in biomarker analysis?

12 Specificity: can specificity be tested in method development and not in validation if the method has not changed?

13 **Parallelism**: what is the general approach used to establish parallelism in LBA experiments?

14 'Required' versus 'best practice': which terminology should be used?

15 Sample treatment: what are generally acceptable processes for sample shipment and handling?

16 Replicates: how many replicates should be analyzed in LBA?

Deliberate discussions, recommendations & consensus points

■ LC–MS topics

1 Reference standards

RSM play a critical role in bioanalysis. The quality of the RSM is essential but the supplied certificate of analysis (CoA) is not always adequate and its content can be quite variable. The CoA for innovator compounds is generally more inclusive than for commercially purchased standards. Although the CoA is usually adequate for small molecules, containing purity and moisture content, the important stability information is often unavailable. Indeed, the duration for which the RSM is stable should be included, or at least a retest date provided. A CoA is probably not as crucial for an IS, and may also be difficult to obtain for metabolites since the quantity available is normally an issue. It is important that there is consistency between lots, since different lots of standards are often used across a series of studies. It should be noted that for significant human metabolites, additional efforts may be needed to provide enough RSM for a comprehensive characterization. Purity assessments are sometimes done by thin-layer chromatography, but should be determined by a more scientifically rigorous method. Other issues to consider for RSM are the shipping conditions, storage temperature and light conditions to which the RSM is exposed and stable. Critical shipping requirements could be indicated on the CoA. It is difficult to reconcile the differences in the first CoA for a standard from subsequent CoAs; thus, a mechanism that ensures traceability and consistency is recommended.

2 Bioanalytical report writing

The content of the bioanalytical report is supposed to facilitate the submission of the bioanalytical information in the eCTDs for regulatory submissions. The most important goal regarding the eCTD format is to facilitate the review of bioanalytical information by regulatory agencies [8]. This could be achieved by using a common format to report the scientific content and not simply a template. The content has to be scientifically driven. This format should be agreed upon by regulatory bodies that should then thoughtfully review for content rather than verify the contents using a 'checklist' approach.

3 Large-molecule bioanalysis by LC–MS

Multiple discussions focused on recent insights in large-molecule bioanalysis by LC–MS:

- Universal surrogate peptide: the following attributes were set for an ideal universal surrogate peptide to enable LC–MS/MS bioanalysis of a diversity of human monoclonal antibody (mAb) and human Fc-fusion therapeutic proteins in preclinical animal studies: 1) The peptide should be present in all human mAb and Fc-fusion therapeutic proteins of interest; 2) The peptide should be reliably produced with trypsin digestion and have desirable amino acid composition; 3) The peptide should not be found in the plasma proteins of animals used in preclinical studies; 4) The peptide should possess favorable LC–MS/MS characteristics. A combination of *in silico* and experimental work was discussed showing that the VVSVLTVLHQDWLNGK tryptic peptide in the Fc region of human IgG1 and IgG4 meets all of the criteria for a universal surrogate peptide. Under the recommended trypsin digestion conditions, deamidation of asparagine was found to be insignificant [9]. An analogous Fc peptide, VVSVLTVVHQDWLNGK, representing a single switch from leucine to valine, was also found to be an equally desirable universal peptide for IgG2-based therapeutic proteins.

- Pellet digestion: this technique involves the isolation of a therapeutic protein in plasma into a pellet and provides a degree of sample cleanup as the supernatant is discarded prior to trypsin digestion of the pellet [9].

- Trypsin digestion: recent developments in process facilitation and cost reduction were discussed. In general, evaluation of sequencing-grade trypsin, purchased in small vials, and 'lower' grade trypsin, purchased as bulk reagent, showed that the use of the latter grade of trypsin, where a large volume of a stock solution can be prepared for easy dispensing, improved the digestion workflow at a much reduced cost.

- Improving sensitivity: low concentrations of signature peptides and the complex matrix constituent present are major bioanalytical challenges to be overcome in order to reach the sensitivity needed to obtain relevant pharmacokinetic data for large-molecule bioanalysis by LC–MS/MS. A recent approach discussed achieving enough sensitivity using offline SPE (with a mixed-mode mechanism) in combination with coupled LC columns. The main conclusion was that selective sample preparation and chromatography is the key to achieve sensitivity and obtaining robust methods for the quantification of large molecules by LC–MS/MS.

- Internal standard: LC–MS/MS methods for quantification of large molecules typically use a SIL signature peptide as the IS. Both synthetic peptide and whole molecule SIL-IS have been reported for mAb LC–MS methods, where an IS has to be generated and qualified in the method for each candidate. The use of a uniformly heavy-isotope-labeled common whole mAb IS can be considered a general LC–MS/MS method approach to quantify mAbs. Recently discussed data showed the quantification of four IgG2 and four IgG1 mAbs in rat plasma with sufficient sensitivity (0.1 µg/ml), good linearity over the dynamic range (0.1–15 µg/ml) [10] and acceptable accuracy and precision. The QC performance of the whole-molecule-labeled IS was shown to be better than those of synthetic SIL-IS peptides. However, there are also extensive data available showing highly accurate/precise quantification using SIL-IS peptides. Nevertheless, use of a whole protein SIL, when possible, is theoretically the ideal IS.

- Oligonucleotides quantitation by LC–MS/MS: LC–MS-based methods are capable of accurately, specifically and robustly analyzing various types of oligonucleotides and their metabolites in different biological matrices. For oligonucleotide quantitation using LC–MS, it

Key Terms

Ligand-binding assay: Laboratory technique that makes use of the binding between a ligand and specific binding reagents (e.g., binding of an antigen to specific antibodies) in order to quantify the specific ligand in a sample.

Specificity: Measure of the extent to which a method can determine a particular compound in the analyzed matrices without interference from matrix components. In ligand-binding assays, this is also the unique differentiation of the respective antibodies. The specificity of an antibody refers to its ability to bind the antigen of interest.

Hemolysis: The rupturing of erythrocytes (red blood cells) and the release of their contents (mainly hemoglobin) into surrounding fluid (e.g., blood plasma). Hemolysis may occur *in vivo* or *in vitro*.

Total error: Concept that expresses the closeness of agreement between a measured test result and its theoretical true value. The term total error describes a combination of systematic (mean bias) and random (precision) error components.

Parallelism: Uses incurred samples to demonstrate that the sample-dilution response curve is parallel to the standard-concentration response curve.

is critically important to establish an efficient extraction (clean-up) procedure, which minimizes potential desulfurization, from biological matrices such as plasma and tissues. A suitable ion-pair reagent (e.g., tributyl ammonium acetate) is also needed to reduce the number of charge states and skew production to a particular charge state to enhance assay sensitivity. These potential problems can typically be readily overcome. Nonetheless, issues such as assay sensitivity limitations (particularly in plasma and for adequately characterizing post-distribution plasma levels that typically provide good insight into target tissue exposure/elimination) and a typically higher per-sample cost than LBAS, remain to be addressed. Moreover, LC mobile phases are limited due to compatibility with MS; extraction recovery and consistency can be an issue depending upon the process used. The compound can bind to containers, and while generally very stable in most matrices, some types of oligonucleotides (e.g., compounds with phosphothioate backbones) may be unstable under certain sample extraction or processing conditions. Regardless, recent data show the possibility of validating rugged LC–MS-based methods with no interference from metabolites, single digits for % bias and %CV, reasonably good sensitivities (i.e., 5–20 ng/ml, although LBAs typically have even better sensitivity), linearity with a dynamic range of three orders of magnitude, throughput of approximately 5 min per sample including multiple analytes and/or metabolites, and the ability to use the same platform to analyze different delivery vehicles (e.g., lipids and PEG) [11].

As large-molecule bioanalysis by LC–MS/MS use increases, an important question is what criteria should be used to make decisions on LBA or LC–MS/MS, and why? In general, large-molecule bioanalysis by LC–MS/MS should be considered a 'hybrid' method since the most commonly used approach involves molecular interaction steps, such as immunochemistry-based separations and enzymatic digestion to peptides followed by peptide analysis by LC–MS/MS using a SIL-IS. In small-molecule LC–MS analysis, replicate sample analysis is not required due to the assay robustness resulting from the addition of an IS. On the other hand, duplicate sample analysis is typical for large-molecule LBAs although this may not be necessary when the method is rugged. When large-molecule bioanalysis by LC–MS uses an IS, replicate sample analysis may not be necessary. However, since this technology is still in its infancy, the consensus is that the traditional LBA acceptance criteria (e.g., ±20% accuracy and precision) could be used initially. As this technology advances and demonstrates applicability, the criteria should be reviewed and could be tightened as needed based on assay performance.

4 Assay specificity & stability with co-administered drugs

The issue of performing matrix stability evaluations in the presence of co-formulated or co-administered drugs was previously debated in the 2010 and 2011 editions of the WRIB, and the discussion continued this year. In 2011, the GCC started collecting quantitative data on matrix stability from numerous CRO laboratories. All the data collected demonstrated no effect on stability caused by the presence of co-formulated or co-administered drugs [12]. The GCC sent a letter to regulatory authorities describing the results of the GCC survey and subsequent recommendation. This letter proposed that further practice of conducting such stability experiments in routine bioanalytical method validation (BMV) should be limited to the situation where the co-administered compound may impact stability due to the collection process. The publication of a GCC's recommendation on this topic is ongoing [13].

The demonstration of specificity when dealing with co-formulated/co-administered drugs is another important aspect to consider, as both the US FDA [14] and EMA [15] guidelines on BMV call for investigation of potential interference of co-medications in the assay. Do co-administered drug(s) or drug–drug interaction compound(s) influence the selectivity of the assay? The potential for interference was shown to be dependent on assay format. It was proposed that the interference testing should follow the assay type. Low-specificity assays, such as LC–UV, would require the most testing, but may also not be specific enough to eliminate the interference. For MS-based assays, interference should be verified by measuring the matrix factors (MF) in the presence of interfering drugs. When using LC–MS/MS, it is generally safe to say that a high degree of assay specificity is reached, especially when SIL-ISs are used. Although the presence of co-medications rarely has an impact on the specificity of these assays, cases of interference may still exist. When an

analog IS is used, it is also important to evaluate the potential for interference due to matrix effect. One can determine the potential for drug interference by measuring the IS-normalized MF. If the IS-normalized MF is close to 1, the potential for any interference has been eliminated. However, specificity of assays may potentially be impacted when the co-administered compounds have the same molecular mass as the analytes being measured (i.e., isobaric compounds). Therefore, in cases where the co-administered drug(s) are isobaric, it becomes obvious that the potential for interference should be carefully evaluated. Whatever the type of assay used, a sound scientific assessment should be made to appropriately evaluate the potential impact of the presence of co-administered drugs on assay selectivity.

5 ISR

The topic of ISR has been discussed in several meetings and publications [16,17] and is now an official requirement in the EMA guideline on BMV [15]. ISR methodology should be designed to detect potential issues in a timely manner such that investigation can occur before sample analysis gets out of control. From that perspective, ISR acceptance criteria on a per-batch basis should also be considered for bioequivalence studies.

The regulatory requirement to perform ISR to evaluate the reproducibility of incurred samples has become a routine in regulated bioanalysis. Through ISR-failure investigations, the need to evaluate not only ISR but also ISS is under discussion. In theory, the use of incurred samples to evaluate analyte stability would be a good scientific practice as differences in sample composition and homogeneity could potentially affect the stability of the analyte of interest. The combined experience of the panelists revealed that the accuracy and stability of the analyte in the matrix may not be completely predicted with the QC samples.

To a certain extent, ISS can be considered to be within the ISR evaluation, but only partially as the storage duration of incurred samples is not always covered by ISR. More so, the trend to conduct routine batch ISR further differentiates the ISR experiment from ISS investigations. A major concern arises when thinking of the design of the ISS: 'How does one obtain a true 'Day 0' concentration value?' For all studies, Day 0 incurred samples are practically impossible to obtain, and the absence of Day 0 samples would enable only the relative assessment of the stability. Although the conduct of an ISS evaluation may allow valuable stability information to be obtained, it is not always possible due to the availability of samples. It was agreed that in most instances, the ISR evaluation gives sufficient insight about the quality of the bioanalytical data, and has the potential to reveal stability issues. Consequently, at this point in time the consensus was that ISS should not be required from a regulatory viewpoint.

6 DBS

The DBS technique has been discussed at length in the last few years [18]. DBS sampling offers some benefits over conventional sampling, especially for certain types of studies (e.g., pediatric, those conducted in developing nations and toxicology). But homogeneity, effect of hematocrit and variable recovery are current challenges that need to be addressed, among other more manageable issues. Although the FDA encourages having DBS as an alternate method, the data obtained are not currently accepted as standalone, and collection of wet and dry samples is encouraged in order to continue to demonstrate bioanalytical concordance. It was also mentioned that if a compound is found to be stable in conventional liquid matrices, this does not extrapolate to that compound being stable on DBS cards. More examples and data are needed to correlate data from standard methods.

The European Bioanalysis Forum (EBF) is currently performing the evaluation of experiments and data focusing on the bioanalytical aspects of DBS (stability, IS, hematocrit and sample dilution), hence, the panelists agreed to wait for this position to be articulated before providing specific recommendations on that topic. In parallel to the EBF effort, a Microsampling Working Group, under the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) Drug Metabolism Leadership Group was recently established in the USA to make recommendations on best DBS practices and seek regulatory input around the topics of demonstrating concordance for dual-sampling approaches and bridging study requirements (e.g., wet/dry, venous to peripheral). It is crucial that the scientific community continues to share learning and engages in open and transparent discussions with the regulatory agencies on the DBS topic.

■ LBA topics

7 Hemolyzed & lipemic samples

The effect of hemolysis on bioanalytical method accuracy and precision has already been extensively discussed in the literature [19]. For LC–MS/MS assays, simple modifications in sample extraction and/or chromatography usually resolve the effect of hemolysis. However, in some cases, hemolysis can impact drug stability, and measurements need to be taken to ensure the integrity of the incurred samples.

It is generally accepted that interference from hemolyzed and lipemic plasma or serum samples is not expected in LBAs using a heterogeneous format; that is, involving one or more wash steps. However, a risk assessment evaluation still needs to be performed in assays that use small peptides, therapeutics that bind to red blood cells/platelets and soluble ligands. Panelists involved in the discussion agreed that scientifically driven discussions and justification need to be clearly stated when including or excluding hemolyzed and lipemic sample testing.

The conclusion for large-molecule analysis from hemolyzed and lipemic samples is to use a fit-for-purpose approach, whereas for small-molecule analysis, the potential impact of these matrices on drug stability should be considered, evaluated and resolved at an early stage of method development to avoid the generation of inaccurate data during incurred sample analysis.

8 Total error

Total error was discussed and it was agreed that the total error of the method (sum of the systematic and random bias) of the validation samples (QCs) should be reported in the LBA calculations to understand the error components. The 4/6/20 rule is recommended for in-study acceptance of QC samples. Stringent criteria for the standard curves are recommended. The criteria could possibly be relaxed based on limitations of the method diligently identified during method development.

9 Dilution QC samples

The dilution of high-concentration QC samples is usually done to demonstrate that diluted samples can be analyzed with acceptable precision and accuracy. It is often done to predict matrix effect problems that can occur with diluted incurred samples. However, QC dilutions do not necessarily reflect sample dilutions. Accordingly, routinely running dilution QC samples is optional. If performed, all predilutions should be done with appropriate matrix and the minimal required dilution should be done to ensure identical final matrix composition.

10 Automation

Automation is widely used as it allows high throughput, reduced costs and eliminates human error. Panelists agreed that automation is an evolving process. Robotic manufacturers are constantly developing new technologies to overcome precision and accuracy challenges due to sample handling. One of the first steps in implementing an automated platform is to carefully define the data flow throughout the entire LBA process. The attendees stressed the need to properly archive the raw data from the instrument logs to ensure the integrity of the data.

11 Use of the term GLP in biomarker analysis

The panelists discussed terminology issues (GLP vs non-GLP, qualified vs validated, exploratory vs regulatory) related to biomarker analysis. It was agreed that not all biomarker work should be done under the rigor of GLP. The term GLP should not be applied to biomarker work unless the analysis is truly being done according to the principles of GLP with all the required GLP attributes [20,21]. To avoid confusion, with the term GLP, it was agreed that either 'regulated' or 'nonregulated' bioanalysis should be used. Both terms can be used to describe bioanalysis being conducted as part of nonclinical and clinical studies.

12 Specificity

The specificity of LBAs is the ability of assay reagents (e.g., antibodies) to distinguish between the analyte and other structurally similar matrix components. It is sometimes challenging for the scientist to determine the experiments that should be performed to show specificity in LBAs.

A common practice is to perform specificity evaluations twice; once in early development to define the assay, and again in validation with the optimal conditions to ensure availability of well-documented data in accordance with regulatory needs. Specificity may need to be re-evaluated in some cases such as the analysis of samples from different disease states (e.g., rheumatoid arthritis), or particular new method requirements (e.g., major change in LLOQ or matrix).

13 Parallelism

Parallelism demonstrates that the sample dilution–response curve is parallel to the standard concentration–response curve. It is often confused with dilutional linearity, which is used as a first-pass estimate of parallelism using spiked QC samples. Parallelism is a performance characteristic that is typically not possible to address during development or prestudy validation because it requires the use of incurred samples. At this time, routine testing of parallelism for LBA has not been widely adopted by the industry.

Active discussion on the relevance of the parallelism evaluation and when it should be performed is ongoing and more feedback from the industry will be required in order to provide appropriate recommendations on this subject.

14 Terms 'requirement' versus 'best practice'

The term 'requirement' is used to define very specific methodologies that must be followed, or regulatory guidance. The panelists agreed that the recommendations discussed in the present White Paper are not requirements, but rather best practices suggested by a panel of expert scientists in the field. As they are suggestions, these recommendations do not bind any scientist or group of scientists.

15 Sample treatment

Sample handling and shipment of incurred samples were discussed first. It was agreed that sample treatment is method- and analyte-dependent. The need for additives at the time of collection (e.g., anticoagulants and protease inhibitors), the stability of the analyte during collection procedures (whole blood, plasma and serum), and post-collection processing and storage conditions (e.g., temperature and vial type) need to be evaluated during method development and applied accordingly. Consensus was not reached as to whether samples should be shipped in one or two different portions to ensure a backup if shipping conditions are compromised. This should not be a regulatory requirement, but based on the risk/benefit analysis by the sponsor. Specifically, the question to be answered is whether the added risk involved in the additional sample handling (preparing aliquots and tracking such portions) is justified based on current experience with shippers?

It was pointed out that unknown and QC samples must be treated identically to ensure method validity. During the discussion, it was also stressed that gentle but thorough mixing of the sample to favour homogeneity is a key factor to ensure method robustness and reproducibility.

16 Number of replicates

Historically, LBAs have routinely been performed in duplicate or triplicate. Owing to the inherent variability of the methods, the mean of the replicates has been considered to be closer to the true value. However, it is now believed that there is not always a scientific rationale behind this practice. With some of the newer techniques that display minimal variability between replicates, it is scientifically justifiable to forgo replicate for singlicate analysis. Although a greater number of replicates should be done during method development in order to investigate and understand the concentration–response relationship in the assay, it is generally accepted to run singlicate analysis once the assay has been demonstrated to be robust. However, since singlicate analysis is currently the exception, it would be advisable to have data and analysis justifying the lack of need for replicate analysis. The panelists recognized that it is a case-by-case decision and that some methods would still be considered too variable for singlicate analysis.

Alignment of multiple bioanalytical guidance/guidelines

One of the major focuses of the 6th WRIB was the updates from multiple regulatory agency representatives on the status of their bioanalytical guidance/guidelines and related regulations. The global harmonization of bioanalytical guidance was at the forefront of the 2010 edition of the WRIB, where the initiative to create the Global Bioanalysis Consortium (GBC) was put in place [6] and several publications were issued on the necessity of harmonization in a global context in 2010 [22–25]. After 2 years of effort made by the industry and various regulatory agencies, the 2012 WRIB section entitled 'Year of the New Guidance in Bioanalysis' was a good opportunity to revisit and evaluate the progress of the harmonization process. The reports from the regulatory agencies present at the 6th WRIB generally indicated that different regulatory guidance/guidelines are aligned with respect to bioanalytical requirements. In parallel, GBC will continue the effort to create one unified consensus document that can be

presented to the different health authorities in the future [103].

- EMA Guidelines. In the textual interpretation of the EMA guidelines, terms such as 'should be performed' or 'must be demonstrated' are considered clear (or 'hard') criteria, whereas wording such as 'it is recommended/advised' or 'may be used' represent open (or 'soft') criteria. While clear guideline criteria are quite easy to incorporate into laboratory SOPs, open guideline items require an adequate documentation on the procedures and criteria to be used, thus having a more substantial impact on SOPs. For instance, EMA guideline topics such as selectivity, carryover, calibration curve requirements, dilution integrity/stability and analytical run criteria are topics containing open or soft items that need to be clearly defined in SOPs.

- ANVISA Resolution RDC 899. The new ANVISA draft guidance describing the minimum requirements for the validation of bioanalytical methods was published for public consultation during the summer of 2011 [26,27]. Many aspects of the new ANVISA guidance are similar to those of the EMA. However, ISR was not mentioned in the draft ANVISA guidance as ANVISA believes that too many primary questions about reproducibility in bioequivalence trials still remain unanswered.

- Health Canada Bioanalytical Guidance. The Canadian Regulatory Agency is adopting the EMA guidelines on BMV; the Canadian authorities did not want to create an additional and potentially conflicting guidance for bioanalytical laboratories to follow. Some requirements found in the EMA guidelines were interpreted considering that the guideline is not the law; alternate approaches may adequately address the regulatory issue. The general message was that common sense and good science should always prevail.

- MHLW Bioanalytical Guidance. Following the 1st Asia-Pacific Bioanalytical Conference [28], the Japan Bioanalysis Forum was formed in early 2011. The Japan Bioanalysis Forum collaborates with the MHLW BMV study group to draft the Japanese guidelines for bioanalysis. The first draft guideline on chromatographic small-molecule analysis is expected to be published in the second half of 2012, and will not be significantly different from the FDA and EMA guidance/guidelines [14,15]. A specific draft guideline on large molecules is scheduled to be issued toward the end of 2012 or beginning of 2013.

- China Pharmacopeia Guidance on Bioanalysis. A review of the draft Chinese guidance (version 2015) components indicates that most of them are similar to those of the new EMA guideline. The draft guidance is presently under discussion in China, and waiting for the input from the new upcoming FDA guidance. The Chinese bioanalytical community is eager to hear from other countries so that they can harmonize their guidance to international regulatory standards [29].

- FDA Guidance on BMV. Although a new draft of the FDA guidance on BMV is not published yet, there is evidence from the topics presented in multiple conferences that the discussions and the best practices used by the bioanalytical industry are well heard by regulatory bodies and may be taken into consideration.

The discussion on global harmonization at the 6th WRIB clearly showed that there is a tangible desire from agencies to render their guidance documents comparable, and to cooperate to ease the flow of global study submission. A single harmonized global guideline does not exist yet; however it is becoming clear that individual guidelines are becoming aligned, and this is for the benefit of all.

Conclusion

Below is a summary of the 16 recommendations (six for LC–MS and ten for LBA) made during the 6th WRIB.

LC–MS recommendations

1 Supplied CoAs for RSM are not always adequate and should contain stability data and shipping conditions. A CoA containing uniform information from all suppliers is desirable.

2 The electronic bioanalytical reports should be harmonized as to the content, not the report format. The 'checkbox' mentality has to be avoided when generating and reviewing reports.

3 As the analysis of large molecules by LC–MS/MS is still relatively new, LBA

criteria could be used until the technology is more advanced, permitting the tightening of the criteria. However, analysis in duplicate, typical for ligand-binding methods, may not be necessary as the LC–MS/MS methods include the use of an IS.

4 When using LC–MS/MS, a higher degree of assay specificity is obtained, and the potential for interference is significantly reduced, except when the molecular weights of the co-administrated drug(s) and the analyte are similar.

5 Presently, the evaluation of ISS is not a regulatory requirement. ISR and stability using QC samples usually give sufficient information to detect a potential for stability problems. The discussion on ISS is ongoing within the bioanalytical community.

6 DBS is recognized as a valuable technique in certain instances. The limitations and challenges of DBS should continue to be explored and the community should take note of the outcomes of EBF, IQ working group, and others who have current effort directed at the DBS topic.

■ LBA recommendations

7 The decision of including or excluding hemolyzed and lipemic sample testing in LBAs needs to be scientifically driven and should be addressed as part of validation.

8 The use of total error is recommended to understand error components. The recommended criteria may be modified based on the limitations of the method and the intended use.

9 The evaluation of dilution QCs in every assay is optional.

10 Automation is quickly evolving and offers several advantages for LBAs.

11 To avoid confusion with the term GLP in biomarker analysis, either 'regulated' or 'nonregulated' bioanalysis should be used as part of nonclinical and clinical biomarker studies.

12 Specificity should be established during method development and may be repeated during validation as a convenient means to document specificity data.

13 More feedback from the industry is required in order to make appropriate recommendations on parallelism.

14 The recommendations provided in this paper are 'best practices' and should not be considered as 'requirements'.

15 Samples should always be treated in a way to preserve their integrity.

16 It is acceptable to run singlicate analysis if method robustness is proven.

Multiple but harmonized bioanalytical guidance/guidelines

The key outcome of this year's workshop was the noticeable effort from major regulatory agencies to align their respective bioanalytical guidance documents/guidelines. This effort represents a major step forward in the achievement of bioanalytical global harmonization by having **multiple**, but harmonized, bioanalytical guidance documents/guidelines. This tangible progress in global harmonization was greatly appreciated by the international audience attending the 6th WRIB as an important achievement for the whole industry to facilitate global submissions of studies.

Appendix: 6th WRIB Conference Report

The 6th WRIB successfully met its objectives by sharing information, stimulating discussions and providing answers on various topics of great importance for the bioanalytical community. More detailed information is available on its website [101].

■ Recent regulatory findings from the industry

The first session of the workshop focused on sharing recent regulatory findings from a scientific viewpoint. Mario Rocci (President, ICON Development Solutions, USA), as a GCC representative, shared, in an open discussion, the GCC views and recommendations on some topics of global interest to the pharmaceutical industry and regulators: the acceptability of preparing calibrants and QC from a single preparation of stock solution; the interpretation of 'freshly prepared' calibrants and

QC; the use of a predefined criteria for samples employed for system conditioning and system suitability; the stability of compounds stated as light sensitive by suppliers; the approach for performing whole-blood stability; findings regarding IS variability; carryover and reporting of validation evaluations [12,30–34,104]. An FDA untitled letter issued following FDA inspections of the bioanalytical facility of Cetero Research reporting dates/times falsifications, manipulation of samples and lack of documentation regarding system equilibration was discussed by Roger Hayes (Vice President and General Manager of Laboratory Sciences, MPI Research, USA). Several key points and corrective actions outlined by Hayes included the importance of avoiding the use of equilibration samples that can be substituted in the official run, to have pre-established written equilibration procedures, and to implement controlled practices and in-process checks to ensure contemporaneous documentation. This situation was also discussed as part of a recent GCC meeting [12]. Eric Woolf (Senior Director, Merck Research Laboratories, USA) presented a thorough reflection on ISR failures, their root causes and the potential impact on data reliability. Steve Lowes (Vice President Scientific, Advion Bioanalytical Labs, a Quintiles Company, USA) presented the results of the GCC survey showing no impact on stability evaluations due to the presence of co-formulated or co-administered compounds. Surendra Bansal (Research Director, Bioanalytical R&D, Non-Clinical Safety, Hoffman-La Roche, USA) continued on the topic of co-formulated or co-administered drugs by exposing the specificity perspective.

■ Recent regulatory findings from the agencies

The 6th WRIB was also the occasion for regulatory agency representatives to present their views and concerns on recent findings and observations. Sam Haidar (Chief, Bioequivalence Branch, Division of Bioequivalence and GLP Compliance, Office of Scientific Investigations, FDA, USA) presented a recent analysis made by the FDA of the inspectional experience involving bioanalytical work over 6 years. João Tavares Neto (Head of Bioequivalence Department from General Office of Drugs, ANVISA, Brazil) shared some statistics regarding the audits performed on CROs by the ANVISA Bioequivalence Department. Olivier Le Blaye (Inspector, Clinical Trials Inspection Unit, French National Agency for Medicines and Health Products, ANSN, France) expressed his views on recent French findings; and Brian Booth (Deputy Director, Office of Clinical Pharmacology, FDA) illustrated some examples of situations observed where the quality of the bioanalytical method plays a critical role in regulatory applications.

■ Innovations in bioanalysis

Following its success last year, a session specifically dedicated to innovation was again included this year. This session shared the latest advancements in instrumentation used for quantitative bioanalysis, from drug discovery to drug development and was chaired by Gabriella Szekely-Klepser (Vice President, Drug Safety Evaluation, Allergan, USA). Mohammed Jemal (Senior Research Fellow, Bristol-Myers Squibb, USA) presented the identification and evaluation of an Fc universal surrogate peptide to enable LC–MS/MS bioanalysis of a diversity of human mAbs and human Fc-fusion therapeutic proteins in preclinical animal studies. Jean Lee (Scientific Director, PKDM, Amgen, USA), described several advantages in developing a general LC–MS/MS method to quantify mAbs and quickly provide preclinical pharmacokinetic (PK)/pharmacodynamic data for candidate selection. Magnus Knutsson (Director, Bioanalysis LC–MS/MS, Ferring Pharmaceuticals, Denmark) reported the different challenges encountered with the LC–MS/MS bioanalysis of peptides, such as multiple-charged ions, extensive MS/MS fragmentation, chromatography issues and adsorption problems. Kevin Bateman's (Director, Merck Research Laboratories, USA) presentation first compared the broad knowledge and experience people have on triple-stage quadrupole versus high-resolution mass spectrometers for quantitative applications. Bateman claimed that the HRMS can be used to provide accurate and precise quantitative results from very complex biological matrices. With a few examples, he demonstrated the benefits of mass accuracy and the stability that HRMS offers. He acknowledged the hardware and software challenges of HRMS that need to be improved. Bateman also highlighted the new Triwave technology available on the Waters G2-S system that looks to be very promising.

■ Method development challenges in small molecules

Many challenges are encountered in the bioanalysis of small molecules. Method transfers, DBS and their associated regulatory challenges, impact of hemolysis on drug stability, challenges and solutions

for antisense oligonucleotides (ASO), and the bioanalysis of ASO therapeutics using LC–MS/MS were discussed at this year's 6th WRIB. Mark Arnold (Group Director of Bioanalytical Sciences Department, Bristol-Myers Squibb, USA), with more than 20 years of experience in outsourcing and thus method transfers to contract research laboratories, stated that the transfer process involves the reference compound, including structural information, and all methods being supplied to the CRO. Only minor adjustments to the method are permitted, except if the laboratory is unsuccessful in replicating the method. Arnold continued by presenting recommendations for the cross-validation of a method. He suggested that each QC or study sample pool should be measured using five replicates, and a statistical analysis of the results should be compared with pre-established acceptance criteria. Eric Yang (Worldwide Head of Bioanalytical Sciences and Toxicokinetics group at DMPK, GlaxoSmithKline, USA), discussed the scientific and regulatory challenges that the bioanalysis community needs to overcome in order to employ DBS sampling techniques over conventional sampling. The effect of hemolysis on bioanalytical methods precision and accuracy has already been discussed in the literature [19], but remains a significant challenge. To demonstrate the effect of hemolysis on drug stability, Fabio Garofolo (Vice President, Bioanalytical Services, Algorithme Pharma Inc., Canada) presented the case of morphine extracted from hemolyzed versus non-hemolyzed plasma. The possible impact of hemolysis on stability was clearly demonstrated. To ensure the integrity of the hemolyzed incurred samples, the stability of a compound in that particular matrix must be investigated. John Grundy (Vice President, PK & Clinical Pharmacology, Isis Pharmaceuticals, USA) talked about the use of LC–MS for quantification of antisense oligonucleotides and concluded that LC–MS analysis is suitable for ASO quantitation in many cases, although applicability (e.g., trough/post-distribution plasma levels) for clinical sample testing is still somewhat limited at present. Laixin Wang (Method Development Group Leader, Tandem Labs, USA) talked more specifically of LC–MS/MS and the challenges of developing an assay for oligonucleotides. He demonstrated that most of these challenges can be overcome and rugged GLP methods could be validated for quantitation of oligonucleotides, and potentially used for sample analysis.

■ Method development challenges in large molecules & LBA

A section pertaining to large molecules and LBA was introduced at the WRIB 2 years ago for the first time, and the focus on this area was expanded considerably in last year's meeting. With the increased demand in this particular field, the 2012 6th WRIB once again further extended its large-molecule and LBA section with more extensive and advanced short courses, a plenary session and roundtable discussions dedicated to this field. This plenary LBA section was chaired by Binodh DeSilva (Executive Director Bioanalytical Sciences-Biologics, Bristol-Myers Squibb, USA), and Russell Weiner (Executive Director, Clinical Development Laboratory, Merck Research Laboratories, USA). Dominique Gouty (Senior Director, Intertek/ALTA Immunochemistry, USA) kindly gave Joseph Marini's (Associate Director, Janssen Research and Development, USA) presentation as he was unable to attend the 6th WRIB. Key scientific considerations and recommendations expressed in the FDA Biosimilar draft guidance released in February 2012 were discussed. The importance of post-translation modifications in the regulation of Biosimilars was outlined, clarifying that glycosylation is one of the most common and complex of all post-translation modifications associated with therapeutic proteins. The PK and anti-drug antibody assessments of biosimilar test versus reference products were presented as different scenarios and discussed. Gouty also presented the new technologies to overcome drug tolerance in immunogenicity assays. She first discussed the impact of immunogenicity on PK for therapeutic proteins and then described the various assay platforms available to test for immunogenicity. She focused her talk on a new advanced technology, the solid-phase extraction acid dissociation (SPEAD)/biotin extraction acid dissociation (BEAD) assay. According to Gouty, this assay is complex as it involves multiple dilutions and steps, but offers several advantages, including drug tolerance up to 5 mg/ml.

Surinder Kaur (Group Leader Bioanalytical Sciences, Genentech, USA) gave an overview of the bioanalytical strategies and challenges associated with antibody–drug conjugates (ADCs). These ADCs have molecular characteristics of both small and large molecules and are used for targeted delivery of a cytotoxic drug to tumor targets. She explained that the ADC structural complexity includes a range of drug–antibody ratio (DAR) distributions and how additional complexities are often generated *in vivo* due to catabolism. She indicated that there are currently no regulatory

guidelines specifically for ADCs, and Genentech is using a fit-for-purpose approach. She talked about the potential to elicit immune responses *in vivo* with ADCs and the importance of measuring anti-therapeutic antibodies (ATAs) to all components (antibody, linker, cytotoxic drug and so forth). Lauren Stevenson (Principal Scientist, Development Translational Medicine, Biogen Idec, USA), focused on the challenges encountered with large-molecule biomarker assays. She specifically talked about the difficulties of purified/recombinant proteins versus endogenous analytes, the characterization of critical reagents, and the 'unique challenges' encountered with most commercial kits. Stevenson's last comment was that you need to understand your assay and its limitations and that the data need to be interpreted accordingly.

Sherri Dudal (Senior Investigator, PK/Pharmacodynamic Bioanalytics, Novartis Pharma AG, Switzerland) presented the Gyrolab™ technology as an alternative tool to the traditional ELISA. She then discussed the points to consider for method development, such as choosing the right antibody pair combination, reagent optimization, the serum effects on the standard curve, the carryover, the precision and reproducibility, the preclinical sample analysis and the microsampling of these samples. In general, the Gyrolab format offers several advantages compared with the ELISA format. However, key differences in the assay acceptance criteria were pointed out and still need to be evaluated. Dudal ended her presentation by comparing the Gyrolab with other platforms and highlighted the major advantages of this platform: the use of small sample volume and the semi-automation.

■ GBC large-molecule harmonization teams update

A parallel roundtable session was specifically dedicated to presentations and discussions from the LBA harmonization teams for common bioanalytical approaches. As part of the GBC, six harmonization teams of scientific experts are specifically focused on the bioanalysis of large molecules. Each team leader or a representative of these teams presented the summary of their recent meeting and discussions. DeSilva encouraged the audience to ask questions and comment on the recommendations during this session, and was successful in getting people to participate and give their opinions on the different topics so that the team can further update the GBC recommendations.

■ Updates of the new guidance/guidelines

Several regulatory representatives addressed the new or upcoming bioanalytical guidance documents from their respective agencies. João Tavares Neto (Coordinator of Bioequivalence from General Office of Drugs, Brazil ANVISA) presented the main items comprised in the new draft ANVISA bioanalytical guideline, and compared them with the previous ANVISA Resolution RDC 899 issued in 2003 [35], as well as to the EMA guideline on BMV effective since February 2012 [15]. An update on the status of the bioanalytical guidance in Japan was presented by Toshinari Mitsuoka (Senior Analyst, Evaluation and Licensing Division, Japan MHLW). Eric Ormsby (Manager, Office of Science, Bureau of Policy, Science and International Programs, Health Canada TPD), informed the group of the two upcoming new Canadian bioequivalence guidance documents: 'Conduct and Analysis of Comparative Bioavailability Studies' and 'Comparative Bioavailability Standards: Formulations Used for Systemic Effects'. These two documents will replace 11 current guidance documents and notices found on the Health Canada website [105]. Jan Welink (Senior Pharmacokinetic Assessor, Medicines Evaluation Board, The Netherlands), representing the EMA, provided his views on several components of the new EMA guideline on BMV. Daniel Tang (General Manager Asia-Pacific, ICON Development Solutions, China) presented the new draft China SFDA guidance on bioanalysis and compared it with the current FDA guidance and EMA guideline on BMV.

Acknowledgements

The authors would like to acknowledge the US FDA, EMA, TPD (Canada), ANSM (France), MEB (The Netherlands), ANVISA (Brazil), MHWL (Japan) and AGES (Austria) for supporting this workshop. B DeSilva (Bristol-Myers Squibb [BMS]), F Garofolo (Algorithme Pharma), G Szekely-Klepser (Allergan), R Weiner (Merck) and M Rocci (ICON) for chairing the workshop. All the workshop attendees and members of the bioanalytical community who have sent comments and suggestions to complete this White Paper. Speakers/panelists: M Arnold (BMS), S Bansal (Hoffmann-La Roche), K Bateman (Merck), R Bauer (AGES), B Booth (FDA), S Davis (PPD), B DeSilva (BMS), S Dudal (Novartis Pharma), F Garofolo (Algorithme Pharma), D Gouty (Intertek), J Grundy (Isis Pharmaceutical), S Haidar (FDA), R Hayes (MPI Research), M Jemal (BMS), S Kaur (Genentech), M Kelley (MKelley Consulting LLC), M Knutsson (Ferring Pharmaceuticals), O Le Blaye (ANSM), J Lee (Amgen), S Lowes (Advion), M Ma (Amgen), T Mitsuoka (Japan MHLW-NIHS), J Tavares Neto (ANVISA), R Nicholson (PPD), E Ormsby (TPD), M Rocci (ICON), J Sailstad

(Sailstad and Associates), L Stevenson (Biogen Idec), G Szekely-Klepser (Allergan), D Tang (ICON), J Welink (Medicines Evaluation Board), R Weiner (Merck), CT Viswanathan (CT Vishwanathan & Associates), L Wang (Tandem Labs), E Woolf (Merck) and E Yang (GlaxoSmithKline). S Martinez, I Dumont, F Landry and C Dicaire (Algorithme Pharma) for collecting the workshop minutes and preparing the first draft of this White Paper. W Garofolo (Meeting Coordinator) for the logistic organization of the event. Workshop sustaining sponsors: Waters, Pharma Medica Research, GYROS, Biotage, Tandem Labs, Intertek Pharmaceutical Services, Thermo Scientific, Worldwide Clinical Trials, mSpec Group, JCL, WIL Reseach Company, Zef Scientific, PPD, Celerion, EMD Millipore, CPR Pharma Services, ICON, Supelco Analytical, PRA International, Advion, Cerilliant, AIT Bioscience, Chemtos, XenoBiotic Laboratories, MSParts, BioPharma Services, MicroConstants, Alliance Pharma, Algorithme Pharma, iStudy Reporter Bioanalytics, Altura Analytics, AB Sciex, MPI Research, Radix Biosolutions, Nuvisan Pharma Services, Randox, Bioanalysis Journal, Peak Scientific, ICF. Media Partner: Future Science Group.

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Financial & competing interests disclosure

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

No writing assistance was utilized in the production of this manuscript.

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