

Acronyms	
Abbreviation	Definition
ADC	Antibody–drug conjugate
BMV	Bioanalytical method validation
CDR	Complementarity determining region
DAR	Drug to Antibody Ratio
ELISA	Enzyme-linked immunosorbent assay
ISR	Incurred sample reanalysis
LBA	Ligand binding assay
LCMS	Liquid chromatography mass spectrometry
LLOQ	Lower limit of quantification
mAb	Monoclonal antibody
MD	Method development
MDB	multi-domain biotherapeutic
MOA	Mechanism of action
MRD	Minimum required dilution
NAb	Neutralizing antibody
PEG	Polyethylene glycol
Pre-Ab	Pre-existing Antibodies
PD	Pharmacodynamic
PK	Pharmacokinetic
QC	Quality control samples
SPE	Solid phase extraction
WRIB	Workshop on Recent Issues in Bioanalysis

White Paper, the exchanges, consensus and resulting recommendations on 36 recent issues ('hot' topics) in bioanalysis are presented. These 36 topics are distributed within the following areas:

- Small molecules by LCMS:
  - Emerging technologies (three topics);
  - Bioanalytical challenges (height topics).
- Hybrid LBA/LCMS:
  - Large molecules by LCMS (five topics);
  - Antibody–drug conjugates (three topics);
  - Protein biomarkers by LCMS (five topics).
- Large molecules by LBA:
  - Immunogenicity (five topics);

- PK LBA bioanalytical challenges (six topics).

- Electronic Laboratory Notebooks (one topic).

Following the recommendations on the above topics, an additional section of this White Paper focuses specifically on several key inputs from regulatory agencies.

Due to its length, the 2014 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 3) covers the recommendations for Large molecules bioanalysis using LBA and Immunogenicity. Part 1 (Small molecules bioanalysis using LCMS) and Part 2 (Hybrid LBA/LCMS, Electronic Laboratory Notebook and Regulatory Agencies' Input) were published in the *Bioanalysis* issues 6(22) and 6(23), respectively.

## Large molecules by LBA discussion topics

### Immunogenicity

Characterization of immunogenicity responses towards antibody–drug conjugates (ADCs)

Do immunogenicity assays need to be specific for each species (different payload levels)? What is the industry experience with anti-drug antibody (ADA) domain specificity evaluation? Do regulatory agencies expect domain specificity evaluation? Should the industry conduct domain specificity evaluation going forward?

Characterization of Pre-existing Antibody (Pre-Ab) responses

What data are available concerning the specificity of naturally existing antibodies that cross-react with therapeutic proteins and treatment-emergent ADA? What correlative data are available to link IgG-mediated hypersensitivity reactions to treatment-emergent ADA? When is determination of ADA isotype and domain specificity testing merited? What strategies are used to manage Pre-Abs in preclinical and clinical phases?

Challenges with bioanalysis of anti-polyethylene glycol (PEG) antibodies and ADA specific to PEG

What are the challenges encountered when developing assays to detect anti-PEG antibodies (i.e. cross-reactivity of detergents used in assay buffers, matrix interference including human IgG or PEG analogs)? What is the incidence of anti-PEG antibodies among human populations? Does it vary with age, condition and ethnicity? What is the predominant isotype of the anti-PEG antibodies reported in the clinic and in the literature? What mitigation strategies could be used to reduce their potential impact in safety/efficacy? What type of mitigation strategies should be in place when

anti-PEG antibodies are detected in a large number of drug-naïve patients?

#### Neutralizing Antibody (NAb) assays

When is it important to have an assay that identifies NABs? Under what circumstances is a cell-based assay warranted rather than an LBA? What functional endpoints make reasonable NAb assays? What are the most important characteristics for a strong NAb assay?

#### Impact of circulating drug on ADA measurement

What are the industry standards on drug tolerance, its impact on interpretation of ADA results, and mitigation strategies? What are the new approaches to speed up early preclinical immunogenicity assessments?

#### PK LBA bioanalytical challenges

##### Bioanalysis of multifunctional biotherapeutics and biotransformation of biotherapeutics

What are the proper methods to fully characterize the PK of multifunctional domain molecules? What is the fit-for-purpose immunogenicity strategy that should be implemented for this type of molecule? How to evaluate the long-term stability of this type of molecule *in vitro* and *in vivo*? With regards to biotransformation of biotherapeutics, what needs to be measured and why? How to distinguish between charge variants? Does it depend on PK/PD exposure–response relationship? What is the impact of biotransformation (safety, efficacy, stability/half-life)?

##### Challenges with bioanalysis of PEGylated proteins by LBA

What are the assay development and validation considerations for PK assays when there are limited options for specific reagents against PEG molecules? What is the industry standard? What is the effect of PEG on free and total forms?

##### Transition from early to later PK assays

Are there challenges in the transition from early to later assays? What is the current industry practice when changing from valency insensitive assays to assays that measure a dominant/active species? How to manage unexpected PK profiles related to changes in assay format?

##### Overcoming interferences in PK LBA

What are the current practices for initially evaluating LBA interference during assay development and then mitigating these interferences? Are free or bound assays really necessary, especially for preclinical studies? What are the pros and cons of LCMS versus LBA?

#### Key terms

**Immunogenicity:** The ability of a particular biotherapeutic substance to provoke an immune response, either by humoral production of anti-drug antibodies or through cellular-based immune responses.

**Payload:** In antibody–drug conjugates, the payload represents the biologically active cytotoxic drug that is conjugated with the antibody.

**PEGylated proteins:** Proteins modified through covalent attachment of polyethylene glycol polymer chains to improve their pharmacological properties, such as improved drug solubility, increased drug stability, extended circulating life and enhanced protection from proteolytic degradation to achieve reduced dosage frequency without diminished efficacy, and potentially reduced toxicity.

Specifically, do interference issues with LBA justify switching to LCMS methods?

#### PK assay and immunogenicity

What is the impact of ADA on measuring serum drug levels? Is it possible to have a complete dissociation of the antibody/drug complex using a pre-treatment? Will it allow interpretation of an accurate concentration? How are data from free assays being handled, knowing that the equilibrium may be disturbed with dilution? Should ADA interference in PK assays routinely be examined? Do strategies need to be employed to mitigate ADA interference? Should ADA tolerance for PK LBAs be examined/reported?

#### Follow-up on LBA part of the 2013 draft FDA Guidance on bioanalytical method validation (BMV)

How should the nominal concentrations of protein drugs be determined? Is it useful to include additional quality control (QC) samples when study sample concentrations are clustered in a narrow range of the standard curve? For sample analysis using LBA, are we ready to use a single replicate for sample analysis?

#### Large molecules by LBA discussions, consensus & conclusions

##### Immunogenicity

##### Characterization of immunogenicity responses toward ADCs

ADCs belong to the second generation of biotherapeutic compounds characterized by complex multi-domain structure and multistep mode of action. Other examples of multi-domain biotherapeutics (MDBs) are bi-specific antibodies, PEGylated proteins/peptides and fusion proteins. MDBs, including ADCs, are capable of inducing an immune response similar to

any other type of biotherapeutic. Antibody response to ADCs should be carefully evaluated non-clinically and in human studies. A recent publication [7] addressed potential MDB-related risk factors and approaches to be considered when evaluating and characterizing antibody responses to MDBs. Potential compound-related immunogenicity risk factors that are specific for ADCs based on their multi-domain nature are as follows:

- Novel linear and structural epitopes, for example non-human amino acid sequences found on the tether or the linker, inter-domain and linker-domain interface structures and surfaces. Such novel non-human epitopes have an elevated antigenic potential;
- Small molecule toxin attachment to an antibody molecule may result in increased half-life of the toxin and therefore has the potential to increase the incidence or magnitude of an immunogenic response through the improved presentation of small molecule antigen to antigen-presenting cells (hapten effect) [7,8];
- Formation of large immune complexes based on the multivalent nature of antibodies and availability of multiple epitopes on the ADC compound. Large circulating immune complexes may potentially form and lead to risk of immune complex toxicity;
- Plants or bacterially derived toxins can have significant immunogenicity potential.

As ADC compounds are commonly developed for oncology indications, a relatively low patient immune competence should be considered for the overall immunogenicity risk assessment.

During anti-ADC antibody testing, a tier-based approach should be applied, which minimally includes screening and confirmatory tests [9]. Anti-ADC assays with sufficient sensitivity, selectivity and drug tolerance are typically expected. Neutralizing activity of anti-ADC antibody should be considered specifically in the clinical phase of development. Additional characterization of domain specificity of anti-ADC antibody should be considered as dictated by the immunogenicity risk-based analysis for the specific compound. Specificity of the anti-ADC responses to the mAb, the small molecule drug or the drug/linker domains can be determined. Although ADCs are expected to be a heterogeneous mixture of components with variable drug loading, a separate testing of immunogenicity potential for various drug to antibody ratio (DAR) components is not proposed.

Various domain specificity tests have been described including competitive inhibition and separate domain

specific screening tests [10,11]. Typically, availability of a positive control (PC) specifically developed for the respective domain specificity test is expected. Competitive inhibition-based domain characterization assays are typically set up as a confirmatory test where an excess of the domain in question is spiked into the sample. A reduction in the assay signal indicates domain specificity of the anti-ADC antibody. As with any typical confirmatory assay, domain specificity tests require a confirmatory cut-point to be set up during assay development or validation phase [9]. Since the anti-ADC response is polyclonal, a mixed type of specificity should be expected and has been reported [10].

Antibody domain specificity testing enables a better understanding of the nature of the anti-ADC immune response including the identification of critical contributing factors. This understanding could also enable the ability to design improved testing strategies during later compound development phases and could provide valuable information to be applied during compound re-engineering. This testing might also improve the prediction of potential clinical sequelae.

For example, anti-ado-trastuzumab emtansine (T-DM1) antibodies were evaluated in a tiered-based approach [12] with approximately 5% of the patients testing positive. Domain specificity characterization tests showed that majority of the anti-T-DM1 antibodies were directed to epitopes not found on the antibody component of the ADC, but were directed toward the linker-drug or conformational alterations introduced at the point of conjugation. Importantly, there was no significant impact of the anti-T-DM1 antibodies on safety, pharmacokinetics or efficacy [12].

Overall, anti-ADC antibodies with various domain specificities may be induced. The value and requirement for the domain specificity characterization should be dictated by the immunogenicity risk evaluation of the compound driven by specific risk factors and requirements of the development phase of the biotherapeutic. Domain specificity characterization may be introduced in the early clinical studies in order to learn the possible differences in the impact from antibodies specific to individual domains of the ADC. Alternatively, a decision to conduct domain specificity evaluation may be made based on the totality of evidence, including immunogenicity incidence, safety and efficacy observations. A well-defined strategy for anti-ADC immunogenicity evaluation, including domain specificity assessment, is strongly suggested.

#### Characterization of Pre-Ab responses

Pre-Abs are a topic of considerable interest given their prevalence and potential impact on therapeutic proteins. Pre-Abs are defined herein as antibodies that are

reactive to a biotherapeutic and exist in circulation prior to the patient receiving any dose of the biotherapeutic of interest. Detection of Pre-Ab is especially relevant in populations of subjects that have received therapy with similar products (e.g., TNF inhibitors) since cross-reactive antibodies can modify the safety and efficacy of the second (structurally similar) therapeutic. Antibodies developed upon treatment with similar product(s), although pre-existing in nature, belong to a different category. These antibodies are usually categorized as treatment-induced antibodies and represent a different immunogenicity risk level from that of (auto) antibodies detected in treatment-naïve subjects. A Pre-Ab, regardless of its origin, could serve as a base for a memory immune response.

A higher frequency of Pre-Ab is reported in patients suffering from autoimmune conditions, for example, rheumatoid arthritis patients, suggesting that disorders with sustained inflammatory responses can have an impact in the ADA pool. Additionally, antibodies directed against bacterial, viral and yeast antigens pre-exist and can be detected in human sera regardless of their immune status. Although these antibodies are considered part of the natural antibody pool needed to protect the organism against different pathogenic agents, they could potentially cross-react with epitopes present either in the recombinant protein, for example, yeast glycans, or recognize residual impurities derived from the manufacturing process, e.g., bacterial host cell proteins, leading to unwanted antibody-mediated responses.

Autoantibodies are antibodies directed against self-proteins. Commonly detected autoantibodies are antibodies directed against cytokines involved in modulation of the immune system, for example, anti-TNF antibodies in MS patients [13], but can also be antibodies implicated in autoimmune disorders (e.g., rheumatoid factor in rheumatoid arthritis patients, anti-dsDNA antibodies in SLE patients). Cross-reactive Pre-Ab is those that may be induced by exposure to one compound and can cross-react with another compound of similar structure or to an endogenous counterpart. Examples include TNF- $\alpha$  inhibitors and IFN- $\beta$ . In addition to having cited examples of cross-reactivity between structurally similar therapeutics (e.g., betaseron, rebif, avonex), autoantibodies to interferons have also been reported [13,14].

Pre-Ab positive samples are those that confirm positive in the ADA assay according to the conventional multi-tiered approach [9]. It is common for ADA assays to utilize a bridging format. Therefore, in addition to ADA, it is possible for multimeric target proteins to create the bridge and produce false positive results. Since bridging assays that rely on dimeric binding have

the potential to produce false-positive results due to their ability to bridge dimeric or multimeric targets [15], potential for target interference should be examined in assay development. A non-bridge assay format to confirm antibody positive status may be needed. This aspect of assay evaluation is important for both Pre-Ab and post-treatment antibody response evaluations.

When establishing the screening assay cut-point to detect ADA, it is important to differentiate between naïve patients with and without Pre-Ab. The assay cut-point selection is challenging when high ADA signals in the pre-dose samples exist, in particular when there is a significant difference between the assay validation cut-point and the in-study (population specific) cut-point. In these cases, monitoring of treatment emergent titer changes becomes crucial.

Whether Pre-Ab prevalence should be reported in the label is a question still under active discussion. However, there is some consensus that the initial immunogenicity risk assessment should be used as guidance to determine the relevance of the detected pre-Ab. It is agreed that the prevalence of the detected pre-Ab should be described in the final clinical reports. If no post-treatment antibody titer boosting is observed, the clinical relevance of these pre-Ab will depend on the type of adverse events observed in the clinic and whether or not they can be associated to the presence of these antibodies. It is generally recommended that these pre-Ab are reported since they may help understand the nature of the immune response observed in response to the biotherapeutic.

While the same assays are usually employed to assess the presence of ADA in pre- and post-dose samples, in some instances, greater characterization may be required. For example, further characterization may be merited when an unusually high prevalence of pre-Ab is detected during the course of compound development or when significant differences are observed between validation and pre-dose sample analysis. Pre-validation method development should examine and mitigate for interferences mediated by circulating target and cross-reactivity events mediated by rheumatoid factor, soluble dimeric receptors, heterophilic antibodies, anti-carbohydrate antibodies and antibodies to therapeutic components that are analogous to naturally occurring antigens from virus, bacteria and plants. Part of this mitigation may include greater characterization beyond the typical two-tier analysis. All supplemental characterization should be justified and supported by an immunogenicity risk assessment specific to the compound and patient population.

As with all ADAs, Pre-Ab should be understood according to the dosing time course (i.e. titer increases and other maturation events including neutralization)

and monitored for impact on safety and efficacy [16]. Treatment-induced antibody titers should be examined in relation to Pre-Ab titers before treatment to understand 'boosting'. In general, a treatment-induced titer can be defined as an increase in titer over the pre-dose sample titer that is above the variability of the titration assay. However, the clinically relevant titer cannot be determined until clinical immunogenicity data are integrated with the PK, safety and efficacy data.

Another consideration discussed in relation to detection of Pre-Ab responses is the relevance of Pre-Ab response in the context of new technologies and our ability to develop assays with little analytical variability. Researchers and regulators should be aware that development of assays with little analytical variability on highly sensitive technology platforms will discern slight increases in signal above the background noise of the assay as positives. Therefore, when interpreting immunogenicity data, especially in the pre-dose samples, it is imperative that attention is paid to strength of the signal and increase over time after treatment, and factor this into evaluation of clinical impact of immunogenicity. Overall, the risk analysis should provide the most appropriate rationale for the strategies to be used for Pre-Ab characterization, management and approaches to mitigate the potential impact to safety and efficacy of immunogenicity.

#### Challenges with bioanalysis of anti-PEG antibodies & ADA specific to PEG

The prevalence and relevance of anti-PEG antibodies have been a topic of discussion for over a decade during presentations by various institutions at national and international meetings, various publications and the recent inclusion in the 2013 US FDA Guidance on the immunogenicity of therapeutic protein products [17]. PEGylation of therapeutic proteins is a strategy used to diminish the immunogenic potential of certain therapeutics since the presence of this polymer can mask potential antigenic epitopes present in the protein structure. Nevertheless, the extensive use of PEG in the food, cosmetic and over-the-counter treatments are believed to be the reason for the higher incidence of anti-PEG (pre-existing) antibodies among the human population [18–20].

While there still appears to be debate on the best practices for measuring anti-PEG antibodies and the clinical relevance of them, many companies are investing in a diverse set of bioanalytical approaches including the utilization of surface plasmon resonance, direct LBA, bridging LBA [21] and various technologies aimed to amplify signal, IgM, IgG and IgE isotyping assays, commercial PCs, custom PC including humanized PC and more. The one subject that was agreed upon was

that measuring anti-PEG antibody has posed technical challenges due to the repeating motif structure of PEG, the relative low affinity of many anti-PEG antibodies, the comparability of PEG structure to many detergents and the difficulty in producing a relevant PC [20]. There is evidence of clinically relevant anti-PEG antibodies of the IgM and IgG isotype [22–24]. Thus, as with conventional ADA assay formats, anti-PEG antibody assay formats should be able to detect a diverse set of isotypes.

Several PEGylated biologics have been approved and are used as standard-of-care therapeutics for over a decade (e.g., Pegasys and Neulasta), with significant patient benefit and with no direct evidence of adverse events or loss of efficacy attributable to the presence of anti-PEG antibodies. For these and comparable programs, it may not be valuable to launch a full-scale investigation on anti-PEG antibodies. Nevertheless, there is additional clinical evidence showing loss of efficacy and increased incidence of hypersensitivity reactions associated with detectable levels of anti-PEG antibodies of the IgM and IgG isotypes (e.g., pegasparginase and pegloticase) [22–24], suggesting that an adequate bioanalytical strategy may be pertinent to higher risk compounds. As with all ADAs, the bioanalytical strategy should be justified by an immunogenicity risk assessment specific to a given compound and patient population.

It was agreed to follow up in a more concentrated effort to examine the pros and cons of various approaches.

#### NAb assays

Characterization of ADA for neutralizing potential is important to better understand the possible impact ADA can have on the biological function of the biotherapeutic and its endogenous counterpart if applicable. The timing for implementing a validated NAb assay was discussed (e.g., Phase I–III). While it is crucial to have a NAb assay by Phase III clinical development, a risk-based approach must be used in determining the stage-specific needs for any particular program. In the case of high-risk biotherapeutics, it may be important to have an NAb assay in place as early as Phase I program, whereas Phase III may be more suitable for lower risk programs. Risk is defined by the potential for incidence of ADA and the potential severity of the clinical adverse event mediated by them. High incidence alone does not drive risk. Severe consequences, however, could be a major driver in defining risk as in the case with non-redundant compounds where loss of efficacy and/or cross-neutralization of endogenous counterparts could result in severe adverse events (e.g., erythropoietin). If risk and/or mechanism

of immune response is/are not fully understood, it is recommended to collect and bank samples at appropriate time intervals so that analysis can be conducted if needed.

The next topic discussed was when LBA might be considered in place of the more variable and labor intensive functional cell-based assays. The consensus for when LBA may be implemented in place of cell-based assays included: when the biotherapeutic mechanism was based upon a binding event as is the case with most of antagonists. This approach can also be extremely useful in bridging the gap in sensitivity of the binding ADA assay versus a cell-based NAb assay (which typically has significantly less sensitivity). In this case, a researcher is able to utilize an LBA assay format to evaluate the neutralizing potential of the binding antibodies that were detected in the more sensitive binding antibody assays. Activity assays for enzyme therapeutics were not discussed. In any case, an assay format that closely mimics the mechanism of action (MOA) in humans is recommended. Bi-specifics may require two (or more) NAb assays depending on the cumulative MOA of the biotherapeutic. Potency assays frequently provide a good basis for the cell-based NAb assay and many endpoints are suitable, including proliferation, viability, apoptosis and the use of luciferase reporter systems.

This team also discussed whether it was categorically required to have an independent assay for NAb assessment. For example, cumulative PK, PD and ADA data can be informative as to the presence of NAb and could provide as much or even more value than a standalone NAb assay which is typically less sensitive. This strategy might be relatively rare, such as in cases with a definitive absence of immune-mediated safety or clinical consequences, and should be comprehensively described in a well-developed risk assessment.

The last item for discussion concerned an NAb assay confirmatory tier. While only a few laboratories routinely do this, cases were cited where samples are run over Protein A/G columns to isolate the antibodies and remove matrix factors and are then run in NAb assay. This type of confirmation was not thought to be necessary for all programs but should be considered on a case-by-case basis.

#### Impact of circulating drug on measuring ADA

An apparent limitation of ADA assays that commonly use drug to capture the ADA is the potential interference of free drug circulating in the system. This interference is much more relevant to relatively high dosed therapeutics with long half-lives (e.g., mAb, Fc fusion proteins). In nonclinical safety studies and higher dose clinical situations, high concentrations of circu-

lating drug will adversely impact ADA detection. It was agreed that drug tolerance should be thoroughly assessed using a surrogate PC during the ADA assay validation. While the surrogate PC is understood to have limitations in that it is not the best representative of the affinity and avidity of antibody found in the diverse nonclinical and clinical populations, it is presently the best source available for engineering assays that can achieve the greatest tolerance to circulating drug. More common assay strategies used to improve drug tolerance include the implementation of acid dissociation which may be followed by solid phase extraction (SPE) (e.g., SPEAD, BEAD, angiotensin-converting enzyme). A solution ELISA format also has proven to offer much higher drug tolerance even without acid treatment, a step that can sometimes compromise the ability to detect ADAs [25]. This assay format offers advantages as it can be set up using standard immunoassay platforms and is amenable to transfer across laboratories without the need for specialized equipment and training. Others include the use of biosensor systems that do not rely on two arm binding (e.g., Octet, Biacore). Additionally, immune-complex assays, such as that published by Stubenrauch *et al.* in 2010 [26], are innately tolerant to the presence of circulating drug and could serve as useful tools for better understanding of drug and ADA disposition. The last approach discussed was Universal Indirect Species Specific Assay (UNISA), which can be used to accelerate early pre-clinical immunogenicity assessment [27]. If possible, it is always recommended to collect samples for ADA assessment after therapeutic has cleared.

Several therapeutics on the market currently have post-marketing requirements centered around improved drug tolerance. It is questionable whether the increased levels of antibodies that are being detected with these improved assays are yielding clinically relevant results. These data will have to continue to be gathered and compiled to enable general conclusions to be drawn.

Finally, it was the general consensus of this team that data should be reported as 'positive' or 'negative' with the assay caveats discussed in the bioanalytical and/or nonclinical/clinical reports. As assays become more sensitive and drug tolerant, the reporting of 'inconclusive' should no longer be acceptable or standard practice as it pertains to drug tolerance. There was a general consensus that the term 'inconclusive' to report a result adds little value in this case and causes confusion in the minds of other stakeholders of the immunogenicity data (i.e. clinicians who need to interpret safety and efficacy impact, and pharmacokineticists). It should be understood that data from any analytical assay should

be interpreted in the context of limitations of that assay as validated.

#### PK LBA bioanalytical challenges

The PK LBA bioanalytical challenges' discussion spanned a variety of topics and the conclusions are elaborated below. Throughout the discussions, some common themes emerged, many of which ultimately focused on critical reagents; from the practical implications of running more generic assays early in development to the challenges that arise when only suboptimal reagents are available (e.g., lack of high quality, high affinity anti-PEG antibodies).

#### Bioanalysis of multifunctional biotherapeutics & biotransformation of biotherapeutics

The discussion on the bioanalysis of bispecific and multifunctional biotherapeutics focused on the correct choice of method(s) to fully understand the PK concentration–time profiles of multi-functional domain molecules and the application of appropriate immunogenicity strategies. The general recommendation was to adopt the approaches currently employed in the bioanalysis of ADCs, which were discussed in the 2013 White Paper in bioanalysis [6]. For these types of complex molecules, multiple PK assays may be initially required in order to build an understanding of the moiety or moieties that impart clinical impact. This approach requires the generation of multiple critical reagents to enable detection of the multiple domains and potential species. While at the very earliest stages not all assays can be deployed and as reagents become available, additional assays are implemented to enable an understanding of which species are critical to monitor [28]. Once an understanding has been established, measurement of only those relevant species can be performed moving forward. It was also noted that when there are limitations on availability of critical reagents for LBA development, other bioanalytical platforms that are reagent-independent (e.g., LCMS) should be considered for PK- and ADME-related characterization purposes.

A similar approach is recommended for the deployment of immunogenicity assays. Initially, reagent availability limitations may allow only the measurement of ADA for the whole molecule. Once appropriate reagents become available, then the specificity of the ADA response can be tested by performing competition assays to understand the domain(s) to which immunogenicity is directed. Later in development, as for PK assays, the focus can be on clinically relevant measurements.

Moreover, the topic of biotransformation of biotherapeutics and the employment of orthogonal techniques to understand the complexities of biotransformation

were discussed. In this arena it was also agreed that the stage of development dictates which analytes are important to track and the primary goal is to understand the moieties that are active and relevant to safety and efficacy. For example, if metabolites are the active species, then they will need to be measured throughout drug development.

#### Challenges with bioanalysis of PEGylated proteins by LBA

Bioanalysis of PEGylated proteins by LBA highlighted additional challenges associated with complex biotherapeutics. Details and case studies on PEG-specific analyses were reviewed in depth during the workshop. Considerations for PK assay development and validation were similar to those for other complex molecules, with multiple assays employed during the discovery/early development phases as characterization tools to understand relative levels of intact molecule, total protein, free PEG and so on. Once it has been determined that free PEG is not an issue, for example, then there is no need to continue to routinely monitor PEG moving forward, although periodic verifications can certainly be performed if desired.

Critical reagents pose a particular challenge in this arena as PEG reagents are notoriously suboptimal, which limits their utility. No single anti-PEG antibody works well for all PEGs, although some may work well for a subset of applications. There is therefore a need to generate multiple anti-PEG antibodies with no guarantee that they will be adequate to achieve the desired assay sensitivity. Therefore, once characterization assays have been completed, a single assay that measures only the total protein is preferred when moving forward into the regulated environment. If deemed prudent, then verification of PEG integrity can be performed if desired.

There were no specific recommendations for preferred assay formats in the analysis of PEGylated proteins. Instead, it was highlighted that the assays employed during characterization may span multiple formats/orthogonal methods.

Lastly, it was discussed whether free PEG is considered as source of potential interference in the PK assay. The agreement was that to date, this has not been considered an issue, generally because free PEG levels relative to intact protein levels are believed to be extremely low. It was also noted that if significant levels of free PEG were a concern, this would have been identified when characterization experiments were performed during the discovery phase.

#### Transition from early to later PK assays

Discussions on complexities associated with transition from early to later stage assays again began with

an acknowledgment that changes in assay formats are inevitable as drug development progresses, particularly for complex modalities. Assays in support of earlier phases will typically be restricted by the availability of critical reagents. For example, preclinical PK studies may be supported using a generic PK assay, or in the case of an ADC, the total molecule assay may be the only available assay. As development progresses and reagents are generated, more specific or multiple assays may be employed. For complex biotherapeutics, a series of assay changes may occur, starting with fewer assays based upon available reagents, then transitioning to multiple assays while building an understanding of the most relevant biotherapeutic species, and then finally scaling back to fewer assays again once the most relevant species to monitor throughout later stages of development have been identified. It was agreed that it is likely that changes in PK profiles will occur as new, more specific critical reagents become available with the evolution of methods. In fact, this should be expected when the final assay format moving forward is focused only on measuring the specific species of interest rather than multiple species. The consensus view was that it is not necessary to have the same result from different PK assays, but rather that there should be a scientific understanding of the species that the assays are measuring so that results can be interpreted appropriately. It was noted, however, that when feasible, keeping the dose-ranging assay and the first-in-human assay as similar as possible will simplify interpretation.

#### Overcoming interferences in PK LBA

A comprehensive overview and specific case studies on overcoming assay interferences were discussed, which mainly focused on predicting interferences, evaluating them during assay development and validation (both pre- and in-study), and strategies to mitigate interferences. Certain interferences can be predicted based on the assay format and available critical reagents, with examples being interferences from soluble target or ADA. These predicted interferences therefore play into reagent and assay format selection. The recommendation was to be aware of what may potentially be encountered and evaluate accordingly. However, since these pre-study evaluations will be imperfect, close attention should be paid to data from 'real' samples and any unusual or unexpected results should be investigated accordingly. For example, evaluating potential interference of soluble ligand with recombinant soluble ligand may not accurately reflect the effects of endogenous ligand, especially if the therapeutic has been raised against the recombinant ligand. When unpredicted interferences arise from matrices, different assay platforms may need to be evaluated to mitigate

the nonspecific binding. For example, switching from a conventional plate-based format to a microfluidic system was discussed during the workshop.

Robust discussion occurred and no clear consensus was achieved regarding whether free (measuring unbound and partially bound, or active, therapeutic) or total PK assays [29] are preferred within the scope of interference-related issues; especially in the context of preclinical studies. In the discovery and preclinical spaces, some companies are primarily using free assays while others are running total assays. Interestingly, some companies have switched their practices in recent years, indicating that this is an evolving area. However, there was an agreement that availability of reagents frequently drives the decision on assay format selection as do the opinions of the PK scientists, PK/PD modelers and toxicologists. It was also agreed that there are pros and cons associated with both total and free assays. The group also highlighted the need to be cognizant of the perturbations in equilibrium caused by dilution of samples. For example, high concentration samples requiring large dilutions may drive bound drug to the unbound state and measurements derived from a free assay format may therefore include a proportion of drug that was in a bound state *in vivo*.

The pros and cons of employing LCMS versus LBA and whether interference issues with LBA justify switching to LCMS methods were also discussed. It was agreed that there may be multiple approaches that could be applied to mitigate interference issues, including LCMS. Whether it is justified to switch to LCMS would need to be determined on a case-by-case basis. In the panelists' personal experience, LCMS has more frequently been employed as a tool to facilitate understanding of the interferences. With that understanding in hand, then the appropriate assay format is chosen and implemented. It was agreed that it is desirable to deploy only one assay, rather than multiple assays, especially in later stages of clinical development.

#### PK assay & immunogenicity

During the discussion on interferences, particular attention was given to ADA interference in PK assays [30,31]. Dissociation techniques, such as acid treatment, are frequently employed in ADA assays to increase the drug tolerance of the assay. Such techniques can also be used to mitigate ADA interference in PK assays. While it is theoretically possible to achieve complete dissociation, it was agreed that maintenance of this dissociation is questionable. In addition, acid treatment may inactivate the therapeutic and result in reported drug levels being lower than they actually are. For example, acid treatment may fully dissociate complexes, but some re-association will occur upon neutralization.



Therefore, the recommendation was to be aware of the limitations of both the dissociation method and the assay. It was also noted that, practically speaking, differences in measurement of samples that are nearly completely dissociated versus truly completely dissociated may be negligible if the differences are within the assay's bioanalytical variability.

Given that PK assays often employ anti-idiotypic antibodies or soluble target as capture reagents, it is widely acknowledged that ADAs have the potential to interfere in the assay [31]. In addition, ADA to other epitopes of the therapeutic can also interfere with binding of the detection reagent or cause steric hindrance. However, routine assessment of ADA interference in PK assays is not recommended. Instead, it is important to understand the PK assay and what it is capable of measuring and to also consider data from other relevant assessments (e.g., PD and efficacy). Although there may be times where there is good scientific rationale to evaluate for ADA interference, there are caveats with these evaluations since the PCs that are typically employed are imperfect surrogates for genuine ADA. The assessments are therefore not expected to provide meaningful information with respect to *in vivo* responses. Additionally, there is no need to routinely employ strategies to mitigate ADA interference, although there should be an understanding of how ADA may impact PK results.

#### Follow-up on LBA part of the 2013 draft FDA Guidance on BMV

The appropriate calculation of the nominal concentration of protein therapeutics was one of the points discussed in relation to the draft FDA Guidance. It was agreed that protein concentrations should be derived from mass-based methods only. It was indicated that this recommendation is in alignment with best practices at their companies. While protein purity and activity are typically assessed as part of lot release processes, no adjustment in nominal protein concentration should be made.

The question on whether it is useful to include additional QC samples when study sample concentrations are clustered in a narrow range of the standard curve was debated. At the 2013 AAPS Workshop (Crystal City V), it was agreed that ideally the bulk of sample results will be bracketed by QC samples with an acknowledgement that, generally speaking, LBA QC samples tend to already be 'close' to each other. Additionally, when sample dilutions are chosen to target sample results in the middle of the curve then it is expected that data will cluster there. It was further clarified that clustering of sample concentrations in the middle of the calibration curve is actually desirable.

Furthermore it was agreed that there is no scientific rationale for inclusion of additional QC samples in PK LBAs as it is not possible for the entire standard curve and all the QC samples to pass criteria and yet have a subsection of the curve be inaccurate. It was agreed that adding more QC samples would neither add value nor alter the assay results. However, special consideration was given for cases where the bulk of the data fall between Low QC and the LLOQ. This would occur rarely for LBA PK assays and only if all samples from a given study were tested at minimum required dilution only and the results were unexpectedly low. It was agreed that in this case, the suitability of the assay's range would be in question. In contrast, exploratory biomarker assays may commonly have sensitivity challenges that will result in data clustered at the lower end of the standard curve. In these cases, the level of decision making supported by the data would be impacted. Improvements in the assay would be required if the biomarker continued to be pursued into later stages of drug development and was intended to inform clinical decisions.

As new technologies and automation capabilities become available, and as assays evolve and better reagents become available, the question arises as to whether the industry and regulators are ready for the implementation of singlet analysis for LBAs. This topic was also discussed at the 2013 AAPS Workshop (Crystal City V) and the recommendations herein are consistent with the expressed industry position at that conference. Essentially the decision to employ singlet analysis should be approached in a risk-based manner and implementation left to the judgment of the bioanalytical team. There was consensus that singlet analysis should be acceptable if scientifically justified (e.g., based on the performance of the assay used). It was also noted that the generation (and presentation) of data to support singlet analysis will help to gain traction as an acceptable practice.

The overarching conclusion on LBA bioanalytical challenges was that good science must drive decision making whereby strong scientific rationale and relevant data sets should be generated to justify the decisions made. In effect, good science wins the day. Over the course of discussions, the recommendation of "*Do what you have to do to understand what you need to understand to ensure you do the right thing*" was developed. There is some merit in this superficially simplistic view. To illustrate:

- Do what you have to do (e.g., run multiple assays/formats/orthogonal methods);
- To understand what you need to understand (e.g., clinical relevance – efficacy/safety);

- To ensure you do the right thing (e.g., measure the relevant analytes);
- At the right time (needs in the discovery phase differ from needs in late stage drug development).

Another theme that arose through multiple discussions over the course of the workshop was “*don't do something just because you can*”. The recurring recommendation was to be scientifically driven. When anomalous data arise, bioanalytical scientists need to ensure that they not only understand the assays and their limitations but also the biology of the pathway the therapeutic molecule is targeting. Although any issues that may be attributed to assay limitations will need to be mitigated, consideration should be given also that the assay may not need to be fixed. It may in fact be imparting important information related to the biology.

### Conclusion

Below is a summary of the recommendations made during the 8th WRIB.

### Large molecules by LBA recommendations

#### Immunogenicity

- ADCs are only one of many classes of multiple functional domain biotherapeutics. As with other compounds with complex multi-domain structures and potentially multistep modes of action, ADCs require a series of immunogenicity tests to fully understand the immunogenic profile. The current thinking is that specificity testing should be conducted for each domain with a functional endpoint, for example, the antibody and payload. Further testing may be merited based upon a customized risk-assessment.
- Pre-Abs are antibodies that bind to therapeutic before the patient has undergone therapy. Prevalence and impact should be examined during the course of immunogenicity testing. In some circumstances, additional characterizations may be required (e.g., antibody verification, isotyping, epitope reactivity and/or functional domain reactivity).
- The measurement of anti-PEG antibodies has been addressed as being technically challenging due to the repeating motif structure of PEG, the relative low affinity of many anti-PEG antibodies, the comparability of PEG structure to many detergents and the difficulty in producing robust and relevant PCs. Although the bioanalytical methods remain

imperfect and non-harmonized across the industry, enough case study data exist where anti-PEG antibodies have been shown to have adverse effects on therapeutic efficacy such that routine assessment is needed for all PEGylated biotherapeutics. The bioanalytical community should continue to publish and share data on this topic since PEG is a common structure used by many companies and industries to yield various advantages.

- NAb assays are required to support Phase III clinical testing and, depending on the customized risk assessment for a given compound, may be merited earlier. A functional bioassay is frequently preferred but there may be several situations where a binding assay may be more suitable, for example, when the MOA is based upon binding or there is large difference in the sensitivity of the binding ADA assay versus a cell-based NAb assay. Additionally, it can be quite useful to have PD markers which also can indicate the presence of NAb and to establish therapeutic activity *in vivo*.
- Drug tolerance is a frequently encountered challenge amongst relatively high dosed therapeutics with a long half-life. However, a great deal of progress has been made within the bioanalytical community in the last decade concerning the technical hurdles, possibly due to the number of post-marketing requirements from health authorities, thus making drug tolerance a routine objective for all clinical testing.

#### PK LBA bioanalytical challenges

- For the bioanalysis of bi-specific and multifunctional biotherapeutics, multiple PK assays using several critical reagents may be required at an early stage in order to build an understanding of the moieties that impart clinical impact. Once an understanding has been established, measurement of only those relevant species can be performed moving forward. The stage of development dictates which analytes are important to monitor and the primary goal is to understand the moieties that are active and relevant to safety and efficacy.
- The assays employed during characterization of PEGylated proteins by LBA may span multiple formats/orthogonal methods. While still in the discovery phase, multiple assays should be employed as characterization tools to understand relative levels of the different free and bound forms present. Because of the difficulty in obtaining anti-PEG antibodies that allow for desired assay sensitivity, once characterization assays have been completed,

a single assay that measures only the total protein is preferred when moving forward into the regulated environment.

- Changes in assay formats are inevitable when moving from early to later stage assays. Assays in support of earlier phases will typically be restricted by available critical reagents. It is likely that changes in PK profiles will occur as new, more specific critical reagents become available with the evolution of methods. It is not necessary to have the same result from different PK assays, but rather that there should be a scientific understanding of the species that the assays are measuring so that results can be interpreted appropriately.
- Certain interferences in LBA can be predicted based on the assay format and available critical reagents, therefore one should be aware of what may potentially be encountered and evaluate/investigate accordingly. There are pros and cons associated with both total and free assays, and there may be multiple approaches that could be applied to mitigate interference issues, including the use of LCMS. It is desirable to deploy only one assay, rather than multiple assays, especially in later stages of clinical development.
- Dissociation techniques, such as acid treatment, are frequently employed in ADA assays to increase the drug tolerance of the assay. As the maintenance of this dissociation is questionable, the limitations of both the dissociation method and the assay should be considered. Moreover, acid treatment may inactivate the therapeutic and result in reported drug levels lower than they actually are. Routine assessment of ADA interference in PK assays is not recommended; however, it is important to understand the PK assay and to also consider data from other relevant assessments (e.g., PD and efficacy). Additionally, there is no need to routinely employ strategies to mitigate ADA interference, although there should be an understanding of how ADA may impact PK results.
- In relation to the LBA section of the 2013 draft FDA Guidance on BMV, while protein purity and activity are typically assessed as part of lot

release processes, protein concentrations should be derived from mass-based methods only. Regarding the inclusion of additional QC samples in PK LBAs, this is not considered to add any scientific value nor alter the assay results. Finally, the decision to employ singlet analysis for LBAs should be approached in a risk-based manner. Singlet analysis should be acceptable if scientifically justified (e.g., based on the performance of the assay used).

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## 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 2 – hybrid LBA/LCMS, ELN & regulatory agencies' input)

The 2014 8th Workshop on Recent Issues in Bioanalysis (8th WRIB), a 5-day full immersion in the evolving field of bioanalysis, took place in Universal City, California, USA. Close to 500 professionals from pharmaceutical and biopharmaceutical companies, contract research organizations and regulatory agencies worldwide convened to share, review, discuss and agree on approaches to address current issues of interest in bioanalysis. The topics covered included both small and large molecules, and involved LCMS, hybrid LBA/LCMS, LBA approaches and immunogenicity. From the prolific discussions held during the workshop, specific recommendations are presented in this 2014 White Paper. As with the previous years' editions, this paper acts as a practical tool to help the bioanalytical community continue advances in scientific excellence, improved quality and better regulatory compliance. Due to its length, the 2014 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 2) covers the recommendations for Hybrid LBA/LCMS, Electronic Laboratory Notebook and Regulatory Agencies' Input. Part 1 (Small molecules bioanalysis using LCMS) was published in the *Bioanalysis* issue 6(22) and Part 3 (Large molecules bioanalysis using LBA and Immunogenicity) will be published in the *Bioanalysis* issue 6(24).

The 8th Workshop on Recent Issues in Bioanalysis (WRIB) was hosted in Universal City, CA, USA on March 10-14, 2014. The workshop included three sequential core workshop days and six training courses which together spanned an entire week in order to allow exhaustive and thorough coverage of all major issues in bioanalysis. This gathering brought together close to 500 professionals, representing over 200 companies, to share and discuss current topics of interest in the field of bioanalysis. Attendance included a wide diversity of industry experts from pharmaceutical and biopharmaceutical companies, contract research organizations (CROs) and multiple international regulatory agencies.

The actively contributing chairs in the 2014 edition of the WRIB were Eric Fluhler (Pfizer, USA), Olivier Le Blaye (ANSM, France), Dawn Dufield (Pfizer, USA), Lakshmi Amaravadi (Biogen Idec, USA), Lauren Stevenson (Biogen Idec, USA) and Fabio

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Acronyms	
Abbreviation	Definition
ADME:	Absorption, distribution, metabolism, and excretion
BMV:	Bioanalytical method validation
CRO:	Contract research organization
CoA:	Certificate of Analysis
DAR:	Drug to Antibody Ratio
ECM:	Electronic content management
ELN:	Electronic laboratory notebook
eTMF:	electronic trial master file
ISR:	Incurred sample reanalysis
LBA:	Ligand binding assay
LCMS:	Liquid chromatography mass spectrometry
LLOQ:	Lower limit of quantification
mAb:	Monoclonal Antibody
MIST:	Metabolites in safety testing
MD:	Method development
MOA:	Mechanism of action
PEG:	Polyethylene glycol
PD:	Pharmacodynamic
PK:	Pharmacokinetic
QC:	Quality control samples
SIL IS:	Stable isotope-labeled internal standard
SPE:	Solid phase extraction
WRIB:	Workshop on Recent Issues in Bioanalysis

As with prior WRIB editions [1–6], a substantial number of topics were addressed during the workshop and distilled into a series of relevant recommendations. In the present White Paper, the exchanges, consensus and resulting recommendations on 36 recent issues ('hot' topics) in bioanalysis are presented. These 36 topics are distributed within the following areas:

- Small molecules by LCMS:
  - Emerging technologies (three topics);
  - Bioanalytical challenges (eight topics);
- Hybrid LBA/LCMS:
  - Large molecules by LCMS (five topics);
  - Antibody–drug Conjugates (three topics);
  - Protein biomarkers by LCMS (five topics);
- Large molecules by LBA:
  - Immunogenicity (five topics);
  - PK LBA bioanalytical challenges (six topics);
- Electronic Laboratory Notebook (one topic).

Following the recommendations on the above topics, an additional section of this White Paper focuses specifically on several key inputs from regulatory agencies.

Due to its length, the 2014 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 2) covers the recommendations for Hybrid LBA/LCMS, Electronic Laboratory Notebook and Regulatory Agencies' Input. Part 1 (Small molecules bioanalysis using LCMS) was published in the *Bioanalysis* issue 6(22) and Part 3 (Large molecules bioanalysis using LBA and Immunogenicity) will be published in the *Bioanalysis* issue 6(24).

### Hybrid LBA/LCMS discussion topics

#### Large molecules by LCMS

**Immunoaffinity & sample preparation/enrichment**  
What are the most promising non-antibody affinity technologies, and what challenges exist to their wider application (e.g., Protein A and G, immobilized metal affinity chromatography [IMAC])? What affinity enrichment platform gives the best flexibility, cost and throughput (e.g., beads, tips or plates)? Will plates ever be a viable platform for affinity enrichment? What is the industry experience? Is one affinity technology more compatible to automation?

#### Advances in protein digestion & universal peptide for Fc containing biotherapeutics

The need for pre-digestion treatment seems to follow two schools of thought: some believe that pre-digestion treatment is essential to getting good assay reproducibility while others say they have had good success with the pellet digestion without any pre-digestion treatment. What is the recommended approach? In what type of studies should the universal peptide approach be used rather than developing a new assay? What are the recommendations on the use of a secondary peptide? Is a secondary peptide necessary for monoclonal antibody (mAb) assays, or is there sufficient data to use a single surrogate peptide?

#### Cross-validation LBA/LCMS

What are the primary reasons for conducting a cross-validation between LBA and LCMS? At what stage(s)

of development should LBA/LCMS cross-validation be conducted? In cross-validating between LBA and LCMS, what samples should be used? What acceptance criteria should be applied? As molecular complexity increases, should certain molecules always be investigated using both LBA and LCMS? Should the form of the molecule that is being quantified (i.e. free/total, catabolites/metabolites) always be determined? How much validation work should be done on the secondary method (LBA or LCMS) to establish selectivity of the primary method (LBA or LCMS)? What is the best strategy for understanding why two methods don't agree? What should the acceptance criteria be to define agreement?

#### BMV of LM by LCMS

What are the recommendations on the validation criteria to be applied in current LM by LCMS work and why (i.e. LBA versus LCMS)? Should validation-dependent flexible criteria be applied? How does the application of immunopurification to LCMS methods impact the validation criteria? Should immunocapture-coupled methods use less strict criteria (e.g., LBA criteria)? What are the steps currently recommended to ensure that the LCMS method is measuring the intact protein molecule? What is the industry experience and recommendation on the impact of ADAs on LCMS methods? What are the steps that may be used in validation to measure the resistance of an LCMS protein method to inhibition by competitively binding circulating ligands and ADAs that may be in the matrix at various levels during a study? What is the recommendation on methodologies for determining and evaluating digestion efficiency during the validation of a method for a large molecule by LCMS?

#### PEGylated protein quantification by LCMS

How to overcome the interference of PEGylation at target protease cleavage sites? PEGylated protein solubility in organic solvents allows protein precipitation for extraction of PEGylated proteins. Is it always possible to use this approach? What is the industry experience? Is intact PEGylated protein quantification by LCMS a viable strategy? When are denaturation and/or reduction of PEGylated proteins recommended?

#### Antibody–drug conjugates

##### General considerations for bioanalysis of antibody–drug conjugates (ADCs)

What are the biggest challenges with ADC bioanalysis? What are the most commonly accepted approaches? What regulatory guidance should be followed for ADC quantification? What is the naming convention for different analytes? Can different matrices be used for different assays or for the same assay used at different stages of drug discovery and development (preclinical and clinical)?

**Payload quantification by LCMS in ADC bioanalysis**  
Is the payload considered a 'small molecule'? What specific analytes should be quantified? How should pre-existing concentrations of unconjugated payload in reference materials be handled? How should co-existing high concentrations of ADCs and unconjugated payloads be dealt with in *in vivo* samples in stability experiments? Does Metabolites in Safety Testing (MIST) guidance apply to measuring unconjugated payload in pharmacokinetic (PK) samples after ADC administration? Which types of IS are suitable for unconjugated payload quantification? Are there specific Incurred Sample Reanalysis (ISR) considerations? Comparing/cross-validating LBA versus LCMS data for payloads. Are the acceptance criteria for payload LCMS assays the same as for small molecules or can they potentially be widened if scientifically justified?

#### 'Hybrid' assays for ADC bioanalysis

What are advantages and disadvantages of 'hybrid' versus LBA assays for ADCs? Which types of IS are suitable for 'hybrid' conjugated payload quantification? What are the regulatory requirements or preferences for ADC quantification by 'hybrid' versus LBA assays?

#### Protein biomarkers by LCMS

##### Choice of platform for bioanalysis of biomarkers

When should an LCMS assay be considered rather than a LBA for the measurement of large molecule biomarkers? Or, when is it better to use LBA? Can guiding principles around when to select one platform over the other be established? Is the driver sensitivity or selectivity? What data will be needed in the study? What resources are available? What prior experience is available with either platform for this biomarker?

##### Bioanalysis of biomarkers in tissues

Does LCMS provide a unique workflow that is more suitable for tissue bioanalysis of protein biomarkers compared with LBA? What is the current precedence in this area? Have comparison studies been reported

#### Key terms

**Electronic Laboratory Notebook:** A computer-based electronic system designed to replace paper laboratory notebooks used for documenting research, experiments and procedures performed in a laboratory.

**PEGylated proteins:** Proteins modified through covalent attachment of polyethylene glycol polymer chains to improve their pharmacological properties, such as improved drug solubility, increased drug stability, extended circulating life and enhanced protection from proteolytic degradation to achieve reduced dosage frequency without diminished efficacy, and potentially reduced toxicity.



between LCMS and LBA? Is there a difference in bioanalytical approaches for soluble and membrane associated proteins?

#### Biomarker measurement by co-immunoprecipitation

How do novel co-immunoprecipitation approaches allow access to measurement of bound biotherapeutic or endogenous binding partners of the target? What are the new opportunities that can be explored in this area? What synergies with other measurements can be expected?

#### Biomarker assay sensitivity

What are the novel techniques the industry should consider to boost LCMS sensitivity to develop and validate large molecule biomarker assays? This includes sample preparation workflows and options for instrument configurations. Are there any emerging trends or best practices?

#### Comparison of LCMS & LBA measurements for biomarkers

What is the recommended protocol design to compare biomarker values obtained by LCMS versus LBA? What is the project driver to compare results from different platforms? What criteria are used to judge comparability of results?

### ELN discussion topic

#### ELN implementation

What would be the recommended approach for the transition from a paper-based to an electronic system to optimize workflows? What is ELN's actual impact in improving management review and compliance? Would data integrity increase due to ELN? How to establish requirements that are meaningful for the scientist and programmer? What is the recommendation on how to select a technology platform that fits with the corporate culture? What to consider when planning for ELN system upgrades?

### Hybrid LBA/LCMS discussions, consensus & conclusions

#### Large molecules by LCMS

##### Immunoaffinity & sample preparation/enrichment

Increasingly, immunoaffinity sample preparation is playing a role in the bioanalysis of large molecules by LCMS. There have been many recent publications that use various monoclonal or polyclonal antibodies as well

as ligands either online or offline for enrichment at both the protein and peptide levels. It was highlighted that anti-human Fc is used as a more 'generic' pull down enrichment approach for Fc-containing biotherapeutics, especially when used in preclinical species. There was significant interest and discussion on the advantages and utility of affinity enrichment steps. This particular area has gained a lot of attention in recent years and many are leveraging the power of this hybrid approach. In addition to antibody-based immunoaffinity enrichment platforms, several groups are using some non-antibody affinity technologies. Protein A and Protein G are two of the more commonly used supports to enrich antibodies as well as IMAC for enrichment of phosphorylated species prior to LCMS quantification.

The topic over which enrichment platform offers the best efficiency, cost, throughput, etc. was discussed. Many had experience with and preference for bead-based enrichments, as these seemed to offer significant flexibility with respect to capacity, automation and vendor choices. Many groups like the magnetic bead approach utilizing either streptavidin or Protein A or G coupled beads, while others preferred agarose bead-based solid supports. The streptavidin bead was noted as being simple to use with high binding efficiencies that were considered superior to Protein A/G beads; however, it was highlighted that the use of streptavidin beads required either purchasing biotinylated antibodies or performing a biotinylation step, sometimes making the Protein A/G approach more attractive. Furthermore, there was some discussion of the need for crosslinking when using Protein A/G. It seems more people did not cross-link when using this approach on beads; however, cross-linking is necessary when utilizing a regenerating column-based approach.

The other platforms discussed were column-based approaches, affinity tips and plate-based approaches. It was highlighted that column-based approaches work very well for peptide enrichments. However, protein immunocapture is typically not carried out using an online column-based approach, but tips and plate-based techniques have been used offline at the protein or peptide level. There was not enough experience reported with the tip-based approaches, except to highlight they were considered very costly when compared with alternative technologies. Some groups have experience with plate-based enrichments and found them useful in certain situations, as these approaches utilize already existing immunoassay based workflows but may have limited capacity and dynamic range for a more global utilization. In general, there is no preference over which platform is the most compatible to automation, as beads, tips, columns and plates can all be easily automated.

### Advances in protein digestion & universal peptide for Fc containing biotherapeutics

The bioanalysis of proteins by LCMS typically requires the protein to be digested into smaller peptide fragments and quantified with a surrogate peptide approach, as peptides are more amenable to current LCMS workflows. Over the last few years, several researchers have identified a universal set of peptides from the human Fc region of an antibody, which can be quantitated as surrogates. Therefore, these peptides represent a universal or generic LCMS assay to quantify human mAbs or fusion proteins containing the human Fc region in nonclinical studies (e.g., rat, dog, mouse, etc.), where the exact peptides are not present. The consensus on the application of this approach is that it is useful in early discovery and non-GLP studies. However, when projects move to the clinic, it was recognized that a more specific assay, such as utilizing peptides from the CDR region, would be necessary.

There has been much debate over how many peptides are necessary for quantification of a protein via the surrogate peptide approach. This concept was discussed and it was agreed that typically only one peptide should be used for quantification; however, other 'monitoring' peptides would be useful in structural characterization of the analyte as well as troubleshooting the assay, particularly during validation. The specific question was whether there are enough data collected on mAbs specifically that would support quantification by a single surrogate peptide only. The general consensus was that there were enough data that one surrogate peptide would be sufficient for a typical mAb. However, it was noted that a non-typical mAb or other biotherapeutics could benefit from additional peptides as needed for characterizing the assay or pharmacokinetics.

Enzymatic or chemical digestion is a critical step in large molecule LCMS quantification. There are many approaches and different choices. Trypsin is commonly used as a first choice to generate peptides of reasonable sizes and charges for LCMS analysis. The need for a pre-digestion step (e.g., denaturation, alkylation, etc.) was discussed to understand its necessity in achieving reproducible and efficient digestion. Many people use different digestion approaches which include varying digestion times and temperatures as well as whether to digest in solution or on bead. In general, it is recommended that pre-digestion should be applied only when digestion efficiency or reproducibility are not sufficient.

### Cross-validation LBA/LCMS

As the use of LCMS technology for large molecule bioanalysis continues to increase, there is significant discussion on how and when to use LCMS versus LBA. This

strategy is currently being developed and many groups choose the appropriate technology based on a fit-for-purpose approach and factors such as reagent availability, assay complexity, sensitivity, unique challenges, etc. The need to develop an LCMS-based assay and compare its performance to a LBA may arise from a desire for greater analytical specificity, for instance, when there may be conflicting data. It is critical prior to any cross-validation that every effort is made to fully understand what analyte each assay measures, and confirm that the two assays aim to measure the 'same' analyte. It is important to keep in mind that the analyte may be present in different forms, such as bound/free/total, catabolites, post-translational modifications, etc. As a general rule, it is not necessary to measure an analyte with multiple approaches; however, there are situations where multiple technologies may be pursued. An example of this situation may be when one assay or technology encounters issues such as matrix interference. Another example is when early data are generated using one platform and as the molecule progresses in the development, another platform is used. It should be emphasized that the platform should never be switched in the middle of a study. In many instances, both technologies may be utilized when the molecular complexity necessitates additional characterization by both LCMS and LBA, as is the case in many ADC analyses.

Assuming cross-validation is determined to be necessary as in the case when a set of data has initially been generated with one technology and there is a need to switch the platform to the other technology, certain acceptance criteria should be applied. It is felt when comparing two technologies that the acceptance criteria need to use the less stringent of the two. The general acceptance criterion that should be applied when cross-validating between LCMS and LBA is that two-thirds of sample results be within 30%. If the two methods do not agree, the best strategy is to understand exactly what each method is measuring and then use the assay that best addresses the questions (fit-for-the purpose of the study approach). There is no single strategy that can be applied for comparing platforms. It is recommended to utilize different samples sets including QC samples and incurred samples. There was also some discussion about how much validation work should be done on a secondary method to establish selectivity of the primary method. This should be determined on a case-by-case approach. One must fully characterize the assay using whatever technology and characterization is necessary.

### BMV of LM by LCMS

Large molecule LCMS for PK analysis continues to expand beyond discovery into more regulated environments. Typically the analysis of proteins or large

molecules utilizes complicated assays requiring several processing steps during sample preparation, such as enrichment and digestions. Many of these assays involve a hybrid approach which utilize an immunoaffinity or capture step followed by digestion to a surrogate peptide and/or monitoring peptide. When validating such assays for the PK analysis of biotherapeutics, it is recommended to follow the LBA precision and accuracy acceptance criteria of 20% (25% at the LLOQ) rather than the typical small molecule chromatographic criteria of 15% (20% at the LLOQ). Wider acceptance criteria are recommended due to the complexity of the assay and since this is still an emerging field. As the technology matures and more data become available, these criteria may be revised. There was some discussion around flexibility on the criteria and that if validation data indicate that tighter criteria can be applied, then that may supersede the general acceptance criteria suggested.

One question that is often asked when quantification is done via a surrogate peptide is “how could one ascertain that the intact protein molecule is measured?” LCMS methodologies can be readily multiplexed to measure several peptides of interest to confirm that the intact protein molecule is measured and understand its structural integrity, assuming the peptides are unique to the analyte protein and possess the required stability/analytical properties. In cases where there are any structural modifications, one or more signature peptides can be monitored in the region(s) of interest and multiplexing enables several regions of interest to be assessed.

As LCMS methodologies increase in complexity, additional characterization steps may be necessary during method development (MD) and validation. Since most methods rely on some type of enzymatic or chemical digestion, the digestion efficiency should be evaluated. The general consensus was that digestion efficiency should be as high as possible, but, more importantly it should be reproducible and sufficient to ensure the sensitivity (accurately quantify the LLOQ) of the method. It is also important that the IS used resembles the endogenous peptide as much as possible (i.e. labeled proteins) to try to account for differences that may arise from incomplete digestion. A 3-step process for determining digestion efficiency using low, medium and high QC levels of the analyte (protein or signature peptide) was discussed. Step 1: spike samples with the intact protein pre-digestion matrix; step 2: spike samples with the peptide analyte pre-digestion matrix (at equimolar concentrations to the protein spiked QC samples); and step 3: spike samples with the reference peptide added into a blank post-digestion matrix (also at equimolar concentrations to the protein spiked QC samples). The ratio of the peak area signal of step 1 QC samples versus those

of step 2 QC samples gives the digestion efficiency. In addition, for a simple digestion method, the ratio of the step 2 peak areas to that of the step 3 peak areas yields the analytical recovery of the signature peptide for the following digestion.

ADAs may have an impact on LCMS assays depending on the assay format. There are some strategies or steps that can be taken to try to investigate whether an LCMS method is resistant to inhibition by the circulating ADA that may be present in the matrix. The general consensus was to try to eliminate the interference by applying acid or organic (acetonitrile) dissociation or to spike various individual patient samples containing ADAs with the analyte and verify for recovery [7].

#### PEGylated protein quantification by LCMS

The use of PEGylation is becoming a viable approach to enhance the desired PK properties of a molecule. PEGylation provides additional analytical challenges due to its physicochemical properties and complexities. The addition of a PEG molecule may render a protein unsusceptible to cleavage by a certain enzyme *in vivo*, which can pose a challenge also to the enzymatic digestion step as part of the assay procedure. This can be overcome by either trying alternate enzymes or different surrogate peptides that the PEG does not interfere with. It has been noted that PEGylating a protein increases its solubility in organic solvents such that extraction of the PEGylated protein with protein precipitation is typically a viable enrichment strategy. The consensus is that it is currently very difficult to quantify an intact PEGylated protein due to the complexity and general properties of PEG. It is recommended to denature and reduce the PEGylated protein as needed for an assay.

#### ADCs

##### General considerations for bioanalysis of ADCs

The general goals and approaches to ADC bioanalysis are described in recent publications [8,9]. The biggest challenges for ADC bioanalysis include heterogeneity of the reference material containing molecules with different Drug to Antibody Ratios (DARs), changes in ADC composition in biological samples *in vitro* and especially *in vivo* over time and lack of availability of adequately characterized reference materials with different DARs for characterizing assay performance.

The complexity of ADCs requires multiple bioanalytical methods. The most common assays include large molecule assays (by LBA and more recently also by hybrid LCMS), unconjugated payload assays (usually by LCMS and historically by LBA) and ADC conjugate assays (by either LBA or hybrid LCMS). It is recognized that no regulatory guidance specific for the bioanalysis of ADCs currently exists.

The nomenclature of the analytes is critical and may require additional clarification and standardization. The name ‘free payload’ is frequently used interchangeably with the name ‘unconjugated payload’ although it can be misleading, especially taking into account the fact that the ‘unconjugated payload’ (not covalently bound payload) may be non-covalently bound to the circulating proteins in the blood stream, similar to unbound and bound small molecules.

It is acceptable to use different biological matrices (e.g., serum or plasma) for different ADC-related assays (e.g., mAb, ADC and payload assay) and for the same assay used at different stages of the drug development (e.g., between preclinical and clinical assays).

#### Payload quantification by LCMS in ADC bioanalysis

The structures of the ADC payloads vary with some being close to the features of small molecules and the others close to natural products. The complexity of ADC conjugation chemistries, and the ADC catabolism and metabolism may lead to the formation of a broad spectrum of structurally related compounds. The decision about the most relevant payload analytes to quantify requires a collaborative effort of different specialists such as chemists, biologists, ADME experts, pharmacologists, toxicologists, clinicians and regulatory colleagues.

Depending on the ADC conjugation chemistry, purification techniques and stability of ADC, there may be some pre-existing levels of the payload and related molecules in the clinical drug product. These pre-existing levels may be acceptable from the toxicology/safety standpoint but may exceed the LLOQ of the required payload LCMS methods. There are two generally acceptable practices for this type of situation: 1- to further purify the clinical materials to reduce the levels of the pre-existing payload to the levels acceptable for the LCMS assays, and 2- to use the measured difference between pre-existing payload level and spiked levels for determining the accuracy/precision and stability.

The unconjugated payload in *in vivo* samples typically co-exists with the high concentrations of ADCs, which may break down over time to generate additional unconjugated payloads. The absolute amount of the conjugated payload in ADCs is typically vastly higher (3–4 orders of magnitude or even higher) than the concentration of the unconjugated payload in the matrix. This may represent a significant challenge as cleavage of ADCs may result in the artifactual formation of unconjugated payload during *in vivo* sample collection, storage, processing and analysis. Thus, it

may be challenging or even unfeasible to meet the small molecule method validation acceptance criteria for the unconjugated payload assays. Thus, acceptance criteria for payload LCMS assays may need to be widened based on the experimental scientific results.

Due to the potential toxicity of unconjugated payloads, there is a common understanding for the need to continue measuring their levels in the *in vivo* studies, despite the current lack of evidence of correlations of the safety observations with the payload exposures *in vivo* in humans. The MIST guidance does not apply to unconjugated payload quantification *in vivo* due to their typically low abundance relative to the respective ADCs (much less than MIST guidance’s threshold of 10%) if the payloads are treated as the small molecule catabolites of the ADCs.

Similar to small molecule methods, it is agreed that both SIL and analog IS can be used for unconjugated payload quantification. Comparing LBA versus LCMS data for payloads may be difficult or not feasible due to the differences in selectivity, dynamic range, etc. Although the comparison between the two types of assays may be informative, cross-validation between them may not be necessary or meaningful. In addition, performing ISR for payload assays may not always be feasible with incurred samples having lower than 3× LLOQ concentrations, and therefore less strict ISR criteria may need to be used based on scientific judgment.

#### ‘Hybrid’ assays for ADC bioanalysis

The ‘hybrid’ LCMS assays for quantification of ADCs may have some advantages compared with LBA assays: hybrid LCMS assays may be more selective and in some cases more sensitive, may have a generic format and be applicable to different ADCs with similar payloads, and often require less time for MD and validation. Another feature is that ‘hybrid’ LCMS assays measure the antibody-conjugated payload concentration and do not discriminate between ADCs with different DARs, and thus allow accurate quantification even when there are DAR changes *in vivo*. This may be considered an advantage or disadvantage depending on the purpose of the use of the assay data.

Similar to unconjugated payload assays discussed above, both SIL and analog IS can be used for ‘hybrid’ conjugated payload quantification. Cross-validation of LBA and LCMS platforms may be challenging or not even feasible if DAR changes *in vivo* over the course of PK measurements. There is a regulatory endorsement to use LCMS or LBA for measuring ADC conjugates (multiple filings) but the decision is ultimately based on the sponsor’s scientific judgment and project needs.