6th GCC focus on LBA: critical reagents, positive controls and reference standards; specificity for endogenous compounds; biomarkers; biosimilars

The 6th Global CRO Council for Bioanalysis (GCC) Closed Forum was held on 27 March 2012 in San Antonio, TX, USA, the day before the start of the 6th Workshop on Recent Issues in Bioanalysis. The attendance consisted of 45 bioanalytical CRO senior-level representatives on behalf of 37 CRO companies/sites from six countries. In addition to following up on the issue of co-administered drugs stability and on recommendations regarding the European Medicines Agency guideline, this GCC Closed Forum discussed topics of current interest in the bioanalytical field with focus on ligand-binding assays, such as lot changes for critical reagents, positive controls and reference standards, specificity for endogenous compounds, qualification and validation of biomarker assays, approach for biosimilars and criteria for LC–MS assays of small versus large molecules.

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The Global CRO Council for Bioanalysis (GCC), a global independent group of CRO leaders conducting bioanalytical work, was formed in September 2010 [1]. Since then, the representatives of the member companies have met periodically in Closed Forums to openly discuss bioanalysis and regulatory challenges unique to the outsourcing industry [2–4]. Tying this session of the GCC to a major bioanalytical event such as the Workshop on Recent Issues in Bioanalysis (WRIB) contributed to the participation of a high number of CRO member companies.

The 6th GCC Closed Forum was chaired by Robert Nicholson, who began with the usual official admonition statement [1]. Then, the participants were introduced, and the GCC's common vision and mission were reviewed, as traditionally done during the opening of all GCC meetings [1,101].

The agenda of the 6th GCC Closed Forum included the following seven topics:

- Bridging and qualification strategies for critical reagents, positive controls and reference standards following lot changes;
- Demonstrating convincing specificity for endogenous analytes;
- Bioanalytical approach for biosimilars;
- Acceptance criteria for LC–MS-based assays for small versus large molecules;
- Qualification and validation of biomarker assays;
- Updates on the GCC Open Letter sent to regulatory agencies on co-administered drugs stability;
- Feedback on the GCC recommendations on the EMA guideline.

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Bridging & qualification strategies for critical reagents, positive controls & reference standards following lot changes

The characterization of critical reagents used as part of ligand-binding assays (LBAs) and the need to bridge or qualify reagent lot changes represent a large and complex topic. Proteins, antigens, bioanalytical matrices and cell lines in cell-based assays need to be obtained from reliable sources, and require constant attention to ensure assay consistency and reproducibility. A need for lot bridging may occur for various reasons, such as lot expiration, poor commercial kit quality and linking results from multiples studies. When the same assay is transferred to different CROs, for instance LBA cell-based assays for biosimilars, bridging becomes crucial. In addition to the number and complexity of situations that may be encountered, the absence of clear guidance increases the challenge of determining an acceptable approach.

Case studies were presented to stimulate discussion regarding the acceptability of a bridging study versus requalification of all critical reagents: consideration was given to the application of correction factors based on the ratio of the mean concentrations between lots. This option is frequently used in immunogenicity studies; however, for home-brewed kits, a revalidation is often conducted, which is more costly and time consuming. For each case, the needs for bridging and qualifying reagents should be evaluated, considering that these needs are primarily client-dependent and may involve significant additional costs. A pertinent article on this topic, which includes a table containing multiple reagents, was recently published [5].

A question was raised during the discussion: is the supplier asked to provide their acceptance criteria when selling critical reagents? Although significant differences obtained in spiked QC samples or in endogenous levels may be an indication of a reagent's performance, it is often very difficult to obtain useful information from vendors regarding performance criteria. In addition, a high-quality reagent lot provided by the vendor does not automatically imply that its performance will remain constant over time when using subsequent lots. These are examples of challenges faced by CROs that sponsors may not be aware of. A procedure for vendor qualification could be put in place in order to help ensure consistency for key reagents, or the robustness of a given reagent could be tested with multiple vendors.

Although revalidation is an acceptable approach to confirm lot suitability, the ideal bridging strategy for LBAs would be the use of incurred samples to evaluate lot-to-lot variability. However, issues are associated with the use of incurred samples such as matrix stability and differences in matrix from patient populations between various countries.

Demonstrating convincing specificity for endogenous analytes

Specificity is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically these might include impurities, degradants, matrix and so forth [6]. Specificity is a concept that cannot be measured directly. It is evaluated empirically in cross-reactivity experiments involving structurally related substances.

Selectivity, on the other hand, is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products and, in the actual study, concomitant medications and other exogenous xenobiotics [7,8].

The results of a GCC survey were presented where GCC members answered questions regarding different aspects of specificity testing for large molecules. Two types of assay interference were defined: 'specific nonspecificity' (i.e., structurally related to the analyte and analyte-dependent) and 'nonspecific nonspecificity' (i.e., structurally not related to the analyte). Through the survey, it was determined that most companies test the specificity during method validation and/or during prevalidation using structurally similar compounds when available. However, the types of interference and cross-reactivity tested to ensure specificity varies significantly among the survey respondents.

Cross-reactivity with structurally related compounds such as protein precursors, endogenous homologs and metabolites is sometimes verified but not in the majority of cases. In most cases, they are not performed due to unavailability of the structurally related compound. The question was raised on how to choose which potentially interfering substances to test? The EMA guideline refers to 'related compounds', which is interpreted as a substance sharing a similar structure, having known similarities and clinically relevant (i.e., endogenous compounds similar to the reference standard), but could also mean a drug expected to be co-administered.

Cross-reactivity to co-administered compounds is more commonly tested by the majority of the respondents. For those performing these experiments, no change in assay performance was noted but, to be on the safe side, testing will continue, even though members were not aware of regulatory comments on this issue.

Through the survey, it was determined that most companies test selectivity in buffer and normal matrix. However, selectivity using diseased matrix is rarely tested. Selectivity is usually tested typically using QC samples at low and high concentrations. However, it was discussed that cross-reactivity should be tested only at medium or high concentrations due to variability of this testing at the LLOQ. Another question raised was how to choose which potentially interfering substances to test? The EMA guideline refers to 'structurally related compounds', which is interpreted as a substance sharing a similar structure, having known similarities and clinically relevant (i.e., endogenous compounds similar to the reference standard), but reference is also made to 'anticipated concomitant medication'.

Regarding the matrix effect (nonspecific nonspecificity), most do not test with lipemic or hemolyzed matrix plasma despite Form 483s issued by US FDA. Some respondents are reactive; that is, will test only if a significant number of samples are found to be hemolyzed or lipemic. More definitive requirements are anticipated in the upcoming draft FDA guidance. According to some of the participants, no impact has been seen on numerous tests except in the case of methods for Alzheimer's disease therapies. It was reiterated that matrix effect from hemolysis and lipemia should be evaluated on a case-by-case basis and a decision be made based on good science.

Lastly, specificity for antidrug antibodies (ADAs) was discussed. According to the survey, they are rarely tested (67% of respondents do not test interference due to ADAs). Of those performing the test, a parallelism approach is used as per regulations. The question was raised as to when this testing should be done. There was no consensus answer other than when it makes sense scientifically, and that results generated need to be reliable to ensure patient safety.

Bioanalytical approach for biosimilars

Biosimilars are complex molecules that are copies of reference products (innovator biopharmaceutical products), which are similar but not identical. Though some differences between the two molecules may not be critical, some of those differences may affect safety and efficacy. It is therefore important that a bioanalytical approach is defined to identify the dissimilarities between biosimilars and innovator's drugs. Three draft guidances on biosimilars were issued in February 2012 by the FDA for comments [9–11].

During the discussion, it was acknowledged that bioanalytical approaches for supporting the immunogenicity studies are more complex than that for pharmacokinetic (PK) studies. Typically, only one assay can be used for PK support, whereas two assays (using both biosimilar and innovator drug) are required for immunogenicity evaluation to evaluate if similar immune responses are observed from the innovator and the biosimilar.

The results of the GCC survey indicate that the majority (69%) of the GCC respondents believe that the current analytical techniques are insufficient to fully characterize biological products as they may not fully characterize protein molecules. But these techniques are thought to be good enough to compare two molecules (innovator vs biosimilar) as they are adequate for the assessment of primary, secondary, tertiary and quaternary structures, detection of post-translational modifications (e.g., glycosylation) and for testing biological potency (*in vivo* and *in vitro*). However, a majority of respondents (85%) felt that current bioanalytical techniques are adequate to support PK/pharmacodynamic (PD) and immunogenicity evaluations of biosimilars.

It was also acknowledged that many challenges have to be overcome when using the analytical and bioanalytical assays for biosimilars. This may include: lack of acceptance criteria for immunogenicity assays as well as lack of a guidance from FDA; statistically defining how similar is similar; and lack of access to detailed product characterization data for the innovator drug, which can lead to some issues during the development of assays (concentration for the innovator not accurate). Therefore, it is recommended to characterize both innovator and biosimilar drugs.

In the survey, the participants were asked how they determine comparability between biosimilars and innovators for qualitative assays (e.g., immunogenicity assays). Generally, it is dealt with on a case-by-case basis. However, the answers were varied and not definitive.

The conclusion drawn from the survey and the discussion established that the current analytical techniques are insufficient to fully characterize biological products. The current bioanalytical techniques can adequately provide

support for PK/PD and immunological evaluations of biosimilars. However, there remain a number of bioanalytical challenges that need to be addressed.

On this last topic, bioassays to assess any difference in the biological activity between the biosimilar and the innovator monoclonal antibody drug were discussed. The requirements from the EMA guideline and the FDA draft guidance documents on bioassay for biosimilars were discussed, followed by a detailed list of the different assays used in comparative *in vitro* studies. A short discussion ensued regarding which of these assays were used by the GCC members. Some members are using assays to evaluate both Fab-associated functions and Fc-associated functions. Others are only testing Fab-associated functions. It was noted that the EMA guideline requires that both Fab- and Fc-associated functions be tested. The FDA guidelines do not have any specific requirement for testing biological activity of monoclonal antibodies. It was concluded that it would be interesting to have the regulatory agencies' feedback on this topic.

Acceptance criteria for LC–MS-based assays for small versus large molecules

As an introduction to stimulate discussion on acceptance criteria for LC–MS-based assays for small versus large molecules, a table on 'Routine Drug Analysis Process and Run Acceptance Criteria', which previously appeared in the 2007 Crystal City III White Paper [12], was presented. Although the different topics presented in the table have been discussed numerous times, discussion has generally been in the context of LC–MS-based assays for small molecules and LBA for large molecules. This discussion focused on which criteria to apply when using LC–MS for large molecules.

The first topic discussed was the use of a single stock solution or two different ones to spike calibrants and QC samples. For LBAs, a single stock is often used. Although this practice has resulted in the issuance of FDA Form 483s for small-molecule studies, LBAs often employ certified solutions for the reference material whereas small-molecule standards are often weighed out in the laboratory running the assay. Many members indicated that for LBA studies they will use a single stock but will prepare two different intermediate solutions from that same stock. Alternatively, two different stocks or two different vials of the same lot are used. It is recognized that the practice of weighing two stocks is easier for small molecules, but it was also acknowledged that the use of two stocks may introduce a bias between calibrants and QC samples. For the LC–MS analysis of large molecules, the members present were leaning towards a consensus on using a single stock but would like further clarification from regulatory agencies as to their reasons for recommending the use of two stocks.

The second discussion was regarding the number of calibrants used in a run and the use of anchor points as often included in LBA methods. Members appeared to agree on the use of six to eight calibrators (or more for multiplex assays). However, this led to discussions on the use of blank samples as part of the calibration curve. In LBAs, blanks are used to monitor plate background and can be used to demonstrate that calibrants are properly diluted by ensuring the lowest calibrator has a response above the background. Anchor points beyond the calibration range are commonly applied to improve curve fit for nonlinear regression models. It was agreed that LC–MS methods tend to use linear equations and, when used for large-molecule analysis, the use of blanks and calibrants should follow the same practices applied to small molecules.

In LBAs, the acceptance criterion for calibrants is a percent deviation of 20–25% from their nominal concentration. Depending on the quality of the reagents, this is sometimes difficult to attain, especially for LLOQ and ULOQ, where 35% relative error is not uncommon. For LC–MS of large molecules, as long as it is demonstrated that the assay is under control, there should be flexibility in the acceptance limits following a fit-for-purpose approach.

Regarding acceptance criteria for QC samples (15–20% for small molecules, 20–25% or more when total error in validation approaches 30–40% for large molecules), wider acceptance criteria for LC–MS analysis of large molecules may be justified if procedures such as immunoprecipitation and/or digestion are used, thereby adding additional complexity to the assay.

Finally, the use of replicate samples was debated. In LC–MS, it is customary to conduct single analysis of samples since internal standards are generally employed to control assay variability. With large molecules, however, manual methods, often requiring high degrees of dilution, have led to the common practice of duplicate analysis. However, if an automated LBA method is used and high precision and accuracy are demonstrated, a single analysis may be considered acceptable. In immunogenicity assays, triplicates are normally assayed for neutralizing antibody determinations due to

the inherent variability of the assay. It was generally agreed that for LC–MS analysis of large molecules, single analysis should be acceptable.

The other topics presented in the aforementioned table (placement of samples, number of QC samples in a batch, multiple analytes in a run, rejected runs and number of runs for validation) were consistent between large and small molecules, and did not bring comments from the CRO community, thus demonstrating agreement on those subjects.

Follow-up on qualification & validation of biomarker assays

The validation of biomarker assays has been discussed in different GCC forums both in Europe and North America over the last few years. Discussions started at the 3rd GCC meeting held in Guildford, UK, in July 2011, continued at the 5th GCC meeting held in November 2011 in Barcelona, Spain, and was also on the agenda of the 6th GCC meeting. The challenges of biomarker validation have been identified and the need to generate an official recommendation for the CRO bioanalytical community confirmed. A subgroup of CROs specialized in biomarker assays has further expanded, discussed and reached consensus and proposed a recommendation on the biomarker validation topic.

At the 6th GCC meeting, a survey on the different aspects of validation for biomarkers sent to European and American CROs was presented and discussed. A White Paper has been prepared containing the GCC recommendations regarding biomarker validation where the three recommended tiers of validation are detailed depending on the type of methods (LBA, clinical analyzer methods and LC–MS methods) [13]. Furthermore, recommendations for selectivity for those methods as well as for a screening method are included.

Updates on the GCC Open Letter sent to regulatory agencies on co-administered drugs stability

The issue of performing matrix stability evaluations in the presence of coformulated or co-administered drugs was debated in the 2010 and 2011 editions of the WRIB [14,15], and the discussion continued at this year's WRIB [16]. This topic, portrayed at the 6th GCC meeting, was also part of the 4th GCC meeting in October 2011, where the results of a follow-up survey initiated by the GCC in August 2011 to collect quantitative data on matrix stability from multiple CRO laboratories were presented. These data suggested a general lack of an impact on analyte stability caused by the presence of coformulated or co-administered drugs [4].

The stability data collected as part of this initiative, which included long-term, short-term and freeze–thaw stability results, indicated no discernable impact upon stability of the primary compound by the addition of any co-administered drug parent compound into QC samples. As a follow-up, the GCC sent a letter to multiple regulatory authorities on December 2011 describing the background of the issue, the results of the GCC survey, the conclusion and subsequent recommendation. The agencies to whom the letter was addressed were Canada's TPD, FDA, France's AFSSAPS, Brazil's ANVISA and Netherlands' MEB. Although the conclusion of the survey suggested that the presence of such compounds has no impact on analyte stability, the letter acknowledged that scenarios may occur where stability could be impacted; for instance, when the presence of the compounds induces significant changes in the matrix. The concluding comment in the 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 GCC members discussed the restriction of these stability experiments only to bioequivalence studies for fixed-dose formulations, since that seems to have been the area of greatest regulatory concern. A clear answer from regulatory bodies would help in defining specific requirements.

Following discussions held at the 6th WRIB between GCC members and regulatory agencies, the GCC members have further discussed this issue and published a White Paper exclusively on this topic [17].

Feedback on the GCC recommendations on the EMA guideline

The EMA guideline on BMV, effective 1 February 2012 [18], was thoroughly evaluated by the GCC as part of previous GCC meetings. The GCC's interpretation of the EMA guideline and a summary of their corresponding recommendations were presented in a White Paper published earlier this year [19]. The 6th GCC Closed Forum was the occasion to provide further feedback on some topics of the EMA guideline that were considered relevant by the GCC.

As per the EMA guideline, dilution integrity should be demonstrated in method validation

using matrix spiked at a concentration above the ULOQ and then diluted (precision and accuracy calculated on at least five values for a given dilution factor). It is not clear whether this is considered validation of a specific dilution factor or validation of the highest concentration that can be diluted with accuracy and precision. One possible methodology discussed by GCC members was the validation of two dilution factors to bracket all expected dilution factors. The EMA guideline also states that the dilution integrity should cover the dilution applied to the study samples, but is unclear as to whether or not the concentration of the dilution QC tested in validation must cover the highest concentration obtained in incurred samples.

Regarding the reanalysis of study samples above the ULOQ, attendees reported having received deficiency letters from agencies in Europe for diluting study samples for reanalysis, despite the fact that the dilution integrity was demonstrated in validation. While the analytical range is established to be as wide as possible to cover the expected study sample concentrations, it is not always possible to anticipate precisely the magnitude of all concentrations that will be obtained in a study.

For the calibration curve range, the EMA requires that it should adequately reflect the concentrations of the study samples and that at least two QC levels should fall within the range of concentrations measured (curve range or QC concentrations may be adjusted, or new QC levels added as appropriate). The EMA defines the medium QC as being approximately 50% of the curve range. Does this refer to the arithmetic or the geometric mean? The use of geometric mean rather than arithmetic mean would result in the medium QC sample having a lower concentration and therefore more likely to cover the incurred sample range in some instances. It was agreed that the requirements for medium QC placement should not be too strict; placement within the range should remain flexible to cover the needs depending on the analytical method and the situation.

Another aspect of the EMA guideline that was discussed was the source of hemolyzed plasma destined for matrix-effect assessments. Such matrices can be purchased from a supplier, but can be expensive and may be hard to obtain in a timely manner. Thus, preparing them in-house is often the preferred option. However, the establishment of the percentage of hemolysis to be tested is challenging; various preparation procedures and hemolysis levels can be selected based on method development and experience. Whatever the hemolysis level tested, there will likely be cases whereby study samples will exceed the tested level. Other analytical parameters may be considered to evaluate if the study sample result can be reported, for example, the response of the internal standard.

Furthermore, hemolysis may potentially affect the analyte stability differently depending on the reason for hemolysis (due to sample collection at the clinical site or during subsequent sample processing). Selecting hemolyzed samples as part of the incurred sample reanalysis evaluation may help to obtain useful stability data. The source of lipemic plasma, prepared in-house or purchased from an external supplier, was also briefly discussed. One attendee suggested that lipemic plasma prepared in-house should be prepared with a level of triglycerides that would be significantly above a 'normal' level, for example, ten-times a normal level, in order to reflect an extreme case.

The issue of claiming compliance to the principles of GLP and GCP for method validation and sample analysis was also discussed. The need for clinical studies in humans and their associated method validations to follow the principles of GCP is mentioned in the EMA guideline. The latter also states that nonclinical studies should be performed in conformity with GLP, and validations used to support these nonclinical studies should follow the principles of GLP. A question was raised about if the terminology 'following the principles of' corresponds to claiming compliance (as appropriate to bioanalysis), or does it mean that it is just used as a quality standard? Although validation may be done in accordance to some aspects of GLP, it is not mandatory to claim GLP compliance for validation work performed to support clinical studies in humans, as these studies are out of the scope of GLP.

The interpretation of these requirements seems to vary widely from one sponsor to another. A case example was reported wherein some sponsors would direct the CRO to stop the analysis of human volunteers that withdraw during an ongoing study, whereas the analysis of the collected samples was allowed via informed consent. This highlights the importance for CROs to carefully examine the requests from sponsors and to make educated decisions towards them.

Future perspective

The GCC will continue to provide recommendations on hot topics in bioanalysis of global interest and expand its membership by

coordinating its activities with the regional and international meetings held by the bioanalytical industry.

The new draft FDA guidance on BMV is not yet available for review, but is expected sometime in the second half of 2012. As soon as this draft guidance is released, the GCC will form teams to review each topic of interest. Each team will then submit their recommendations/interpretations in preparation of the FDA/American Association of Pharmaceutical Scientists Crystal City V workshop on BMV. Seven categories have been defined:

- LC–MS/LBA biomarkers;
- LBA/LC–MS acceptance criteria for large molecules;
- LBA reagents and stability;
- LBA immunogenicity;
- LC–MS/LBA method transfer and cross-validation;
- LC–MS/LBA incurred sample reanalysis and repeated analysis;
- LC–MS/LBA FDA versus EMA guidance evaluation.

The next GCC Closed Forum will therefore be scheduled before or just after Crystal City V in Crystal City, Arlington, VA, USA. Please contact the GCC for the exact date and time of the aforementioned meeting, and for all membership information.

Acknowledgements

The Global CRO Council for Bioanalysis (GCC) would like to thank the following: R Nicholson (PPD) for chairing the 6th GCC Closed Forum. R Nicholson (PPD) for chairing the session 'Acceptance criteria for LC–MS-based assays for small versus large molecules'. A Safavi (BioAgilytix Labs) and D Mamelak (Custom Biologics) for chairing the session 'Bridging and qualification strategies for critical reagents, positive controls and reference standards following lot changes'. R Islam (EMD Millipore at time of the meeting, but presently at Celerion) for chairing the session 'Demonstrating convincing specificity for endogenous analytes'. D Gouty (Intertek) and M Khan (KCAS) for chairing the session 'Bioanalytical approach for biosimilars'. R Houghton (Quotient Bioresearch) for designing the survey, collecting answers and preparing survey results on 'Qualification and validation of biomarker assays', and J Allinson (ICON Development Solutions) for chairing this session. S Lowes (Advion Bioanalytical Labs, a Quintiles company) for chairing the session 'Updates on the GCC Open Letter sent to regulatory agencies on co-administered drugs stability'. T Harter (Covance) for designing the survey, collecting feedbacks on 'GCC recommendations on the EMA guideline', and M Cruz Caturla (Anapharm Europe) for chairing this session. I Dumont and S Martinez (Algorithme Pharma) for taking the minutes of the 6th GCC Closed Forum and drafting the first version of this conference report. All the member company representatives who filled in the numerous surveys used to prepare the discussion of the 6th GCC Closed Forum. All the member companies who have sent comments and suggestions to complete this report. W Garofolo (GCC) for organizing the logistic of the meeting.

Financial & competing interests disclosure

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

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