White Paper

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Bioanalysis

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 editionof 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 Garofolo (Algorithme Pharma, Canada). The numerous regulatory agency representatives who contributed to the 8th WRIB included Sam Haidar (US FDA), Amy Rosenberg (US FDA), Susan Kirshner (US FDA), Laura Salazar-Fontana (US FDA), Mark Bustard (Health Canada), Jan Welink (Dutch Medi-cines Evaluation Board [MEB] and European Medicines Agency [EMA]), Olivier Le Blaye (French National Agency for Medicines and Health Products Safety [ANSM], France), Ronald Bauer (Agency for Health and Food Safety [AGES], Austria), Katalina Mettke (Federal Institute for Drugs and Medical Devices [BfArM], Germany), Emma Whale (Medicines and Healthcare products Regulatory Agency [MHRA], UK), Jason Wakelin-Smith (MHRA, UK), Noriko Katori (Ministry of Health, Labour, and Welfare - National Institute of Health Sciences [MHLW-NIHS], Japan), and Akiko Ishii-Watabe (MHLW-NIHS, Japan).

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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.

Protein biomarkers by LCMS

Choice of platform for bioanalysis of biomarkers

There is agreement that the underlying biology, pharmacology and project knowledge should contribute to the selection of the most appropriate platform for measurement of a biomarker. Platform selection is driven by project requirements (what exactly needs to be measured), existence of reagents, prior knowledge, existing infrastructure and so forth. For example, the lack of suitable LBA reagents that provide the desired specificity can be a major driver for selecting an LCMS approach, even when an immunoaffinity capture step is needed prior to LCMS analysis. The capture antibody in immunoaffinity-LCMS assay fulfills several purposes including the needed enrichment for enhancing sensitivity. However, the high measurement specificity of an immunoaffinity-LCMS method is based not only on the antibody used for enrichment but also on the mass spectrometric detection. It is recognized that increasing number of protein biomarker measurements are being made using LCMS approaches harnessing the advantages of high measurement specificity based on *m/z* and multiplexing capabilities.

Bioanalysis of biomarkers in tissues

Tissue bioanalysis of biomarkers and therapeutic targets is becoming an area of increasing importance, for example for establishing the PK/PD relationship of biotherapeutics using mechanistic site-of-action modelling or to pursue patient stratification strategies for clinical trials. An increasing array of bioanalytical tools is needed to be able to address the diverse bioanalytical questions for protein biomarkers and targets in tissues, and there was consensus that LCMS is well suited for this work as it is typically less dependent on reagents that could be potentially interfered with by the tissue matrix. It is recognized that one particular bioanalytical approach based on LCMS is emerging that appears to be particularly suited to the analysis of membrane proteins that are not readily accessible by traditional or established quantitative bioanalytical methods. Such an approach employs tissue disruption and harsh denaturing conditions for extraction followed by proteolytic digestion prior to LCMS analysis. Depending on the abundance of the analyte, either physicochemical sample preparation steps (such as SPE) or peptide immunoaffinity can be incorporated prior to LCMS analysis. This offers the potential to overcome challenges associated with extraction efficiency and potentially compromised anti-protein protein capture from tissue lysates (irrespective if LBA or protein immunoaffinity LCMS is used). While this is an emerging area, it is anticipated that this approach will be tested more broadly including the measurement of proteins from small clinical biopsies.

Biomarker measurement by co-immunoprecipitation

Multiplexing possibilities offered by LCMS create the possibility to quantify more than one protein (via its surrogate peptide) in the same sample and the same analytical run. Protein immunoaffinity strategies can be designed that allow for co-immunoprecipitation of binding partners of the protein of interest by selection of an appropriate capture reagent. Linking such an approach to LCMS analysis can facilitate the simultaneous quantification of two binding proteins, such as a soluble target and its shed receptor. This is an evolving area of bioanalytical science and no industry standard exists at this point.

Biomarker assay sensitivity

Instrumental sensitivity has been incrementally increasing over recent years with new generations of mass spectrometers offering improved assay performance. This trend is expected to continue over the coming years and is anticipated to include not only triple quadrupoles but also high resolution mass spectrometry platforms. In order to boost sensitivity, microflow and nanoflow LCMS are also being used, as sensitivity is typically inversely correlated with flow rate. Additional significant sensitivity boost can be obtained by incorporating enrichment steps. Options include the use of antibody-based reagents for proteins and proteolytically derived peptides. All enrichment approaches can be conveniently automated.

Comparison of LCMS & LBA measurements for biomarkers

It has been recognized that a direct comparison of a biomarker measurement with these two platforms is challenging and requires careful interpretation of data. If a comparison is necessary, it is recommended to use similar reagents for both the LCMS assay and LBA. This includes the use of the same standard for assay calibration and the same capture reagent for both assays (should the LCMS include a protein immunoaffinity step). Where applicable, similar buffers, dilution factors, etc., should also be used as well. However, it is conceivable or even expected that a reagent or an assay condition that is optimal for one assay format is not optimal for the other, which needs to be considered during assay development. Finally, the underlying biology needs to be understood to evaluate if the assay format to compare is measuring the same analytes, include/exclude certain forms (such as a pro-form), post-translationally modified

versions of the protein, the portion of the protein bound to an endogenous binding partner, related proteins and so forth.

ELN discussions, consensus & conclusion

It was agreed that the introduction of ELN for capturing contemporaneous bioanalytical records has been slower than expected, whereas the bioanalytical data and reports are already almost exclusively electronic. Initial resistance to change by bioanalysts, regulatory implications and lack of a leading bioanalytical ELN platform can be named as some of the major reasons why ELNs have not advanced in the bioanalytical field. The absence of a leading bioanalytical ELN or a harmonized process means that every implementation has to be custom designed and thereby the laboratory becomes a pioneer in the field. For a successful design and implementation of ELN, consideration should be given on how it will be used from start to finish. One must consider how the ELN will be integrated with instruments, Laboratory Information Management System (LIMS) and other databases in the laboratory, how data will be reviewed by supervisors and QA, how interim and regulatory reports will be prepared, how archiving will be managed and how the regulators will audit data in the ELN. Successful implementation of a bioanalytical ELN may also require an associated Electronic Content Management (ECM) for the storage, linking and archiving of electronic data. Some laboratories define archived.pdf files created from bioanalytical records as official raw data, whereas others use locked databases and electronic files as the raw data. If.pdf files are used to archive a representation of the ELN data, it should be ensured that all required information is preserved with the.pdf files, including audit trails, and that the files are adequately locked and protected against possible modifications.

It is highly recommended that bioanalysts closely collaborate with IT specialists, and take an active role in the design and implementation of the ELN forms and templates. Achieving clarity with the IT team around bioanalytical specifications – for both laboratory practices and regulatory requirements – is essential to the quality and usability of the final system. They should be advised to not compromise the bioanalytical requirements with the system requirements since the bioanalytical requirements are unique. If these requirements are not met, the final system will not be useful for regulatory implementation.

When current studies conducted through an ELN are submitted to regulatory authorities, how these studies will be reviewed by the regulators during inspections must be considered. Regulators prefer (and sometimes must require) unescorted access to study data during inspection. This would require a user-friendly interface of the ELN that would be easy to use by a casual user of the system. A formal but concise training routine for casual users, like auditors and regulators, should be considered while the ELN is being developed with the focus on access and review of ELN data. Significant inspection findings associated with the introduction and use of electronic trial master files (eTMFs) have been given by regulators and there are many lessons to be learned from this. Laboratories intending to implement ELNs should consider guidance associated with eTMFs such as the EMA 'Reflection paper on GCP compliance in relation to trial master files (paper and/or electronic) for management, audit and inspection of clinical trials' [10] or DIA TMF and electronic document management reference models [11] as these may provide additional information on regulatory expectations for the use of e-document based systems.

Other key inputs from regulatory agencies

The 8th WRIB was also the occasion for numerous regulatory agency representatives to share their views on other various topics of interest for the global bioanalytical community attending this event, in order to provide some clarification on unresolved issues or imprecise expectations.

A clarification was shared in relation with the application of the draft FDA Guidance on BMV issued in 2013 [12]. It was confirmed that there should be no changes in regulatory expectations until the FDA Guidance is finalized. FDA encourages organizations to keep their SOPs up-to-date; however, recommendations in the draft Guidance are not final. Hence, current audits are not impacted by the recommendations presented in the draft version of the Guidance.

For reference standards, the draft FDA Guidance refers to the inclusion of a certificate of analysis (CoA) or evidence of identity and purity for both the analyte and the IS. For the IS, a CoA is one way to document this; however, the primary interest resides in the evaluation of cross-interference between analyte and IS. The industry also questioned the sentence in the draft FDA Guidance stating that if the reference standard expires, stocks made with this lot should not be used unless purity is re-established. The regulatory feedback on this was that 'daughter' stock solutions, prepared from a pure reference standard before its expiration, should be treated according to the demonstrated stability in the solution.

Other excerpts of the draft FDA Guidance were also discussed. The intent of the inclusion of a minimum of six runs in BMV was to recommend incorporation of run acceptance QC samples in validation

runs other than those intended only for accuracy and precision. With regards to ISR, the mention of 7% of samples in the draft FDA Guidance, although not currently applied, is considered to provide appropriate precision. From a regulatory view, if ever adopted in the final version of the FDA Guidance, this percentage is not expected to create conflict with the current EMA Guideline. With reference to the assessment of the impact of hemolysed and lipemic samples as part of BMV, it was also confirmed that this assessment is of interest to regulators where appropriate. The draft FDA Guidance includes the calculation of accuracy and precision for ULOQ; if the recommendation is ever included in the final version of the Guidance, regulatory inspectors are expected not to contradict it. Another point from the draft Guidance that was clarified was the confirmation of the need to include in the bioanalytical report the details on manual reintegration of chromatograms (e.g., side-by-side comparison of the initial and revised chromatographic integration parameters, chromatograms from the initial integration, initial and final integration data) and any additional intermediate integrations as well.

Although not part of current regulatory documents, regulatory authorities do not discourage having specific requirements for curve slope variation in LCMS assays. Such variation may be observed in the presence of proton-deuteron exchange, matrix effects on detection of analyte and stable isotope-labeled (SIL) IS, and different MS conditions for detection of analyte and IS. Another concern raised by the industry was the level of information the CRO should obtain about the conditions of samples received from other clinics prior to sample receipt. The regulatory feedback concluded that as it is expected that regulatory audits will request information of the conditions for examination of the BMV stability data, it was recommended to describe the conditions in study reports.

The impact of a difference in anticoagulant counter-ions, a topic debated as part of previous White Papers [2,4], was addressed from a regulatory perspective, where it was stated that cross-validation and stability tests would generally not be required for a change in anticoagulant counter-ions (K2EDTA vs K3EDTA) if one was used for validation and study samples were collected in another. This could be acceptable provided that the bioanalytical report indicates that K2 versus K3 is the only difference, or any additional differences should be identified and shown to not affect the reliability of the data. However, cross-validation and stability data may be requested if unexplained discrepancies in results are noted by the reviewer.

The Japanese draft BMV guideline for LBA was discussed [13]. The contents of the guideline was almost consistent with EMA Guideline or FDA Guidance about acceptance criteria of validation or study sample analysis, but included some specific concepts: 1- Full validation should be conducted at a minimum required dilution (MRD), which has been determined in the course of MD. MRD should be identical for all samples; however, it is not necessarily the minimum dilution. When MRD is changed, partial validation is necessary. 2- Partial validation is required when the critical reagent lot is changed. 3- Parallelism is not mentioned in the text of the guideline. Parallelism evaluation is not mandatory.

An overall recommendation from regulatory agencies was that one should always keep in mind that the primary objective of any study should be to pursue sound science and good documentation. Guidance documents do not and cannot address every aspect of every study; they serve to provide best recommendations based on current knowledge. By themselves, guidance documents offer recommendations for complying with regulations; they are not binding on either Agency or Industry.

Conclusion

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

Hybrid LBA/LCMS recommendations

Large molecules by LCMS

- Immunoaffinity and non-monoclonal affinity enrichment steps for large molecule LCMS analyses have well established advantages and utility. Protein A/G as well as IMAC are the most commonly used supports, while anti-human Fc is used as a more sensitive 'generic' pull-down enrichment approach. Bead-based enrichments offer significant flexibility. Column-based and bead-based approaches can be used for peptide enrichment. Protein enrichment is typically not done using an online column-based approach, but tips (although costly) can be used offline at the protein or peptide level. Plate-based enrichment can be useful but may have limited capacity and dynamic range which would hinder their wide-scale utilization.

- In early discovery and non-GLP studies, to quantify human mAbs or fusion proteins containing the human Fc region, a universal set of peptides can be selected as surrogate peptide representatives of the human Fc region. However, when projects move to the clinic, a more specific assay would be necessary. Only one peptide is typically to be used for quantification; however, other 'monitoring' peptides can

be useful in understanding the integrity of different parts of the molecule as well as troubleshooting. One surrogate peptide would be sufficient for a typical mAb; however, a non-typical protein could benefit from additional peptides as needed for characterizing the assay.

- LBA and LCMS techniques are complementary. Prior to any cross-validation, it should be confirmed that both technologies measure the same analyte. It is not generally recommended to measure an analyte with both approaches; however, this may happen when one assay or technology runs into issues such as matrix interference, when early data is generated on one platform and then the platform is switched to the other; or when the molecular complexity necessitates additional characterization. When cross-validating between LCMS and LBA, QC samples and incurred samples should be used, and the general acceptance criteria that should be applied are two-thirds of sample results within 30%.

- When validating LM by LCMS, the general LBA acceptance criteria of 20% (25% at the LLOQ) should typically be applied due to the complexity of the assay. As the technology and data become more mature, this recommendation may be amended to support the overall findings. If validation data indicate that tighter criteria can be applied, then that would supersede the general criteria suggested.

- PEGylation provides additional analytical challenges, such as rendering a protein unsusceptible to cleavage by certain enzymes. This can be overcome by either trying alternate enzymes or different surrogate peptides. 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

- The main challenges for ADC bioanalysis include heterogeneity of the reference material containing molecules with different DARs, changing ADC composition in biological samples and availability of the adequately characterized reference materials for all the molecular species with different DARs. The complexity of ADCs requires multiple bioanalytical methods including mAb assays, unconjugated payload assays and ADC conjugate assays.

- As the content of the conjugated payload in ADCs is typically vastly higher than the concentrations of the unconjugated payload in matrix, it could be challenging to prevent the cleavage of ADCs and artificial formation of unconjugated payload during *in vivo* sample collection, processing and analysis. Thus, it may be difficult or even not feasible to meet the small molecule method validation acceptance criteria for the unconjugated payload assays. Acceptance criteria for payload LCMS assays may need to be widened based on the experimental scientific results.

- For quantification of ADCs, LCMS assays may be more sensitive and selective. They may be applicable to different ADCs with similar payloads, and they often require less time for MD and validation.

Protein biomarkers by LCMS

- Platform selection for bioanalysis of biomarkers (LCMS vs LBA) is mainly driven by project requirements, prior knowledge and existence of reagents, expertise and existing infrastructure. The use of LCMS approaches for protein biomarkers analysis is increasing, considering the advantages of high measurement specificity based on *m/z* and multiplexing capabilities.

- LCMS is well suited for the bioanalysis of biomarkers in tissues as it is typically less dependent on reagents that could be potentially interfered with by the tissue matrix. One particular approach that may be suitable for the analysis of membrane proteins employs tissue disruption and harsh denaturing conditions for extraction followed by proteolytic digestion prior to LCMS analysis.

- There is presently no industry standard in relation with an experimental LCMS approach to co-immunoprecipitate binding partners of the protein of interest by selection of an appropriate capture reagent.

- Many options exist to increase biomarker assay sensitivity, including the use of new generations of mass spectrometric instruments, implementation of microflow/nanoflow LCMS and application of enrichment steps using antibody-based reagents for proteins and proteolytically derived peptides.

- When comparing LCMS and LBA measurements for biomarkers, it is critical to understand the underlying biology to evaluate whether both assay formats measure the same analytes, including/excluding certain forms, post-translationally modified versions of the protein, the portion of the protein bound to an endogenous binding partner, related proteins, etc.

ELN recommendation

For successful design and implementation of ELN, consideration should be given on how the ELN will be integrated with instruments, LIMS and other databases in the laboratory, how it will be reviewed by supervisors and QA, how interim and regulatory reports will be prepared, how archiving will be managed and how the regulators can inspect the data via the ELN. Close collaboration with IT specialists is crucial.

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