2020 White Paper on Recent Issues in Bioanalysis: BMV of Hybrid Assays, Acoustic MS, HRMS, Data Integrity, Endogenous Compounds, Microsampling and Microbiome (Part 1 – Recommendations on Industry/Regulators Consensus on BMV of Biotherapeutics by LCMS, Advanced Application in Hybrid Assays, Regulatory Challenges in Mass Spec, Innovation in Small Molecules, Peptides and Oligos)

Hendrik Neubert^{†,1}, Stephen C Alley^{‡,2}, Anita Lee^{‡,3}, Wenying Jian^{‡,8}, Michael Buonarati^{‡,4}, Anna Edmison^{‡,5}, Fabio Garofolo^{*,‡,6}, Boris Gorovits^{‡,1}, Sam Haidar^{‡,7}, Bill Mylott^{‡,9}, Parya Nouri^{‡,10}, Mark Qian^{‡,11}, Stephen Vinter^{‡,12}, Troy Voelker^{‡,13}, Jan Welink^{‡,14}, Jiang Wu^{‡,11}, Eric Yang^{‡,15}, Hongbin Yu^{‡,16}, Chris Evans^{§,15}, Scott Summerfield^{§,17}, Jian Wang^{§,18}, Kevin Bateman^{§,19}, Jason Boer^{§,20}, Brian Dean^{§,21}, Lieve Dillen^{§,22}, Patrick Faustino^{§,7}, Luca Ferrari^{§,23}, Nicola Hughes^{§,24}, Lina Luo^{§,10}, Timothy Olah^{§,25}, Noah Post^{§,26}, Daniel S. Spellman^{§,19}, Jens Sydor^{§,15}, Hui Zhang^{§,1}, Jenny Zhang^{§,1}, Jinhui Zhang^{§,7}, Christine Fandozzi^{‡,27}, Amanda Wilson^{‡,28}, Daniela Fraier^{‡,23}, Christopher J. Beaver^{‡,29}, Suman Dandamudi^{‡,7}, Arindam Dasgupta^{‡,7}, Rebecca Elliott^{‡,21}, Allena Ji^{‡,30}, Wenkui Li^{‡,31}, Michael McGuinness^{‡,12}, Gustavo Mendes Lima Santos^{‡,32}, Tahseen Mirza^{‡,7}, Natasha Savoie^{‡,33}, Diaa Shakleya^{‡,7}, Sune Sporring^{‡,34}, Susan Stojdl^{‡,5}, Phillip Sundman^{‡,1}, Nilufer Tampal^{‡,7} & Eric Woolf^{‡,19}

¹Pfizer, Andover, MA, USA

²Seattle Genetics, Bothell, WA, USA

³Merck, Kenilworth, NJ, USA

⁴Intertek, San Diego, CA, USA

⁵Health Canada, Ottawa, ON, Canada

⁶BRI, Vancouver, BC, Canada

⁷US FDA, Silver Spring, MD, USA

⁸Janssen Research & Development, Spring House, PA, USA

⁹PPD, Richmond, VA, USA

¹⁰Pfizer, Groton, CT, USA

¹¹Takeda Pharmaceuticals International Co., Cambridge, MA, USA

¹²UK MHRA, London, UK

¹³Covance, Salt Lake City, UT, USA

¹⁴EMA, London, UK

¹⁵GlaxoSmithKline, Collegeville, PA, USA

¹⁶Boehringer-Ingelheim, Ridgefield, CT, USA

¹⁷GlaxoSmithKline, Ware, UK

¹⁸Bristol-Myers Squibb, Princeton, NJ, USA

¹⁹Merck, West Point, PA, USA

²⁰Incyte, Wilmington, DE, USA

²¹Genentech, South San Francisco, CA, USA

²²Janssen Research & Development, Beerse, Belgium

²³F. Hoffmann-La Roche Ltd, Basel, Switzerland

²⁴Biopharma Services, Toronto, ON, Canada

²⁵Bristol-Myers Squibb, Pennington, NJ, USA

²⁶Ionis Pharmaceuticals, Carlsbad, CA, USA

²⁷Merck, North Wales, PA, USA

²⁸AstraZeneca, Cambridge, UK

²⁹Syneos Health, Princeton, NJ, USA

³⁰Sanofi, Framingham, MA, USA

³¹Novartis, East Hanover, NJ, USA

³²Brazil ANVISA, Brasilia, Brazil

³³CFABS, Montreal, QC, CAN

³⁴Novo Nordisk, Maaloev, Denmark

[†]SECTION 1 - Hybrid Assays and HRMS (authors in Section 1 are presented in alphabetical order of their last name, with the exception of the first four authors who were major contributors)

[§]SECTION 2 – Small Molecules Innovation, Peptides and Oligos (authors in Section 2 are presented in alphabetical order of their last name, with the exception of the first three authors who were major contributors)

[#]SECTION 3 – Regulatory Challenges in Mass Spec (authors in Section 3 are presented in alphabetical order of their last name, with the exception of the first three authors who were major contributors)

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*Author for correspondence:
fabio.garofolo@hotmail.com

The 14th edition of the Workshop on Recent Issues in Bioanalysis (14th WRIB) was held virtually on June 15–29, 2020 with an attendance of over 1000 representatives from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations, and regulatory agencies worldwide. The 14th WRIB included three Main Workshops, seven Specialized Workshops that together spanned 11 days in order

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to allow exhaustive and thorough coverage of all major issues in bioanalysis, biomarkers, immunogenicity, gene therapy, cell therapy and vaccine. Moreover, a comprehensive vaccine assays track; an enhanced cytometry track and updated Industry/Regulators consensus on BMV of biotherapeutics by Mass Spectrometry (hybrid assays, LCMS and HRMS) were special features in 2020. As in previous years, this year's WRIB continued to gather a wide diversity of international industry opinion leaders and regulatory authority experts working on both small and large molecules to facilitate sharing and discussions focused on improving quality, increasing regulatory compliance and achieving scientific excellence on bioanalytical issues.

This 2020 White Paper encompasses recommendations emerging from the extensive discussions held during the workshop and is aimed to provide the Global Bioanalytical Community with key information and practical solutions on topics and issues addressed, in an effort to enable advances in scientific excellence, improved quality and better regulatory compliance. Due to its length, the 2020 edition of this comprehensive White Paper has been divided into three parts for editorial reasons.

This publication covers the recommendations on (Part 1) Hybrid Assays, Innovation in Small Molecules, & Regulated Bioanalysis. Part 2A (BAV, PK LBA, Flow Cytometry Validation and Cytometry Innovation), Part 2B (Regulatory Input) and Part 3 (Vaccine, Gene/Cell Therapy, NAb Harmonization and Immunogenicity) are published in volume 13 of *Bioanalysis*, issues 5, and 6 (2021), respectively.

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Acronyms

AAV:	Adeno-associated virus
ADA:	Anti-drug antibody
ADC:	Anti-drug conjugate
ADE:	Acoustic droplet ejection
ADME:	Absorption, distribution, metabolism and elimination
AEMS:	Acoustic ejection mass spectrometry
ASO:	Antisense oligonucleotide
BAV:	Biomarker assay validation
BMV:	Bioanalytical method validation

BSA:	Bovine serum albumin
COU:	Context of use
CP:	Cyclic peptide
CRO:	Contract research organization
DNA:	Deoxyribonucleic acid
DOE:	Design of experiment
EDTA:	Ethylenediaminetetraacetic acid
ESI:	electrospray ionization
EWG:	Expert working group
EV:	Extracellular vesicle
FFP:	Fit-for-purpose
FWHM:	Full width half maximum
GalNAc:	N-Acetylgalactosamine
GC:	Gas chromatography
GCMS:	Gas chromatography mass spectrometry (including triple quadrupole MS)
GCP:	Good clinical practices
GI:	Gastrointestinal
GLP:	Good laboratory practices
HFIP:	Hexafluoroisopropanol
HILIC:	Hydrophilic interaction chromatography
HRMS:	High resolution mass spectrometry
IA:	Immunoaffinity
IaaS:	Infrastructure as a service
IBS:	Irritable bowel syndrome
IP:	Ion pairing
IS:	Internal standard
ISR:	Incurred sample reanalysis
KOL:	Key opinion leader
LBA:	Ligand binding assay
LC:	Liquid chromatography
LCMS:	Liquid chromatography mass spectrometry
LLE:	liquid-liquid extraction
LLOQ:	Lower limit of quantitation
mAb:	Monoclonal antibody
MEW:	Mass extraction window
Microbiome:	Bacteria, fungi and microbes found in the gut of animals and humans.
mRNA:	Messenger RNA
miRNA:	Micro RNA
MRM:	Multiple reaction monitoring
MS:	Mass spectrometry
NAb:	Neutralizing antibody
OPI:	Open-port interface
PaaS:	Platform as a service
PD:	Pharmacodynamic
PK:	Pharmacokinetic
PPB:	Plasma protein binding

PTM:	Post-translational modification
QC:	Quality control
qPCR:	Quantitative polymerase chain reaction
siRNA:	Small interfering RNA
SaaS:	Software as a service
SCFA:	Short-chain fatty acids
SOP:	Standard operating procedure
SIL:	Stable isotope label
SPE:	Solid phase extraction
TE:	Target engagement
VAMS:	Volumetric absorptive microsampling
WRIB:	Workshop on Recent Issues in Bioanalysis
XIC:	Extracted ion chromatography

Introduction

The 14th edition of the Workshop on Recent Issues in Bioanalysis (14th WRIB) was held virtually between 15–29 June 2020 with an attendance of over 1000 representatives from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations, and regulatory agencies worldwide. The 14th WRIB included three main workshops, seven Specialized Workshops that together spanned 11 days to allow exhaustive and thorough coverage of all major issues in bioanalysis, biomarkers, immunogenicity, gene therapy, cell therapy and vaccine.

Moreover, a comprehensive vaccine assays track; an enhanced cytometry track, and updated Industry/Regulators consensus on bioanalytical method validation (BMV) of biotherapeutics by mass spectrometry (hybrid assays, LCMS and HRMS) were special features in 2020.

As in previous years, this year's WRIB continued to gather a wide diversity of international industry opinion leaders and regulatory authority experts working on both small and large molecules to facilitate sharing, reviewing, discussing and agreeing upon best approaches aimed to achieve scientific excellence and increase regulatory compliance on bioanalytical issues.

The active contributing chairs included Dr. Stephen C Alley (Seattle Genetics), Dr. Anna Edmison (Health Canada), Dr. Chris Evans (GSK), Dr. Christine Fandozzi (Merck), Dr. Sally Fischer (Genentech), Dr. Fabio Garofolo (BRI), Dr. Christine Grimaldi (Boehringer Ingelheim), Dr. Lindsay King (Pfizer), Dr. Rocio Murphy (Merck), Dr. Hendrik Neubert (Pfizer), Dr. Manoj Rajadhyaksha (Regeneron), Dr. Catherine Soo (Health Canada), Dr. Susan Spitz (Incyte), Dr. Roland Staack (Roche), Dr. Scott Summerfield (GSK), Dr. Alessandra Vitaliti (Novartis), Drs. Jan Welink (EMA), Dr. Haoheng Yan (US FDA), Dr. Tong-yuan Yang (Janssen), Dr. Hongbin Yu (Boehringer Ingelheim), Dr. Yan Zhang (BMS).

The participation of regulatory agency representatives continued to grow at WRIB [1–25] including the below:

- **Regulated Bioanalysis and BMV Guidance/Guidelines:** Dr. Arindam Dasgupta (US FDA), Dr. Sam Haidar (US FDA), Dr. Mohsen Rajabiabhari (US FDA), Dr. Tahseen Mirza (US FDA), Dr. Nilufer Tampal (US FDA), Dr. Suman Dandamudi (US FDA), Dr. Diaa Shakleya (US FDA), Dr. Jinhui Zhang (US FDA), Dr. Patrick Faustino (US FDA), Dr. Jan Welink (EU EMA), Mr. Stephen Vinter (UK MHRA), Mr. Michael McGuinness (UK MHRA), Dr. Anna Edmison (Health Canada), Dr. Catherine Soo (Health Canada), Dr. Susan Stojdl (Health Canada), Mr. Gustavo Mendes Lima Santos (Brazil ANVISA)
- **Immunogenicity, Gene Therapy, Cell Therapy and Vaccines:** Dr. Susan Kirshner (US FDA), Dr. Daniela Verthelyi (US FDA), Dr. Joao Pedras-Vasconcelos (US FDA), Dr. Haoheng Yan (US FDA), Dr. Meiyu Shen (US FDA), Dr. Mohsen Rajabi Abhari (US FDA), Dr. Isabelle Cludts (UK MHRA), Dr. Elana Cherry (Health Canada), Dr. Lucia Zhang (Health Canada), Dr. Akiko Ishii-Watabe (Japan MHLW), Dr. Sara Gagneten (US FDA), Dr. Andrew Exley (UK MHRA), Dr. Therese Solstad (EU EMA/Norway NoMA), Dr. Richard Siggers (Health Canada)
- **Biomarkers:** Dr. Yow-Ming Wang (US FDA), Dr. Abbas Bandukwala (US FDA), Dr. Kevin Maher (US FDA), Dr. Yoshiro Saito (Japan MHLW)

All the traditional “*working dinners*” attended by both industry key opinion leaders (KOL) and regulatory representatives were held in a virtual format this year, and the extensive and fruitful discussions from these “*working dinners*” together with the lectures and open panel discussions amongst the presenters, regulators and attendees culminated in consensus and recommendations on items presented in this White Paper.

A total of 167 recent issues (‘hot’ topics) were addressed and presented in this White Paper, which are the background on each issue, exchanges, consensus and resulting recommendations on these one hundred and sixty-seven topics.

Due to its length, this comprehensive White Paper has been divided into three parts for editorial reasons. This publication covers **Part 1** recommendations.

Part 1 – Issue 4 – February 2021

Hybrid Assays and HRMS

- BMV of Biotherapeutics by LCMS and Hybrid Assays: Regulatory Rigor & Acceptance Criteria While Waiting for the ICH M10 Draft Guideline (six topics)
- Hybrid Assays for Adeno-Associated Virus (AAV) Gene Therapy & Extracellular Vesicles: Advanced Applications (four topics)
- Hybrid Assays for Target Engagement: Novel Applications (three topics)
- High Resolution Mass Spectrometry for Protein Therapeutic Bioanalysis: Current Developments (three topics)

Small Molecules Innovation, Peptides and Oligos

- Microbiome Contributions to Small Molecule Drug Metabolism and its Impact on Bioanalytical Assays (six topics)
- Acoustic-Mass Spectrometry (MS) for Bioanalytical Applications (four topics)
- High Resolution Mass Spectrometry (HRMS): Small Molecule Method Development Strategies (four topics)
- Design of Experiments for Therapeutic Peptides: Modern Discovery Bioanalytical Laboratories (three topics)
- Oligonucleotides and Chain-Shorted Metabolites: Advanced Strategies (four topics)

Regulatory Challenges in Mass Spectrometry

- Data Integrity and Regulatory Factors to Consider when Using Cloud Computing (three topics)
- Impact of Excipients on Bioanalytical Methods: “What Are Regulators Asking?” (two topics)
- Parallelism Evaluation in Small Molecule Endogenous Compounds – New Considerations on ICH M10 Draft Guideline (three topics)
- Abnormal Internal Standard Response: Compliance with the 2018 FDA Guidance and ICH M10 Draft Guideline (four topics)
- Microsampling in Regulated Bioanalytical Juvenile & Pediatric Studies (three topics)

Part 2A – Issue 5 – March 2021

Biomarker Assay Validation (BAV)

- Need for a BAV Guidance (three topics)
- When Clinical Biomarker Assay Should be Under Clinical Laboratory Improvements Amendments (CLIA)? (three topics)
- Current Applications of Context of Use (COU) in Fit-for-Purpose (FFP) BAV (six topics)
- Advancements in Extracellular Vesicles (EV) (two topics)

PK LBA Regulated Bioanalysis

- Stability Testing of Biotherapeutics: FDA, EMA, & Draft ICH M10 Guidance/Guideline (two topics)
- Critical Reagents: Latest Approaches (six topics)
- Bispecific Monoclonal Antibodies & Bispecific T-cell Redirectors: Unique Challenges in Pharmacokinetic (PK) Assays (three topics)
- Common Issue with Laboratory Information Management System (LIMS) Based Software for Ligand Binding Assay (LBA) Support (two topics)

- Parallelism Evaluation in Regulated Bioanalysis for PK LBA: FDA, EMA, & Draft ICH M10 Guidance/Guideline (four topics)

Flow Cytometry Validation

- Flow Cytometry Validation: Applicability of Clinical and Laboratory Standards Institute (CLSI) H62 Guide-line to Regulated Bioanalysis (four topics)
- Flow Cytometry Validation: Target Engagement and Receptor Occupancy (three topics)
- Flow Cytometry Validation Strategies for Assays Using Challenging Sample Types (two topics)
- Validation Strategies for Image Cytometry Based Assays (three topics)
- Validation of Lower Limit of Quantitation (LLOQ) in Flow Cytometry (two topics)

Cytometry Innovation

- New Insights in Automated Gating (three topics)
- Advantages & Challenges in using Mass Cytometry (CyTOF) (five topics)
- High Dimensional/High Parameter Flow Cytometry (five topics)

Part 2B – Issue 5 – March 2021

Input from Regulatory Agencies on Bioanalysis & BMV

- Input from Regulatory Agencies on Immunogenicity & Biomarkers

Part 3 – Issue 6 – March 2021

Vaccine Clinical Assays and Cell Therapy

- Clinical Vaccine Assay Validation (six topics)
- Quality control (QC) Samples in Chimeric antigen receptor T cells (CAR-T) & Vaccine Flow Cytometry Assays: Current Industry Standards (three topics)
- Quantitative (real-time) Polymerase Chain Reaction (qPCR) Assays for CAR-T Programs (three topics)
- Immunogenicity Strategy for CAR-T Products (three topics)

Gene Therapy, qPCR and ELISpot Validation:

- qPCR, Droplet Digital Polymerase Chain Reaction (ddPCR), and Next Generation Sequencing (NGS) Assay Development & Validation: Best Practices (five topics)
- AAV Capsid Neutralizing Antibody (NAb) Assays Development and Validation (three topics)
- ELISpot & Single Cell Western Blot Assay Validation (three topics)
- Application of Current FDA/EMA Immunogenicity Guidance/Guideline to Gene Therapy (two topics)

NAb Assay Harmonization, Biosimilars and FDA/EMA Guidance/Guideline:

- Cell-based NAb Assays – Sensitivity and Drug Tolerance and the Relevance for Clinical Outcome (four topics)
- NAb Assay Harmonization: Recent Trends and Expectations (four topics)
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Immunogenicity Assay Strategies:

- Lessons Learned from Late Stage Clinical Studies (four topics)
- Circulating Immune Complexes (three topics)
- Multi-Domain Biotherapeutics: Immunogenicity Assay Strategies (four topics)
- Definition of Persistent Anti-Drug Antibody (ADA) Responses and its Clinical Relevance (four topics)

SECTION 1 – Hybrid Assays and HRMS

Hendrik Neubert¹, Stephen C Alley², Anita Lee³, Wenying Jian⁸, Michael Buonarati⁴, Anna Edmison⁵, Fabio Garofolo⁶, Boris Gorovits¹, Sam Haidar⁷, Bill Mylott⁹, Parya Nouri¹⁰, Mark Qian¹¹, Stephen Vinter¹², Troy Voelker¹³, Jan Welink¹⁴, Jiang Wu¹¹, Eric Yang¹⁵, Hongbin Yu¹⁶

Authors in Section 1 are presented in alphabetical order of their last name, with the exception of the first four authors who were major contributors.

The affiliations can be found at the beginning of the article

DISCUSSION TOPICS & CONSOLIDATED QUESTIONS COLLECTED FROM THE GLOBAL BIOANALYTICAL COMMUNITY

The topics detailed below were considered as the most relevant ‘hot topics’ based on feedback collected from the 13th WRIB attendees. They were reviewed and consolidated by globally recognized opinion leaders before being submitted for discussion during the 14th WRIB. The background on each issue, discussions, consensus and conclusions are in the next section and a summary of the key recommendations is provided in the final section of this manuscript.

BMV of Biotherapeutics by LCMS and Hybrid Assays: Regulatory Rigor & Acceptance Criteria While Waiting for the ICH M10 Draft Guideline

Hybrid assays for biotherapeutics have been in use for more than a decade; few have been part of BLA submissions to date. What was the industry and regulator experience? Is industry still hesitant or reluctant to submit hybrid assays? Do regulators have enough experience? How can this gap be closed? What is the scientific and regulatory justification for the use of hybrid assays in clinical applications: unique biotherapeutic modality; lack of appropriate reagents for LBA; matrix interference (ADA, drug, endogenous proteins); *in vivo* drug instability / catabolism; and overall unsatisfactory performance of LBA? Does it still make sense to only add the minimum requirements for a hybrid assay in the ICH M10 Draft Guideline [26], as recommended in the 2019 White Paper in Bioanalysis [24], or is the industry ready for more extensive requirements? After one year, do we still agree that due to the increased complexity of hybrid assays, the LBA acceptance criteria of 20%/25% for precision and accuracy, and 30% for incurred sample reanalysis (ISR) criteria should be adopted for most protein, soluble target and antibody quantification? What about the other minimum requirements: specificity of the surrogate peptide(s); digestion efficiency; dilutional linearity; and capture reagent lot-to-lot variation? What else should be considered for ensuring hybrid assay performance/robustness during the sample analysis in regulated bioanalysis?

Hybrid Assays for AAV Gene Therapy & Extracellular Vesicles: Advanced Applications

Since this topic was discussed in the 2018 White Paper in Bioanalysis [20], what is the current applicability of hybrid assays in clinical and preclinical settings for gene therapy drug development? What experience exists across industry in the analysis of transgene protein expression by LCMS aiding gene therapy development? Tissue analysis by immunoaffinity (IA)-MS is increasingly supporting clinical and preclinical drug development; simplified and automated tissue lysis and sample preparation steps are needed. What approaches are being pursued to enable higher throughput tissue analysis? IA-MS assays are used in gene therapy and target analysis support for biologics. All these emerging areas require an investment in reagents. How can we get anti-protein and anti-peptide reagents for IA-MS faster and with improved quality? What is the next horizon for protein biomarker mass spectrometry from extracellular vesicles?

Hybrid Assays for Target Engagement: Novel Applications

What are the advancements and applications when using hybrid assays for target engagement (TE) since the 2017 White Paper in Bioanalysis recommendations [17]? What are the current industry best practices when using hybrid LBA/LCMS to measure a single assay method for drug exposure and TE at the site of action? What parameters should be considered for human assay FFP validation?

High Resolution Mass Spectrometry for Protein Therapeutic Bioanalysis: Current Developments

Have there been any advancements for sensitivity improvements following the 2018 and 2019 White Paper recommendations [20,23]? Have there been any advancements on other fronts, such as sample preparation, chromatography, native intact MS bioanalysis, or software? Do you think that intact protein bioanalysis will be expanded in the regulated space? Are there other new recommendations on intact protein bioanalysis for the 2020 White Paper?

Has intact protein bioanalysis been exclusively used or used in combination with other assay platforms such as bottom-up trypsin assays, immuno-assays, or activity assays? Are there examples of bridging intact assays with other assay platforms and what are the challenges?

DISCUSSIONS, CONSENSUS AND CONCLUSIONS

BMV of Biotherapeutics by LCMS and Hybrid Assays: Regulatory Rigor & Acceptance Criteria While Waiting for the ICH M10 Draft Guideline

In the past decade, LCMS alone and hybrid assays (also called hybrid LBA/LCMS or IA-MS or IC-MS) have emerged as complementary platforms to LBA and have been applied to the quantification of biotherapeutics that are not amenable to LBA due to matrix or ADA interferences or lack of appropriate reagents. Moreover, LCMS and hybrid assays are powerful tools for determining *in vivo* stability, biotransformation, and characterization of therapeutic proteins. Mass spectrometric techniques have also helped circumvent challenges regarding subtle differences in protein sequences. Sequential immunoprecipitation at the protein and peptide level in conjunction with LCMS offers high sensitivity and enables protein PK measurement at extremely low doses. With appropriate capture reagents, hybrid assays are currently considered the most versatile option to overcome the above bioanalytical challenges.

However, hybrid assays can be challenging due to complex and time intensive sample preparation, as well as potentially long analysis times. Special attention needs to be given to sustainable and consistent assay performance during larger studies such as clinical trials. Planning of how the assay will be utilized for sample analysis is essential for the assay to be sufficiently robust for use in a large clinical trial. Careful consideration is required during assay validation to ensure that the resulting assay meets scientific excellence and full regulatory compliance. These types of assays demand a deep understanding of specific biology and typically need close monitoring of the data which may lead to further optimization using incurred study samples since they may behave differently from the spiked QCs initially used during method development and validation. A hybrid assay is a serial technique that may need up to two days for the analysis of a single sample batch due to lengthy incubation and digestion steps. Determining the correct logistical setup for a high-throughput laboratory is essential for success.

Indeed, current data continue to confirm that more complicated drug modalities drive the need for more complex bioanalysis even in a fully regulated environment. Antibody-drug conjugates (ADC) present significant bioanalytical challenges for ensuring comprehensive PK characterization. In a case study, multiple hybrid assays were developed in order to appropriately characterize the PK properties of a unique ADC, ultimately measuring at least 14 unique species/catabolites [27]. In another case study, this technique was used for ADA impact assessments [28], where hybrid assays were developed to confirm the impact on the PK by ADA and to deconvolute the ADA binding domain. Sugimoto *et al.* demonstrated that the quantification of fusion proteins can be accomplished with hybrid assays [29], which was necessary because the existing anti-IFN Ab used was not assured to work with all construct variants of the drug in the LBA PK assay. Therefore, the hybrid assay was established and orthogonally compared with LBA with success. Another example involved the ADC cyno ADA isotype quantification with hybrid assays, which was then compared with LBA [30]. The integrated hybrid assay and LBA approach were used to successfully confirm the ADA impact on the PK of the anti-CD38-ET fusion protein and the key ADA binding domain on the fusion protein was identified. The results helped advance the project by selecting the right construct. The hybrid assay capability of semi-quantifying immunoglobulin isotypes allows for assessing the ADA level and isotype distribution for the tested ADC. While a hybrid assay is a preferred option, reagent-free LCMS is proven to be an alternative for the hybrid assay when the reagent is not readily available. A recent paper demonstrated that orthogonal liquid chromatography (LC) separation in conjunction with MRM-MS quantification may offer the compatible sensitivity for hybrid assays [31].

Regulators continue to have limited first-hand experience with hybrid assays in submission reviews even if the number of studies that are supported by hybrid assays continues to grow. Overall, as long as the rationale is scientifically sound and the method is validated, regulators have no issues with accepting the data produced with hybrid assays. There are several aspects that should be carefully considered when selecting a PK assay platform (LBA, LCMS or hybrid assay), including:

- Reagent availability
- Potential matrix interference
- *In vivo* drug instability

- Catabolism
- Sensitivity

If any of the assay types are able to overcome any of these challenges, then it is the non-scientific/non-regulatory aspects, such as time and costs, which often determines the assay format. The decision should not be about comparisons to LBA, but the most appropriate assay to answer the question.

There is no BMV regulatory guidance/guideline for biotherapeutics by LCMS and hybrid assays. Considering the variety of different approaches associated with this platform, there is a need for better alignment within the industry on the best practices and regulatory expectations to improve overall confidence in assay performance, especially for those established and validated in support of PK and pharmacodynamic (PD) studies used for setting safety margins, PKPD modeling and dose selection. Prior white paper recommendations have begun to set the framework for drafting a regulatory guidance/guideline [5,6,11,14], and then the 2019 White Paper in Bioanalysis [23] provided a clear statement for the “minimum requirements” that should at least be included in the upcoming ICH M10 Draft Guideline [26]. Building on and supporting these 2019 recommendations, this White Paper assembles them all and provides additional guidance to help industry write Standard Operating Procedures (SOPs) specific for hybrid assays.

Minimum Requirements

There was overwhelming agreement that it will be essential for the ICH M10 Draft Guideline to contain at least the minimum requirements for hybrid assays to help to establish these techniques for biotherapeutics as a mature one that can be used for regulatory submissions, and not necessarily as a supplementary or complementary approach to regulated bioanalysis.

The minimum requirements recommended in the 2019 White Paper in Bioanalysis were confirmed [23]:

- “Specificity of the surrogate peptide(s)
- Digestion efficiency
- LBA (20%/25%) accuracy, precision and 30% ISR criteria should be targeted until more experience with hybrid assays has accrued
- Dilutional linearity
- Capture reagent lot-to-lot variation”

The current regulatory expectation is that if the validated approach used is different from that found in the BMV guidance/guideline [32,33], then documentation should exist a priori to outline the approach and criteria, and the validation should follow this plan.

Precision and Accuracy

The 2019 White Paper in Bioanalysis recommended that precision and accuracy criteria should mimic that of LBA – within 20% (LLOQ QC within 25%) – and this recommendation was upheld until more experience dictates that different criteria may be more applicable. A minimum of 3 runs should be used during the validation. It was noted that a high purity, well characterized SIL-peptide or protein IS should be used to achieve improved assay performance.

Sensitivity

Substantial improvements in the assay sensitivity and selectivity are achieved by enriching the analytes with a variety of technical approaches, such as immunocapture at protein and/or peptide levels, online orthogonal peptide separation, and micro or nanoflow LC separation.

Selectivity and Specificity

Selectivity should be tested in 6–10 lots of blank control and patient matrices, if available. Additional interference from potential binding molecules, soluble targets, ADA or concomitant drugs should be examined [11,14]. The impact of hemolysis and lipemia should also be investigated.

Digestion Efficiency/Recovery

If possible, for PK assays in regulated bioanalysis, the immunocapture step should be performed upstream in order for the surrogate peptide(s) to accurately represent the biotherapeutic [11]. The method of calculating the actual digestion efficiency/recovery could be challenging. It was recommended that those assessments be done in assay development to get a general sense if the recovery is adequate and reproducible. When optimized, multiple lots of matrix should be tested for consistency of recovery, especially if a surrogate peptide IS is spiked at the end of the extraction. It should be demonstrated that the surrogate peptide serves its intended purpose.

Critical Reagents

Hybrid assay critical reagent life-cycle management is important and must be considered (i.e., sourcing, availability, stability) [11]. Critical reagent lots should be bridged when necessary. Capture reagent lot-to-lot variation can be tested using the method as intended (curve and run acceptance QCs). It was recommended to add a QC above the upper limit of the curve to analyze undiluted for the purpose of demonstrating capture capacity.

Parallelism

Depending on the analyte, it is important to establish parallelism for PK hybrid assays as for PK LBA

Additional Considerations

- Duplicate analysis, typically used as part of LBA methods, was not considered required if an internal standard is used [5]
- To ensure hybrid assay performance and robustness during the sample analysis, it was recommended that automation be used to increase reproducibility and throughput
- Additional assay performance testing may also be needed when the assay is applied to new patient populations or matrices
- When biotherapeutics are analyzed by hybrid assays, bridging studies should be performed between discovery and development to ensure interpretation of the results remains consistent as the method evolves [20]

Hybrid Assays for AAV Gene Therapy & Extracellular Vesicles: Advanced Applications*Gene Therapy*

Novel therapeutic approaches in gene therapy are gaining significant momentum to find cures to diseases with unmet medical need, with initial focus on rare diseases. Gene therapy holds the promise to require only a single dose to result in lifelong causal improvement for disorders that today may have no other significant treatment option. There are two modes of action used by the vector. The first is to deliver a normal transgene to compensate for loss of function caused by a gene mutation. The second is to use the transgene to encode RNA to suppress pathogenic gene expression. The most prominent vector is AAV, which is particularly suited to infect both dividing and non- or slowly-dividing cells.

Initial recommendations on the use of hybrid assays for gene therapy have already been issued in the 2018 White Paper in Bioanalysis and sustained in 2019 White Paper in Bioanalysis [20,25]. It was agreed that hybrid assays are becoming a critical tool not only for quantifying but also characterizing the transgene products for which specificity is key that would not be easily available from other types of assays. Equally important and analogous to gene replacement therapy is the application of the hybrid assay to measure the knock-down of the protein of the targeted gene for antisense oligonucleotide (ASO) or small interfering RNA (siRNA) modalities. In this field challenges exist due to different protein forms, post-translational modification (PTM), lack of reagents, low abundance, and so on.

As a new therapeutic modality, gene therapy presents several technical and regulatory challenges. To advance a gene therapy product into clinical trials, a thorough understanding must be gained preclinically about the biodistribution of the vector genome to both target and off-target organs [34]. Biodistribution analysis is typically done by qPCR (quantitative polymerase chain reaction) to measure a specific DNA sequence expressed as vector copy number per mass of total DNA in study samples and may additionally be used to look at the messenger RNA (mRNA) expression in the same tissue using reverse transcription polymerase chain reaction (RT-PCR). The quantification of protein expression, primarily in the target organ, but preclinically also in off-target organs where the vector genome has been detected, may be required. Hybrid assays that use anti-peptide antibodies for enriching surrogate tryptic peptides from tissue lysates are increasingly being used in the quantification of target protein

expressed as a result of the gene therapy. Sensitivity can be a barrier not only when the endogenous target protein is of low abundance, but also when low levels of transgene protein relative to a normal, healthy expression need to be measured. Thus high sensitivity assay configurations are needed. In a hybrid assay format, the high level of specificity of MS is combined with the high sensitivity of anti-peptide antibody capture while avoiding anti-protein antibodies, which are associated with potential capture efficiency challenges in tissue lysates. The necessary harsh, denaturing tissue extraction is compatible with the downstream digestion and anti-peptide capture. The process is suitable for most classes of transgene proteins including membrane and structural proteins.

Results from both qPCR and hybrid assays are used to test for correlation with efficacy. The translation of transgene expression or protein expression data including their correlation with other endpoints requires the consideration of translational assay design in discovery. The preclinical assay should be developed with the clinical assay in mind, and the clinical assay should be applicable to assay preclinical samples if needed. This can be done by selecting an appropriate peptide-antibody pair, a surrogate matrix that can be used across many target tissues and suitable stable isotope labelled peptide or protein internal standards. When identical hybrid assays are used, it enables confidence in concentration assignments across species.

Tissue lysis and sample preparation steps are in need of simplification and automation. Tissue homogenization is one of the primary bottlenecks at the moment. This becomes apparent preclinically, as one collected tissue may result in multiple samples for analysis with the aim to understand the distribution of expression within a tissue. Typically, individual tubes for tissue preparation are still being used. It would be ideal to move to a 96-well format. It was suggested to interface with instrument manufactures to develop better automated tissue handling/disruption/homogenization technologies. Additionally, multichannel homogenizers for microbiopsies are needed.

Like other assay types, hybrid assays also require an investment in reagents. Anti-protein and anti-peptide reagents are needed faster and with improved quality. This requires a good reagent generation team in place, supporting method development. For custom anti-peptide antibody generation, the most appropriate algorithm should be selected to predict the antigenicity of selected sequences. Peptides selected for immunization are conjugated to different carrier proteins that stimulate the host immune system to boost the antigenicity. Obtaining high-affinity anti-peptide reagents is not always guaranteed. When antibodies with lower affinity are obtained, they could be immobilized in a column, rather than a bead format, to pack more reagent in excess to compensate for poor affinity. There was consensus that overall, while there are some successful examples, the experience with aptamer reagents for IA-MS assays has not been very successful. Aptamer reagents may take a slightly shorter time to generate but are very expensive and often do not yield high-quality, high-affinity capture reagents for hybrid assays.

Extracellular Vesicles

In recent years, the contents and functional properties of EVs have been subject to significant multi-disciplinary research. The promise of using EVs and their cargo as clinical biomarkers is tremendous. Particularly appealing is the pursuit of EVs in the context of liquid biopsy, i.e. sampling and analyzing non-solid biological tissue. Because a matrix such as blood can be easily obtained and analysis repeated, it is ideal for screening, staging, profiling and monitoring of tumor biomarkers. Traditional biopsies are invasive, time consuming, may not represent the whole tumor, and therefore cannot be used for routine disease monitoring. Liquid biopsies are minimally invasive, less expensive and easier to obtain. Initial progress has been made mostly in oncology; however, the potential opportunities span many non-oncology diseases.

Although liquid biopsies appear overall advantageous, there are many preanalytical variables like blood collection, handling and processing that can affect EVs, such as the anticoagulant used, the mixing procedure, the time to process the sample, the storage temperature and the shipping and transfer conditions [35]. Standardization of blood collection and processing is therefore considered a key yet unmet prerequisite for validation of EV biomarkers, underlining the need for quality control metrics. Furthermore, hemolysis has a dramatic impact on EV number, content, and quality.

EVs carry a variety of cargo, including micro RNA, mRNA and proteins. Recently, several proteomics studies have been conducted to begin the construction of a comprehensive proteome database for extracellular vesicles [36,37]. Even though thousands of proteins have already been identified, these efforts only constitute the beginning. Further work is needed to understand optimal blood collection methods, and to optimize EV isolation, both to efficiently separate EV proteins from matrix proteins (e.g., serum proteins) or to separate EVs by their origin tissue. Looking ahead, an important requirement enabling biomarker decisions is to make the protein cargo analysis quantitative.

To this end, the EV field can take advantage of hybrid assays that are increasingly employed for the quantification of soluble or tissue-based protein biomarkers. The workflow employing anti-peptide antibody enrichment prior to LCMS based quantification of a surrogate peptide provides a suitable bioanalytical strategy for EV cargo proteins. Together with the needed advances in EV isolation strategies, this method holds significant promise to measure differences in EV protein cargo amount and modification.

There are several EV isolation methods that can be used for LCMS. Selection of the appropriate method should consider the possibility of serum protein contamination, yield, and potential damage to EVs by lysis or aggregation. Ultra-centrifugation, which includes differential and density gradient centrifugation, is a well-established but complex method. Large volumes can be processed, but with low throughput. Size exclusion chromatography is a simple reproducible procedure that preserves the integrity of the vesicles (i.e., no aggregation), but sample volumes with this method are limited. Polymer precipitation is a simple procedure using kits and no complex equipment. However, the cost of the kits is significant, and they often have poor reproducibility with the potential for polymer contamination. Finally, immunocapture offers good selectivity options, however these are dependent on the target protein orientation in the EV membrane and accessibility for the antibody.

The next horizon for protein biomarker mass spectrometry from EVs needs more straightforward processes to enrich and extract EVs. It is necessary to differentiate EVs originating from different tissues (i.e. liver-derived vs. kidney-derived EVs) using surface protein enrichment strategies and to increase yield in order to increase sensitivity.

Hybrid Assays for Target Engagement: Novel Applications

Hybrid assays have become an important bioanalytical tool in the investigation of TE in drug discovery, nonclinical development, and early clinical development. Results from the TE in serum and tissue samples are used to support PK-PD correlation, human dose projection, and proof of drug-target interactions in early clinical development. The target can be either in a free form (not bound to drug) or a bound form (bound to drug). Depending on the reagents that are available for immunocapture, the assay can either detect free target, bound target, or total target.

Recommendations for this topic were last provided in the 2017 White Paper in Bioanalysis [17], and re-evaluated after 3 years of additional industry experience [38–41]. Discussions confirmed that hybrid assays for TE were being used frequently for both small and large molecules for internal decision-making purposes. TE assays for small molecule therapeutics can be developed for covalent binders. The target protein is enriched and the surrogate peptide that covalently binds to the drug (bound) or quencher (free) can be measured [42]. A typical hybrid assay workflow for TE begins with an immunocapture step followed by binding to beads. The analyte is then separated from the beads in a wash step and eluted for digestion or directly digested on the beads. The digest is then injected into an LCMS for target peptide analysis. Quantification of the target is based on its surrogate peptide.

Some TE assays can be challenging to develop. For example, when developing a free assay method, the capture antibody binds to an epitope competitive to the drug. Not only the reagent may cause dissociation of the drug-target complex, other factors include the binding affinity, binding off-rate, incubation conditions, or sample dilution. A small degree of dissociation from the highly abundant drug-target complex can result in significant overestimation of the free target [38,39]. Also, consideration should be given to the endogenous binding partners of the target when measuring free/total target; it is also well established that sample preparation steps can shift the equilibrium of drug binding (just like in LBA).

Surrogate matrix is commonly used for calibration standard preparation for biomarker assays since authentic matrix contains the endogenous biomarker (i.e., target). When surrogate matrix is used, similar matrix effects and extraction recovery must be demonstrated in both the surrogate and original matrices. Furthermore, a parallelism experiment is required to evaluate whether observed changes, when altering the analyte concentration, are equivalent between the surrogate and the authentic matrix [41].

Reference standards and other critical reagents need to be selected or developed carefully. Recombinant protein calibrators may not resemble the endogenous form of the TE biomarker (differences in structure, folding, PTM, binding characteristics, access to binding partners). The calibrator used for reagent antibody screening may not have the same binding properties as the endogenous protein. All these differences can affect recovery. Measuring the target in free, bound, or total forms is completely dependent on the immunocapture reagent (e.g., drug and analyte-specific mAbs or pAbs, antibody fragments, other analyte binding proteins and aptamers) [40,41].

The current industry best practices with using a single hybrid assay to measure site of action drug exposure and TE was discussed. Using the same assay to measure drug exposure and TE is not commonly done since measuring drug concentration eventually falls under GxP, whereas TE can be exploratory. This is currently used more in the

exploratory drug discovery stage. Furthermore, site of action claims can be challenging; if measuring TE in tissue, it is difficult to determine if, for example, TE is in the interstitial space, intracellular of the tissue.

The parameters to consider for FFP validation of a human TE assay depends on many factors, such as technical issues and patient population. It is critical to understand the COU, because depending on intended use of data, validation requirements can be variable. TE is usually used in first-in-human studies and proof of concept to aid in dose selection. When developing the assay, it is important to verify the shift of the drug-target binding equilibrium during sample preparation is minimized or taken into account (at least for soluble TE). Although, this step cannot be validated, mixed QCs can be used to monitor for this (different ratios of free/bound). Stability of the binding and selection of the surrogate matrix should also be considered. It is recommended to utilize endogenous QCs with spiked drug to get a low QC rather than diluting authentic matrix with endogenous target, so as not to change the matrix. Finally, the effect of drug on the assay always needs to be demonstrated.

High Resolution Mass Spectrometry for Protein Therapeutic Bioanalysis: Current Developments

The use of HRMS for intact proteins bioanalysis were discussed in the 2018 and 2019 White Papers in Bioanalysis where it was recommended to assay intact proteins instead of surrogate peptides representative of proteins [20] and to consider the stage of development, the question being asked and the analyte when developing the assay [23]. Since these recommendations, the application of intact analysis is expanding from mAb and ADC to new modalities, providing valuable information for the development of new biotherapeutics such as antibody-peptide conjugates [43], peptide-Fc recombinant proteins and peptide-albumin recombinant proteins.

Sensitivity is a major challenge for intact analysis; the most sensitive published LCMS assay for a large protein (e.g., mAb) quantified by intact mass is 50 ng/mL from plasma [44]. Some strategies to improve sensitivity include deglycosylation, hinge digestion, and top-down parallel reaction monitoring [45–48]. There have been some observations where IS interferences could complicate the spectra and sacrifice detection at the low end, raising the question of whether an internal standard is necessary.

There have been no improvements in chromatography and native intact MS bioanalysis beyond those published by Zhang *et al.* [49]. Furthermore, there remain concerns that software is vendor limited, and in most cases not built for regulated bioanalysis although one has been recently released with deconvolution capabilities that is GxP compliant; Extracted Ion Chromatography (XIC) may be a more adaptable process [50]. Advancements in front-end technology are still needed to more efficiently introduce ions. There have been some reports of reducing charge dispersion via chemical methods, but this approach is still early. Microflow-LC can improve ion efficiency, but, again, more experience is needed.

As an update to previous White Papers, it was recommended that intact analysis be used in the regulated space on a case-by-case basis such as when there are no suitable surrogate peptides available, if there is a clipping/catabolism risk, for example. Intact protein bioanalysis may not currently be mainstream in regulated space due to challenges highlighted previously; it is mostly used as a characterization method, rather than a quantification method, and validation is FFP. If intact analysis is used more for characterization, bridging is mostly used to inform on performance of the validated assays (i.e., to explain discrepancies). Intact mass assays can give similar concentration data as other platforms, given similar capture antibodies/ immunocapture schemes; bridging the intact assay with the bottom-up approach is quite solid if the analyte is stable as in the case of a typical mAb [50]. If the molecule is not stable or biotransformations occur, that could result in discordant results with other assay platforms (i.e. LBA or bottom-up LCMS assay). Use of the same QC samples and incurred samples for bridging the two assays may help ensure differences are due to the molecule and not the platform. Reagent generation for the other assay is usually the main bottleneck when bridging.

RECOMMENDATIONS

Below is a summary of the recommendations made during the 14th WRIB:

BMV of Biotherapeutics by LCMS and Hybrid Assays: Regulatory Rigor & Acceptance Criteria While Waiting for the ICH M10 Draft Guideline

- The ICH M10 draft guideline should contain at least the **minimum requirements** for LCMS and hybrid assays to help establish them for biotherapeutic PK measurements as a mature technique that can be used for regulatory submission, and not necessarily as a supplementary or complementary technique

- The minimum requirements recommended in the 2019 White Paper in Bioanalysis were confirmed [23]
- If the validated approach used is different from that found in the BMV guidance/guideline [32,33], then documentation should exist a priori to outline the approach and criteria, and the validation should follow this plan as long as the rationale is scientifically sound and the method is validated, regulators have no issues with accepting the data
- **Precision and accuracy** criteria should be within 20% (LLOQ QC within 25%). A minimum of 3 runs should be used
 - A high purity, well characterized SIL-peptide or protein IS should be used to achieve improved assay performance
- **Selectivity and Specificity**
 - Tested in 6–10 lots of blank control and patient matrices, if available
 - Interference from potential binding molecules, soluble targets, ADA or concomitant drugs should be examined
 - Hemolysis and lipemia should also be investigated
- **Digestion Efficiency/Recovery**
 - Immunocapture step should be performed upstream in order for the surrogate peptide(s) to accurately represent the biotherapeutic
 - The method of calculating the actual digestion efficiency/recovery should be done in development
 - Multiple lots of matrix should be tested for consistency of recovery, especially if a surrogate peptide internal standard is spiked at the end of the extraction
- **Critical Reagents**
 - Lots should be bridged when necessary
 - Capture reagent lot-to-lot variation can be tested using the method as intended (curve and run acceptance QCs)
 - Add a QC above the curve to analyze undiluted for the purpose of demonstrating capture capacity
- **Additional Considerations**
 - Duplicate analysis is not required if an internal standard is used
 - Use automation to increase reproducibility and throughput
 - Additional assay performance testing may be needed when the assay is applied to new patient populations or matrices
 - When biotherapeutics are analyzed by hybrid assays, bridging studies should be performed between discovery and development to ensure interpretation of the results remains consistent as the method evolves

Hybrid Assays for AAV Gene Therapy & Extracellular Vesicles: Advanced Applications

Gene Therapy

- High sensitivity hybrid assay configurations are needed as critical tools not only for quantifying but also characterizing the transgene products
- Hybrid assays that use anti-peptide antibodies for enriching surrogate tryptic peptides from tissue lysates are increasingly being used in the quantification of transgene target protein
 - The necessary harsh, denaturing tissue extraction is compatible with the downstream digestion and anti-peptide capture
- The preclinical assay should be developed with the clinical assay in mind, and the clinical assay should be applicable to assay preclinical samples if needed. Select:

- An appropriate peptide-antibody pair,
 - A surrogate matrix that can be used across many target tissues
 - Suitable stable isotope labelled peptide or protein internal standards
- Need to move to a 96-well or other higher throughput format for tissue preparation to improve assay throughput, however individual tubes are still being used. This is particularly problematic when one collected tissue provides multiple sample sections
 - Interface with instrument manufactures to develop better automated tissue handling/disruption/homogenization technologies.
 - Multichannel homogenizers for microbiopsies are needed
 - Generally, anti-protein and anti-peptide reagents for hybrid assays are needed faster and with improved quality
 - Generally, the most appropriate algorithm should be selected to predict antigenic sequences for hybrid assays using anti-peptide Abs
 - Anti-peptide antibodies can be used in a column, rather than a bead format, to pack more reagent in excess to compensate for poor affinity

Extracellular Vesicles

- The workflow employing anti-peptide antibody enrichment prior to LCMS based quantification of a surrogate peptide provides a suitable bioanalytical strategy for EV cargo proteins
- Standardization of blood collection and processing is a key yet unmet prerequisite for validation of EV biomarkers, underlining the need for quality control metrics
- Hemolysis has dramatic impact on EV number, content, and quality
- Selection of the appropriate EV isolation method should consider serum protein contamination, yield, and potential damage to EVs by lysis or aggregation
- The next horizon for protein biomarker mass spectrometry from EVs needs more straightforward processes to enrich and extract EVs
- It is necessary to differentiate EVs originating from different tissues (i.e., liver-derived vs. kidney-derived EVs) using surface protein enrichment strategies and to increase yield in order to increase sensitivity

Hybrid Assays for Target Engagement: Novel Applications

- LCMS for TE is being used frequently for both small and large molecules for internal decision-making purposes
- Regulators have not seen the use of hybrid TE assays in submissions
- TE assays for small molecule therapeutics can be developed for covalent binders; the target protein is enriched and the surrogate peptide that covalently binds to the drug or quencher is measured
- Consideration should be given to the endogenous binding partners of the target when measuring free/total target
- Sample preparation steps can shift the equilibrium of drug binding (just like in LBA)
- When surrogate matrix is used, similar matrix effects and extraction recovery must be demonstrated in both the surrogate and original matrices
- A parallelism experiment is required to evaluate whether observed changes when altering the analyte concentration are equivalent between the surrogate and the authentic matrix
- Measuring the target in free, bound, or total forms is completely dependent on the immunocapture reagent (e.g., drug and analyte-specific mAbs or pAbs, antibody fragments, other analyte binding proteins)
- Using the same assay to measure drug exposure and TE is not commonly done since measuring drug concentration eventually falls under GxP, whereas TE can be exploratory.
- When developing the assay, it is important to verify the shift of the drug-target binding equilibrium during sample preparation is minimized (at least for soluble TE). Mixed QCs can be used to monitor for this (different ratios of free/bound)
- Consider the stability of the binding and selection of the surrogate matrix

- A Low QC should be prepared using endogenous QCs with spiked drug rather than diluting authentic matrix with endogenous target, so as not to change the matrix
- The effect of drug on the assay needs to be demonstrated

High Resolution Mass Spectrometry for Protein Therapeutic Bioanalysis: Current Developments

- The application of intact analysis is expanding from mAb and ADC to new modalities, providing valuable information for the development of new biotherapeutics such as antibody-peptide conjugates, peptide-Fc recombinant proteins and peptide-albumin recombinant proteins
- There has been some experience where IS interferences could complicate the spectra and sacrifice detection at the low end, raising the question of whether an internal standard is necessary
- Software is vendor limited, and in most cases not built for regulated bioanalysis although one has been recently released with deconvolution capabilities that is GxP compliant; XIC may be a more adaptable process
- Advancements in front-end technology are still needed to more efficiently introduce ions
- Intact analysis can be used in the regulated space on a case-by-case basis when there are no suitable surrogate peptides available, if there is, for example, a clipping/catabolism risk
- If intact analysis is used more for characterization, bridging is mostly used to inform on performance of the validated assays (i.e., to explain discrepancies)
 - Use of same QC samples and incurred samples for bridging the two assays may help ensure differences are due to the molecule and not the platform
 - Reagent generation for the other assay is usually the main bottleneck when bridging

SECTION 2 – Small Molecules Innovation, Peptides and Oligos

Chris Evans¹⁵, Scott Summerfield¹⁷, Jian Wang¹⁸, Kevin Bateman¹⁹, Jason Boer²⁰, Brian Dean²¹, Lieve Dillen²², Patrick Faustino⁷, Luca Ferrari²³, Nicola Hughes²⁴, Lina Luo¹⁰, Timothy Olah²⁵, Noah Post²⁶, Daniel S. Spellman¹⁹, Jens Sydor¹⁵, Hui Zhang¹, Jenny Zhang¹, Jinhui Zhang⁷

Authors in Section 2 are presented in alphabetical order of their last name, with the exception of the first three authors who were major contributors.

The affiliations can be found at the beginning of the article

DISCUSSION TOPICS & CONSOLIDATED QUESTIONS COLLECTED FROM THE GLOBAL BIOANALYTICAL COMMUNITY

The topics detailed below were considered as the most relevant ‘hot topics’ based on feedback collected from the 13th WRIB attendees. They were reviewed and consolidated by globally recognized opinion leaders before being submitted for discussion during the 14th WRIB. The background on each issue, discussions, consensus and conclusions are in the next section and a summary of the key recommendations is provided in the final section of this manuscript.

Microbiome Contributions to Small Molecule Drug Metabolism and its Impact on Bioanalytical Assays

Given the increased understanding of the impact of the microbiome on human health, what are the most important bioanalytical technologies to gain further knowledge in this field? Considering that drug metabolism and impact on human health can be associated with specific strains, is there a need for better bioanalytical technologies to determine strain differences in the microbiome faster as well as with improved accuracy and precision? How and when should gut metabolism and specific bioanalytical assays be included in drug discovery and development? Is quantification of drug metabolites in feces necessary considering FDA and ICH guidelines are concerned with systemic exposure? In gut metabolism studies, what are the current industry standards in Human Fecal Homogenate for sample collection and cryopreservation? How can preservation of microbial communities existing in the gut be ensured? Can short-term and long-term stability of short-chain fatty acids (SCFA) in samples be improved by reducing volatility of SCFA in stool samples, optimization of sample conditions, storage time, stabilizers, or temperature?

Acoustic-MS for Bioanalytical Applications

What are the current limitations of Acoustic-MS for regulated bioanalysis? How can ionization suppression in Acoustic-MS be reduced? What are the true variability measurements of incurred samples from multiple

measurements with Acoustic-MS? What experiments can be avoided or better performed with Acoustic-MS: chromatographic method development; autosampler stability; column performance evaluation (lot to lot); carryover; method development timelines?

HRMS: Small Molecule Method Development Strategies

Do method development and validation look different when using HRMS? What are the additional challenges and drivers for method development and validation with HRMS beyond what was already discussed in the 2016 White Paper in Bioanalysis [13]? Since the 2016 White Paper in Bioanalysis recommendations, what is still delaying the embracement of quantitative HRMS by the bioanalytical community, especially in the field of small molecules? Is HRMS being used for small molecules, endogenous analytes and/or transporter biomarker quantification? What are the optimal HRMS settings for balancing selectivity with sensitivity and quality with quantity?

Design of Experiments for Therapeutic Peptides: Modern Discovery Bioanalytical Laboratories

What are the current challenges and advances in the modern discovery bioanalytical laboratory? What are the best approaches for developing a modality independent bioanalytical support? What are the best strategies for employing Design of Experiment (DOE) to optimize sample preparation for peptides? What should be considered for rapid bioanalytical method development of sensitive and selective assays for structurally diverse peptide therapeutics?

Oligonucleotides and Chain-Shorted Metabolites: Advanced Strategies

How does the knowledge of chemical modifications in ASO, e.g., understanding the interplay between extraction recovery and ionization efficiency as ASO length decreases, benefit their bioanalytical method development? What are the advantages in using hexafluoroisopropanol (HFIP) in oligonucleotide LCMS bioanalysis? What are the precautions to adopt when using IP-RP LCMS? Is Hydrophilic interaction chromatography (HILIC) really an alternative to the use of ion pairing?

DISCUSSIONS, CONSENSUS AND CONCLUSIONS

Microbiome Contributions to Small Molecule Drug Metabolism and its Impact on Bioanalytical Assays

The human gut microbiome represents a complex ecosystem which has been emerging as a major factor in the disposition as well as efficacy and safety of drugs and other components affecting human health and disease. The gastrointestinal (GI) tract is a highly variable biome which functions as a metabolic organ, containing a vast array of microbes (e.g., bacteria, fungi, yeast). This microbiome collectively encodes >100-fold more genes than the human genome. The most important region for microbial metabolism is the large intestine. Altering the intestinal environment can alter metabolism and therefore the systemic exposure of a drug significantly metabolized by GI tract bacteria [51]. The potential effect of the microbiome on drugs depends on the drug disposition. Drugs that are highly absorbed in the small intestine, due to their physicochemical properties, are not significantly affected by the GI microbiome unless a biliary excreted metabolite undergoes further metabolism in the GI. The largest effects will be on drugs that pass into the large intestine or undergo enterohepatic circulation [52].

The metabolic pathways in the microbiome are very different compared to the metabolic pathways in the liver. Bacterial metabolism in the GI is largely degradative, reductive and hydrolytic in contrast to the predominantly oxidative and conjugative metabolism of the liver and gut wall. Selected groups which can be prone to microbial metabolism include oxadiazole, isoxazole, hydrazine, sulfoxide, and non-aromatic double bonds. In some cases, these biotransformations can have significant effects on PK, efficacy and toxicity through bioactivation, inactivation or toxification [53].

The investigation of these effects poses major challenges for bioanalytical scientists in order to evaluate the modulation of drugs by the microbiome to bioactive, inactive or toxic components. A common approach is the incubation with fecal samples under anaerobic conditions. In addition, GI simulators provide *in vitro* tools to investigate microbiome metabolism [54]. *In vivo* approaches include germ-free rodent studies, which compare metabolite profiles from germ-free animals vs wild-type animals and the ability to transfer human fecal microbiome into animals. Another common approach is the use of bile-duct cannulated studies, which compare metabolite profiles of biliary and fecal excretion in cannulated and conventional animals [55].

In order to better understand drug biotransformations by the gut microbiome, the above-mentioned studies are often performed at a later stage as they are often unknown or overlooked until completion of radiolabeled mass balance studies and associated biotransformation analysis.

Given the increased understanding of the impact of the microbiome on human health, the bioanalytical technologies to gain further knowledge in this field were discussed. It was concluded that one of the biggest challenges centered around tissue measurements and processing. To ensure appropriate preservation of microbial communities existing in the gut, standards for sample collection and cryopreservation are necessary. Use of both fresh and cryopreserved samples has been reported, with a preference for fresh samples, and the inclusion of both positive and negative controls. Regardless of if fresh or frozen samples are used, anaerobic conditions are required. Furthermore, to preserve fecal samples for eventual sequencing, the addition of RNA/DNA stabilizers should be evaluated.

When a major systemically circulating drug metabolite is sourced to the gut microbiome in very rare cases, feces is an important matrix for investigation. However, the quantification of feces samples should not become mandatory as regulatory guidance is concerned with circulating metabolites. SCFAs are a group of organic carboxylic acids with a linear or branched aliphatic tail of two to six carbon atoms in length. Anaerobic microbiota in the human colon metabolize dietary fibers or resistant starches into linear SCFAs while amino acid metabolism produces branched-chain SCFAs. SCFAs are biologically relevant small molecule ligands since they have been shown to modulate glucose, lipid and cholesterol metabolism in peripheral tissues after absorption. They are also known to play a role in gut integrity, appetite regulation and immune function. Disruption in the intestinal barrier has been linked to intestinal diseases such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), celiac disease and colon carcinogenesis, and thus the role of SCFAs have been evaluated in these diseases. As a result, SCFAs have been shown to be important for the maintenance of gut mucosal barrier homeostasis, and recent studies have shown that SCFAs can modulate the immune function by influencing regulatory T-cell generation and intestinal macrophages function through histone deacetylase inhibition.

Due to their important and multifaceted role in human health and biological homeostasis, it is desirable to absolutely quantitatively measure SCFAs in various disease conditions and from diverse biological matrices. Efforts have already been made to demonstrate that SCFAs can be used as surrogate biomarkers to assess gut integrity in disease conditions, such as IBS using human fecal samples. Literature precedent has been established for the direct quantitative measurement of SCFAs in human feces, where they are present in high concentrations. However, SCFAs are also known to be absorbed in the gut and are present in various human matrices such as plasma, serum and urine, albeit at much lower concentrations than feces. After absorption, it is important to determine SCFA concentrations in matrices other than feces as they are known to be involved in the host's biology, for example acting as ligands for histone deacetylases, G protein-coupled receptors (GPCRs) and effecting biological functions such as energy homeostasis and immune function. A direct analysis approach of native SCFAs using existing methods would be limited in sensitivity since, in addition to their much lower levels in nonfecal matrices, SCFAs are acidic, polar small molecules lacking a chromophore and have relatively poor ionization efficiency. In order to comprehensively evaluate the SCFA concentrations in nonfecal matrices, sample preparation procedures including derivatization and solid phase or liquid–liquid extraction (LLE) need to be considered.

Acoustic-MS for Bioanalytical Applications

Significant improvements on both LC separation and MS instrumentation in the past decade have enabled sub-minute analysis speeds of biological samples. However, for various bioanalysis applications it is still desirable to further increase the analysis speed, lower the sample consumption, and simplify the sample processing steps. Toward that goal, a novel MS analytical platform with integration of acoustic droplet ejection (ADE) technology, an open-port interface (OPI) for sampling, in combination with electrospray ionization (ESI) MS was developed. The ADE technology delivers nanoliter droplets in a touchless manner with high speed, precision and accuracy; subsequent sample dilution within the OPI, in concert with the capabilities of modern ESI-MS, minimizes assay development and almost eliminates the laborious sample preparation required in current approaches. Second-per-sample analysis speed can be routinely achieved with desirable sensitivity in many cases (large dilution effect of the OPI may limit high sensitivity for some applications), high reproducibility, wide linear dynamic range, and broad compound coverage. The continuous flow of carrier solvent for the OPI maintains ionization stability and actively cleans the entire flow system resulting in no observed carry-over under most conditions. However, carry-over can be seen depending on carrier solvent and sample type/amount.

The recommendations in the 2019 White Paper in Bioanalysis [23] indicated that while sensitivity and selectivity were challenges of using this approach, it could be a useful tool to employ in drug discovery due to the possibility for high throughput and the benefit of reduced sampling volume. In the past year, additional experience, though limited, was acquired and discussions continued. Until recently, all instruments were prototypes available in a limited number of laboratories. However, Sciex recently launched the Acoustic Ejection Mass Spectrometry (AEMS) Echo[®] MS system (an ultra-fast non-chromatographic direct injection instrument), providing opportunity for broader adoption and experience within the bioanalytical community. Unresolved sensitivity, selectivity and ion suppression issues may initially limit the applications (ruggedness needs to be demonstrated), but the low injection volume may limit carryover, potentially mitigate ion suppression and benefit microsampling studies. Extra sample cleanup may still be required, and a SIL would be advantageous in order to off-set any unresolved matrix effects. Software validation may be a logistical issue. Finally, concerns remain around the potential degradation of phase 2 metabolites or other interferences.

The technology is still in its infancy and further investigation is warranted. It was pointed out that new/different approaches/workflows could be taken for this new technology. For example, there is a potential to analyze multiple replicates ($n = 10$) per sample, as well as to increase the number of standards and QCs that can be analyzed which could increase the analytical rigor. In addition, development of companion technologies (such as special sampling devices for microsampling approaches) would help the implementation of this technology for broader bioanalysis applications. As of this writing, there has been limited experience using this technology in a regulated environment since discovery stage research has been the main focus for applying this technology.

HRMS: Small Molecule Method Development Strategies

The improved performance of HRMS systems has triggered more bioanalysts to switch from triple quadrupole MS to HRMS for quantitative analysis when additional selectivity was required [56–58]. There was consensus that the conclusions reached in the 2015 White Paper in Bioanalysis, i.e., HRMS technology is fit for use in regulated bioanalysis for PK and biomarker assays [10] are still valid, and there have been regulatory submissions already. It had also been concluded that methods must be validated according to BMV guidance/guidelines [10,32,33], but that additional validation experiments may be necessary, especially when considering extraction windows [13,59,60]. It was agreed that there are cases where HRMS provided better selectivity but not necessarily increased sensitivity than traditional triple quadrupole LCMS technologies. HRMS has also been frequently used for improved separation from isobaric interferences as well as improved sensitivity for compounds of very low molecular weight. Alternatively, HRMS can be used to guide method development and facilitate troubleshooting, and then the method is transferred to traditional triple quadrupole LCMS systems for validation and study support.

Because the extraction window has been determined to be important during method development/validation, and as more experience and uses for this technology have evolved, so have the options for data processing. The impact of centroid versus continuum/profile data on the mass extraction window (MEW) selection and quantification was discussed using case studies. There was agreement that “centroid data provide advanced selectivity resulting in cleaner XICs for qualitative analyses but with no effect on quantitative performance (LLOQ, precision, linearity) and the optimal MEW selection for centroid data can be solely defined by the mass accuracy of the system used. For profile data, the improved selectivity provided by narrower MEWs can be counterbalanced by a loss in MS peak area affecting signal-to-noise. Therefore, the full width half maximum (FWHM) of the peak can be the ideal starting point for MEW selection in addition to mass accuracy which becomes gradually more important with increasing MS resolving power. Continuum data can be handled differently by different software packages” [61].

Commercial HRMS instruments and hardware are not a limiting factor; however, system and quantification software may be challenging. Full scan HRMS has the potential to perform data-mining for post-acquisition data extraction. However, this should not be considered for regulated bioanalytical support due to data size and regulatory implications.

Design of Experiments for Therapeutic Peptides: Modern Discovery Bioanalytical Laboratories

The mandate of the bioanalytical laboratory has changed dramatically over the last decade. This has been fueled by the continued diversification of the therapeutic modality space, including monoclonal antibodies, a host of immunoglobulin analogs, fragments and modifications, antibody drug conjugates, fusion proteins, synthetic enzymes, oligonucleotides, peptides and other macrocycles, as well as continued advancement of vaccine technologies. To facilitate the support of such a diverse landscape of candidate therapies, the modern discovery bioanalytical

laboratory has become a multidisciplinary space often blending a host of approaches and technologies such as the integration of traditional immunoassay and LCMS approaches and incorporation of automation. A prime example of a uniquely challenging therapeutic class is peptides, and in particular cyclic peptides (CPs). CPs represent an important alternative therapeutic modality due to binding affinity, unique target selectivity and tolerable toxicity. Support of activities such as PK, PD, and cell-based characterization require high quality, robust quantitative methods with fast turnaround times.

These molecules pose several challenges for quantitative method development. First, often uncharacteristic MS/MS fragments from random ring openings are produced in comparison to linear peptides creating challenges to the traditional multiple reaction monitoring (MRM)-based triple quadrupole quantification of drug molecules. Secondly, generic sample preparation methods are not readily available to support CP, which often have a wide range of chemical diversity. In addition, selectivity is complicated by biological matrices crowded with a broad dynamic range of diverse endogenous peptides leading to challenges of sensitivity as well. The 2017 and 2018 White Papers in Bioanalysis provided useful recommendations on cyclic peptide method development and techniques to mitigate some of these challenges [16,19].

Tools such as DOE and high-resolution mass spectrometry (HRMS) can be used to deliver high quality quantitative data. DOE has been applied in process and manufacturing arenas for more than 25 years. When developing methodology for analytes such as CPs, where extensive empirical assessment must be conducted to identify an ideal approach (e.g., the method cannot be predicted prospectively), DOE is an extremely powerful approach to selecting an optimized solution.

DOE allows for the assessment of a broad experimental space through the testing of a minimal number of key predicted permutations. This data can then be mathematically projected across the full range of theoretical experimental space to allow for one to select optimal conditions across a much larger territory than what is tested. When employing such an approach for CPs one can prioritize the most important parameters to optimize, such as solid phase material and loading, wash and elution buffers in the context of CPs, and efficiently explore an experimental space that would take a tremendous amount of time to execute one at a time. Bioanalytical method development DOE does not need to be limited to peptides, but can be used where there are a large number of parameters to be optimized and it is unclear how, and especially when, there are interplays among the parameters (modality independent approach) [62]. HRMS also can be a powerful tool for CP analytical method development. Given the challenges of fragmenting these molecules, and their larger molecular weight than traditional small molecule drugs, the PPM mass accuracy of HRMS instrumentation can provide sufficient selectivity from full scan (MS1) data.

There are a large number of methods to be developed in drug discovery and parameters to optimize in some regulated drug development methods (primarily using LBA or hybrid assay), therefore it might be less efficient to look for different approaches but to follow a systematic universal approach. In order to rapidly develop sensitive and selective bioanalytical methods for structurally diverse peptide therapeutics, experiments could include evaluation and mitigation of typical issues including nonspecific binding, poor extraction recovery due to high protein binding (diverse extraction techniques might be necessary), poor fragmentation for cyclic peptides, solubility/recovery from aqueous matrices. It was suggested that automation could be used to facilitate DOE (i.e., D300 or liquid handlers), although the level of automation available in each laboratory varies [19]. Sample extraction conditions are often peptide and matrix dependent. Strong anion exchange SPE provides satisfactory clean up results for the majority of peptides with medium to low polarities while the analysis of polar CPs presents a major challenge in terms of selectivity and sensitivity. Mixed mode weak cation exchange SPE has been demonstrated to be a viable alternative method for cationic hydrophilic CPs. Ion suppression and interferences in MS detection are challenges when analyzing samples extracted by protein precipitation. Chemical derivatization and ring opening can be an effective approach to improve LCMS performance for CPs. Additionally, poor MS fragmentation and high polarity could be addressed using careful considerations of sample preparation strategies and selection of most appropriate LCMS systems to achieve different requirements of throughput, sensitivity and selectivity for various types of *in vitro* and *in vivo* CP studies.

Oligonucleotides and Chain-Shorted Metabolites: Advanced Strategies

In order to determine the PK/PD properties of oligonucleotides, sensitive and selective quantitative bioanalytical methods are required. LCMS has emerged as a powerful tool and one of the most specific bioanalytical technologies to quantify a variety of oligonucleotide therapeutics in biological matrices. Recommendations in the 2018 White

Paper in Bioanalysis [19] outline the industry consensus on this topic. One of these states that, due to the requirements of the MIST guidance [63], the analysis of metabolites is a critical step in drug development and should be investigated for oligonucleotides. Compared with hybridization-ELISA assays, LCMS methods can selectively quantify and easily differentiate these full-length oligonucleotides and their major metabolites, having a much wider dynamic range and fewer specific reagents. LCMS is especially advantageous for quantifying relatively small oligonucleotide therapeutics (<20 mer) [64]. Finally, it allows quantification of double-stranded oligonucleotides, such as siRNA, by measuring either sense- or antisense-strand RNA. However, significant challenges may arise and should be taken into account when developing an LCMS assay for oligonucleotides.

For full length ASO method development, it can be technically complex to develop a method to retain metabolites of vastly different molecular weights while removing other interferences. It is reasonable to assume that at least some metabolites will be extracted using the full-length methods due to chemical similarities. Like full length species, their negative backbone also dominates metabolite characteristics, although as length decreases, the following challenges are encountered:

- Extraction recovery decreases
- Chromatographic retention time decreases
- Protein binding decreases (as well as target binding)
- Fragmentation efficiency decreases
- Ionization efficiency increases
- Charge state distribution alters

Base composition and sugar modifications also affect all of the above parameters, resulting in challenges with the absolute quantification of metabolites. Oligonucleotides are polar analytes which are extensively protein-bound, particularly in cells. The very high protein binding and non-specific adsorption to the surface of containers may adversely affect the analyte recovery. In order to achieve satisfactory analyte recovery, displacement of ionic interactions between the oligonucleotide and the basic residues of the protein is necessary, requiring the use of additives and special materials. Oligonucleotide concentrations may also decrease after storage in standard solutions and extracts when the concentrations are low. This is caused by adsorption to the surface of containers rather than by compound instability. In order to prevent this phenomenon, use of glassware should be avoided (if used, ensure it is silanized first), low bind plastic containers can be used, and some competitive components (e.g., bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), or scrambled oligonucleotide sequences) should be kept in the extracts.

The MS sensitivity can also be affected by the ionization suppression caused by sample matrix components; therefore the extraction procedure should be carefully designed to isolate the analytes from the interfering agents. For this purpose, bi-dimensional chromatography or orthogonal extraction techniques can be successfully employed [65]. It was suggested to focus on the extraction first and ensure it is compatible with the detection/quantification method using the overall structure, inherent PK and metabolism to drive bioanalytical strategies. For example, modifications in the ribose (2') or in the strand have not been shown to have an impact; stronger paring may be required for more hydrophilic ASOs (GalNAc (N-Acetylgalactosamine) targeting moiety attached on an ASO). Similarly for Gen 2.5 ASOs (i.e., 3-10-3 cEt moiety [thus leading to high concentrations of metabolites that are only 3 bases long instead of the 5 base long ones from the Gen 2.0]). Truncated ASOs (typically N-1, N-2) can be easily analyzed using the same extraction and chromatography adopted for the parent compound.

The multiple charge states of the analytes can also negatively affect the assay performance, hence the precursor ions should be appropriately selected and the use of additives be considered to optimize the charge distribution with consequent improvement in sensitivity. The lack of retention and poor peak shape are usually observed by reversed-phase chromatography due to the extreme polarity of the analytes, hence there is a need to use IP agents or alternative stationary phases and retention mechanisms (e.g., HILIC) to achieve an adequate chromatographic separation and peak symmetry [66,67]. The formation of cation adducts in the ion source can also reduce the MS response and therefore special precautions should be adopted to offset this phenomenon.

The use of HFIP is the gold standard for improving ionization and reducing adduct formation [68], however each ion-pair combination behaves differently; a thorough evaluation of their affects needs to be performed to select the most appropriate reagent for the application [69,70]. Various HILIC approaches have been published in literature [67]; however, sensitivity was a limitation when panelists explored these techniques in their labs. Overall,

there was consensus that using ion-pairing agents and HFIP is the preferred approach. Some groups have employed LCMS instrumentation for analysis at Cmax concentrations but utilize LBA for trough measurements due to the sensitivity of this platform.

When making these decisions during method development, consider preparing fresh chromatographic mobile phases in small batches due to miscibility of IP and HFIP and high volatility concerns; dedicate specific LC systems due to long equilibration times and avoid cation adducts by using plastic containers rather than glass. Furthermore, oxidation of amines can cause signal loss, therefore consider the preparation of IP mobile phase under a layer of argon and the addition of EDTA, which can also improve mobile phase stability, but comes at the cost of reduced sensitivity.

RECOMMENDATIONS

Below is a summary of the recommendations made during the 14th WRIB:

Microbiome Contributions to Small Molecule Drug Metabolism and its Impact on Bioanalytical Assays

- Available bioanalytical tools:
 - *In vitro*: anaerobic fecal incubations, GI simulators
 - *In vivo*: germ-free rodent studies, which compare metabolite profiles from germ-free animals vs wild-type animals and the ability to transfer human fecal microbiome into animals, and bile-duct cannulated studies, which compares metabolite profiles of biliary and fecal excretion in cannulated and conventional animals
- To ensure preservation of microbial communities existing in the gut, standard processes for sample collection and cryopreservation are necessary. Both fresh and cryopreserved samples have been used, with a preference for fresh samples, and the inclusion of both positive and negative controls
 - Regardless of if fresh or frozen samples are used, anaerobic conditions are required
 - To preserve fecal samples for eventual sequencing, the addition of RNA/DNA stabilizers should be evaluated
- It is desirable to quantitatively measure SCFAs in various disease conditions and from diverse biological matrices. Efforts have already demonstrated that SCFAs can be used as surrogate biomarkers to assess gut integrity in disease conditions, such as IBS using human fecal samples
- In order to comprehensively evaluate the SCFA concentrations in nonfecal matrices, sample preparation procedures including derivatization and solid phase or LLE need to be considered

Acoustic-MS for Bioanalytical Applications

- Until recently, instruments were prototype in nature and available in a limited number of laboratories. However, Sciex recently launched the AEMS Echo[®] MS system, allowing more broad adoption in the bioanalytical community over the next years
- Unresolved sensitivity, selectivity and ion suppression issues may limit the applications (ruggedness needs to be demonstrated), but the low injection volume may limit carryover, potentially mitigate ion suppression and benefit microsampling studies
- Extensive sample cleanup may be required, and a SIL-IS would be advantageous
- Concerns remain around the potential degradation of phase 2 metabolites or other interferences

HRMS: Small Molecule Method Development Strategies

- HRMS technology is fit for use in regulated PK and biomarker bioanalysis
- MEW-centroid is uniquely defined by the mass accuracy of the system
- MEW-continuum is defined by the addition of the mass accuracy of the system and the FWHM
- Methods must be validated according to BMV guidance/guidelines
- HRMS has also been frequently used for improved separation from isobaric interferences as well as improved sensitivity for compounds of very low molecular weight

- HRMS is often used to guide method development and facilitate troubleshooting, and then the method is transferred to traditional LCMS for validation and study support

Design of Experiments for Therapeutic Peptides: Modern Discovery Bioanalytical Laboratories

- Bioanalytical method development utilizing DOE does not need to be limited to peptides, but can be used where there are a large number of parameters to be optimized and it is unclear how, and especially when, there are interplays among the parameters (modality independent approach)
- There are a large number of methods to be developed in drug discovery, therefore it might be less efficient to look for different approaches but to follow a systematic universal approach
- In order to rapidly develop sensitive and selective bioanalytical methods for structurally diverse peptide therapeutics, experiments could include evaluation and mitigation of typical issues including:
 - Nonspecific binding
 - Poor extraction recovery due to high protein binding (diverse extraction techniques might be necessary)
 - Poor MS/MS fragmentation for cyclic peptides
 - Solubility/recovery from aqueous matrices
- Automation could be used to facilitate DOE (i.e., D300 or liquid handlers)
- Strong anion exchange SPE provides satisfactory clean up results for the majority of peptides with medium to low polarities
- Mixed mode weak cation exchange SPE has been demonstrated to be a viable alternative method for cationic hydrophilic CPs
- Chemical derivatization and ring opening can be an effective approach to improving LCMS performance for CPs

Oligonucleotides and Chain-Shorted Metabolites: Advanced Strategies

- LCMS is especially advantageous for quantifying relatively small oligonucleotide therapeutics (<20 mer)
- In order to achieve satisfactory analyte recovery, displacement of ionic interactions between the oligonucleotide and the basic residues of the protein is necessary, requiring the use of additives and special materials
- In order to prevent adsorption issues, use of glassware should be avoided (if used, ensure it is silanized first), low bind plastic containers can be used, and some competitive components (e.g., BSA, EDTA) should be kept in the extracts
- Focus on the extraction first and ensure it is compatible with the detection/quantification method using the overall structure, inherent PK and metabolism to drive bioanalytical strategies
- The precursor ions should be appropriately selected and the use of additives be considered to optimize the charge distribution with consequent improvement in sensitivity
- Use IP agents or alternative stationary phases and retention mechanisms (e.g., HILIC) to achieve an adequate chromatographic separation
- The use of HFIP is the gold standard for improving ionization and reducing adduct formation, however each ion-pair combination behaves differently; a thorough evaluation to select the right one for the application is proposed
- Considerations during method development:
 - Prepare fresh reagents in small batches due to miscibility of IP and HFIP and high volatility concerns
 - Dedicate specific LC systems due to long equilibration times
 - Avoid cation adducts by using plastic containers rather than glass
 - Consider the preparation of IP mobile phase under a layer of argon and the addition of EDTA, which can also improve mobile phase stability, but comes at the cost of sensitivity

SECTION 3 – Regulatory Challenges in Mass Spectrometry

Christine Fandozzi²⁷, Amanda Wilson²⁸, Daniela Fraier²³, Christopher J. Beaver²⁹, Suman Dandamudi⁷, Arindam Dasgupta⁷, Rebecca Elliott²¹, Allena Ji³⁰, Wenkui Li³¹, Michael McGuinness¹², Gustavo Mendes Lima Santos³², Tahseen Mirza⁷, Natasha Savoie³³, Dina Shakleya⁷, Sune Sporning³⁴, Susan Stojdl⁵, Phillip Sundman¹, Nilufer Tampal⁷, Eric Woolf¹⁹

Authors in Section 3 are presented in alphabetical order of their last name, with the exception of the first three authors who were major contributors.

The affiliations can be found at the beginning of the article

DISCUSSION TOPICS & CONSOLIDATED QUESTIONS COLLECTED FROM THE GLOBAL BIOANALYTICAL COMMUNITY

The topics detailed below were considered as the most relevant ‘hot topics’ based on feedback collected from the 13th WRIB attendees. They were reviewed and consolidated by globally recognized opinion leaders before being submitted for discussion during the 14th WRIB. The background on each issue, discussions, consensus and conclusions are in the next section. The key recommendations are summarized in the final section of this manuscript.

Data Integrity and Regulatory Factors to Consider when Using Cloud Computing

Do regulators view cloud hosting facilities or companies as inspectable? What would trigger such an inspection? Do cloud providers view themselves as inspectable? Do they have processes in place to manage regulatory inspections? Is there a regulatory expectation that sponsors know the exact geographic location(s) where their data is stored? Should cloud providers have any fiduciary responsibility regarding the regulated data that they store?

Impact of Excipients on Bioanalytical Methods: “What are regulators asking?”

What are regulators’ concerns regarding excipients used in drug formulations? What is feasible and what is not? How is a fit-for-purpose validation established for unusual bioanalytical instrumentation such as propellant gas analysis (GCMS headspace analysis)?

Parallelism Evaluation in Small Molecule Endogenous Compounds - New Considerations on ICH M10 Draft Guideline

The draft ICH M10 Draft Guideline [26] does not discriminate between the parallelism evaluation for chromatographic assays and LBA for endogenous compounds. Is further clarification needed or would it be too prescriptive since this difference is straightforward and already described in White Papers? What should be considered to evaluate parallelism for chromatographic assays for endogenous compounds? What should be added to the 2018 White Paper in Bioanalysis recommendations [19] on parallelism for chromatographic assays of endogenous compounds? Is it necessary to give a recommendation for slope criteria when conducting parallelism of chromatographic assays for endogenous compounds?

Abnormal Internal Standard Response: 2018 FDA Guidance and ICH M10 Draft Guideline

Should some key points of the 2019 FDA IS question and answer guidance [71] be added to the ICH M10 Draft Guideline [26] to ensure harmonization? If yes, what should be added? What are acceptable and non-acceptable IS variability criteria? Is 80–120% IS variation the industry standard? When is 50–150% IS variation acceptable? What is the impact on the LLOQ signal-to-noise ratio? What is the impact of using a double vs single curve on internal standard variability? Can two curves be used (one front and one back) to compensate for potential IS drift? Can a single curve be used with QCs dispersed throughout the batch if IS drift is ensured during method development? After so many discussions over the years, published White Papers [4,7,10] and now also a specific FDA guidance, is there still a need for further discussion on the evaluation, identification, and impact assessment of abnormal IS response variability in future bioanalytical meetings? If yes, why?

Microsampling in Regulated Bioanalytical Juvenile & Pediatric Studies

ICH S3A [72] recommends a bridging study to prove the equivalence between capillary vs venous sampling within a project whereas the 2017 White Paper in Bioanalysis [16] recommends that no bridging study is needed when only the matrix volume differs and the matrix remains the same. Are bridging studies between capillary vs venous PK data considered good science and performed in industry? If yes, do you have a SOP in place outlining the procedure (e.g., number of subjects/samples/points on the profile)? How does the bioanalytical community overcome the need to conduct ISR when using microsampling if the collected sample volume is not sufficient? Are there any new FDA/EMA interactions on microsampling/patient centric sampling since the 2019 White Paper in Bioanalysis [23]? Are there any new updates on available technologies for regulated bioanalysis since the 2019 White Paper in Bioanalysis [23]?

DISCUSSIONS, CONSENSUS AND CONCLUSIONS

Data Integrity and Regulatory Factors to Consider when Using Cloud Computing

Cloud computing uses a network of remote servers owned and operated by a third-party provider and accessed via the internet to store, manage, and process data, rather than a local server or a personal computer [73]. It is important to note that the third-party cloud provider is independent of the user, data owner or study sponsor. With continued technology advancements and capabilities in mass spectrometry, flow cytometry, and high throughput screening, the costs associated with storing the data using traditional on premises storage technologies are increasing at a rapid rate. Another driver in favor of using cloud computing for regulated bioanalytical work is improved resistance to data loss through redundant storage and automated back-up. These potential benefits are balanced by concerns regarding the potential inability to inspect cloud facilities and ambiguity with respect to exactly where data is being stored.

There are three cloud computing service models available: software as a service (SaaS), platform as a service (PaaS), and infrastructure as a service (IaaS). The SaaS model is comprised of applications, managed by a third party, delivered through the internet. The applications are generally run through a web browser, which eliminates the need for local installation. Technical issues are managed through the vendor, reducing the level of client/sponsor support needed. The PaaS model is based on a platform (i.e., operating system) managed by a third party on which applications are built and installed by the client or sponsor. This service model provides the freedom to concentrate on building the software without having to worry about operating systems, software updates, storage, or infrastructure. Finally, IaaS provides the same technologies and capabilities as a traditional data center without having to physically maintain or manage all of it; it provides a virtual data center where resources are purchased on-demand and as-needed instead of having to buy hardware outright. The client or sponsor is responsible for all aspects of management at the operating system level and above.

Under the various service models, cloud providers do more than provide the physical housing and associated environmental infrastructure for assets owned by the sponsor. The cloud provider owns and is responsible for the hardware on which regulated data resides. Under the IaaS and PaaS models, in an appropriately configured and secure environment, cloud providers do not have access to the data itself, but they do have the ability to limit access to the underlying, generally virtual, infrastructure on which the data resides.

The infrastructure of a public cloud is open and can be accessed from a web address by the public (with proper login credentials). The cloud provider hosts the infrastructure on their premises, and could be either an academic institution, a business or a government organization. Conversely, a private cloud is exclusively used by all the business units of a single organization, accessible only on computers connected to the network of the organization. The infrastructure could be housed either on or off the premises of the organization, depending on if it is owned and operated by them, or by a third party.

Cloud providers point to various certifications (e.g., ISO, FedRAMP) and reviews by third parties in an effort to assure potential clients that their systems are compliant with GxP regulations and are suitable to use for regulated bioanalytical activities. However, all such certifications are voluntary and tend to lump all requirements under the GxP umbrella and do not focus on regulatory nuances specific to Good Laboratory Practices (GLP) (i.e., archive) and Good Clinical Practices (GCP) (e.g., identity protection and sponsor monitoring responsibilities). They do not take the place of the sponsor needing to verify that a cloud provider is compliant with applicable regulations (e.g., GCP, GLP, 21 Part 11). The FDA has not issued an overarching guidance specifically on cloud use, however, existing guidance [74–79] does apply to cloud computing, requiring the application of predicate rule requirements regarding qualification of staff and training records, system validation and data integrity. Records must be retained and available for inspection for as long as the records are required by applicable regulations. Records must be retained and available for inspection for as long as the records are required by applicable regulations, therefore the application should have archiving capabilities. Controlled access to the application and user actions should be verified using authorization checks (e.g., unique username and password). Electronic signature capabilities should have controls in place. Data encryption should be in place, both in transit and at rest. The application should have a computer-generated, secure audit trail that time-stamps all recorded actions or data modifications. The user organization should have access to the vendor's performance records and the electronic service provided as well as have the ability to monitor the vendor's compliance with data integrity controls and security. Risk assessments should be conducted and documented by the sponsor. A service level agreement is required between the sponsor and the cloud service provider. Sponsors should also be aware and conduct appropriate assessments of cloud use

by bioanalytical contract research organizations (CROs) that support their studies. Gathering and maintaining evidence of the above is the responsibility of the cloud user/test facility management and cannot be based solely on the cloud provider attestations.

Some barriers to using cloud storage include certification of cloud storage vendors to meet corporate security standards; the time and network bandwidth required to access and move the data to/from the cloud storage; managing data stored on the cloud in alignment with corporate record retention requirements; security / encryption concerns and issues and addressing cloud storage impact on regulated system validations and data integrity.

The retention requirements are an important consideration when deciding to use a cloud model. A method and system to track and manage archived data is needed that must comply with regulatory expectations, corporate retention requirements and corporate legal holds. The ongoing ability to review and analyze archived data should be considered by ensuring that the software and computing environment are still available when needed.

Another consideration when storing data in the cloud is the use of data compression. Compressing the data can reduce transmission time and storage costs as well as keep related sets of data files bundled together, thereby having fewer files to store and track in the cloud. CRC checks (cyclic redundancy checks) also provide added data integrity checking of the data. Compression has been shown to reduce file storage requirements by anywhere from 50–90% for mass spectrometry and flow cytometry data sets. However, the compression advantages are countered by the time and processing power needed to compress the data, ensuring the availability of decompression software or algorithms in the future when the data may be needed, and the additional temporary storage space needed during the compression process. It is suggested to ensure that the compression server is co-located with data storage for performance, and to split up extremely large data sets into multiple “chunks” for portability reasons.

FDA recognizes that sponsors may choose to outsource electronic services and should consider whether there are adequate controls in place to ensure the reliability and confidentiality of the data [74]. Refer to Part 2 of this White Paper [80] for additionally regulatory inputs on this topic.

There is currently little to no experience from industry or regulators on the use of cloud hosting of bioanalytical data but there is a regulatory expectation that sponsors know the exact geographic location(s) where their data is stored, both primary location and secondary location for business continuity. The cloud provider does not currently take on any fiduciary responsibility. As such, a relevant question to consider is what occurs when a cloud provider wants to decommission the infrastructure without the permission/knowledge of the data owner. Such a situation might potentially occur due to non-payment for services. Perhaps this might be one of the more significant concerns for storing bioanalytical data in the cloud, since the sponsor has little to no control over the infrastructure. Before storing bioanalytical data in the cloud, the sponsor should understand the associated risks with the infrastructure and do whatever is possible to mitigate the risks with a service level agreement.

Impact of Excipients on Bioanalytical Methods: “What Are Regulators Asking?”

The use of delivery vehicles to enhance the PK properties of medicines is a common practice in the pharmaceutical industry and the safety and exposure of many excipients are well characterized. In recent years, the pursuit of patient and environmentally friendly drug administration solutions has led to a renewed interest, from industry and regulators, in the excipients themselves. Seeking alternative delivery approaches for biologics and nucleotide therapeutics traditionally administered by injection has also led to increased interest in the application of oral absorption enhancers.

Measuring the exposure of formulation excipients that are ubiquitous in the environment as a consequence of their routine use as food or product additives can be a bioanalytical challenge. The case of orally delivered macromolecules was discussed in detail. These modalities have a large molecular size, are typically susceptible to break down at gastric pH and have low intestinal epithelial membrane permeability break down at negligible oral bioavailability. The use of permeability enhancers in dosage formulations can assist in transporting nucleic acid and amino acid macromolecules across the intestinal epithelial barrier through the mechanism of opening the membrane intercellular tight junctions. As an example, medium chain fatty acid absorption enhancers are mild surfactants which act as detergents by fluidising the plasma membrane, leading to an increase in intracellular calcium and alteration of tight membrane junctions. An example of a medium chain fatty acid permeation enhancer is C10 (Capric acid/Decanoic acid). C10 is an approved food additive that is also naturally found in some oils and milk from various animals and due to its natural prevalence and use in commercial products it is ubiquitous in the environment. When analysing these types of compounds using LCMS, it can be difficult to achieve acceptable sensitivity and selectivity due to the high levels of ubiquitous C10 in control plasma, reagents and the laboratory

environment. Two solutions were presented. The first used a deuterated form of the excipient (C10-d¹⁹) in the formulation during preclinical testing in order to allow for the management of environmental contamination of C10 in the assay which enabled characterisation of C10 exposure following low dose administration. An alternative approach was required to support macromolecule safety studies that were formulated with unlabelled C10. Due to the high and variable levels of C10 in control matrix, a surrogate matrix approach was used for the preparation of calibration samples and screened plasma was used to prepare the quality control samples. In addition, the surrogate matrix reagents themselves required some screening to manage the environmental impact of C10 in the analytical workflow. By using GCMS, the LLOQ was 15-fold more sensitive compared to using the deuterated approach combined with LCMS, however, the analytical run throughput was significantly impacted by the longer analytical run time. For the fit-for-purpose validations, it was suggested to use a surrogate matrix for LLOQs and the real matrix for high level standards. Standard addition and dilutional linearity should also be investigated.

Formulation scientists also seek to improve the sustainability of inhalation formulations by evaluating next generation near zero global warming product propellants. Quantification of the propellants creates unique challenges for the bioanalytical scientist. One approach used to quantify propellants is GCMS headspace analysis; the analysis of the vapor phase above a biological sample in a closed system. Validation using this method is FFP, and must consider the ability to freeze the samples, use an IS, potential gas leaks from the vial after storage, the ability for repeat analysis of samples as well as handling samples above the upper limit of quantitation. Calibrants and QC samples are prepared using neat gas from a cylinder transferred to a Tedlar[®] gas sampling bag. The neat gas from the Tedlar[®] bag is then diluted by transferring a known amount of the gas (using a gas tight glass syringe) to a Tedlar[®] gas bag containing a known amount of nitrogen. The gas is diluted serially in this fashion to produce spiking standards. Suitable volumes of the spiking standard are added to the sealed GC headspace analysis vials, containing whole blood matrix, through the gas tight septum to prepare QC and calibration samples. Head space gas stability data indicates that samples cannot be frozen as the integrity of the vial seal can be impacted. Samples must be analyzed within 48 h of the in-life sampling to achieve adequate recovery. Samples are typically analyzed via GCMS and the long run times can limit the number of samples that can be analyzed within the established stability time frame.

Close collaboration between *in vivo* scientists and bioanalysts is critical. When designing the study, the time from sampling to analysis may be limiting if more samples are taken than stability will cover during analysis; staggered dosing regimens can mitigate this issue. Blood should be collected directly into GC headspace vials to minimize gas loss. Animal and clinical technicians need to be in control of the volume taken and prepare samples quickly using a suitable sampling syringe or by measuring blood spike with weight by difference. Due to the stability time constraints, the in-life work must be physically close to the bioanalytical facility and the necessity for quick turnaround headspace analysis often requires project support being run in series not in parallel.

During the discussion it was noted that there is limited GC instrumentation at the CRO available to support the pharma industry, leading to GCMS becoming a niche area. Demand and competition for these instruments are high, despite much longer run times than current LCMS methods.

Parallelism in Small Molecule Endogenous Compounds – New Considerations on ICH M10 Draft Guideline

There are similarities between endogenous compounds and biomarkers since both have an endogenous counterpart. However, they are not exactly the same since endogenous compounds follow BMV guidelines/guidance for PK assays [32,33] whereas there is not yet a BAV guidance. Hence, only some biomarker principles can be applied to endogenous compounds.

For ligand-binding biomarker assays, because of a non-linear response equation, parallelism is assessed by diluting a biological sample with the buffer or surrogate matrix used for calibration curve preparation, then measuring the analytical recovery of the diluted sample. If the diluted sample meets the accuracy acceptance criteria (i.e., $\pm 20\%$ or $\pm 25\%$ bias from nominal values), then the response of the analyte in the surrogate matrix is parallel to the response in the authentic matrix. Therefore, the surrogate matrix can be used for calibration curve preparation. The matrix effect is evaluated according to the 2018 White Paper in Bioanalysis recommendation [19], in which a low QC level analyte is spiked into surrogate matrix, medium level samples are from authentic matrix without spike (endogenous analyte only), and high level samples are made by spiking the analyte into a medium level authentic matrix, tested in the same run. Six different authentic biological matrix lots should be used. The purpose

of evaluating the matrix effect is to test variability among authentic biological matrix lots and to determine if spiked analytes can be accurately recovered from authentic matrix with a biomarker.

The purpose of assessing parallelism of small molecule endogenous compounds that are assayed using LCMS is quite different. Quantification of biomarkers and endogenous compounds by LCMS is complicated by the presence of an endogenous counterpart. A common way to overcome an endogenous counterpart in the calibration curve is to spike the analyte standard into a surrogate matrix devoid of the target analyte. The parallelism between the calibration curve of the target analyte in biological matrix and in surrogate matrix must be demonstrated in order to ensure the accuracy of biomarker results. Amongst the many small molecule biomarker by LCMS recommendations in the 2018 White Paper in Bioanalysis [19], it was recommended that the parallelism experiment for the surrogate matrix approach is not required during validation. However, it is good practice to always investigate it along with other key parameters like matrix effect during method development in order to optimize the method. There was consensus that no additional recommendations are needed at this time, although it was confirmed that surrogate matrices should be demonstrated to be adequate for use.

The ICH M10 Draft Guideline [26] does not discriminate between the parallelism evaluation for chromatographic assays and LBA for endogenous compounds. It was clarified that regulators want to see that the use of the surrogate matrix is justified (e.g., lack of authentic matrix and limited quantities), and that the surrogate matrix is “similar” to the authentic matrix. In this context, it was requested that the guideline define “similar” but there is apprehension to do so as it may result in language that is too prescriptive. It was recommended to separate the LCMS parallelism section from the LBA one in the ICH M10 Draft Guideline and to also follow the recommendation for small molecule biomarkers by LCMS discussed in the 2018 White Paper in Bioanalysis [19] for small molecule endogenous compounds.

It was also discussed to potentially use an approach where slopes generated from curves prepared with authentic matrix and surrogate matrix are compared. These slopes should be in the same analytical run for at least two runs. The slope acceptance criterion for surrogate matrix is based on % bias for concentration. If concentration is allowed $\pm 15\%$ bias, then % difference in slope of the surrogate matrix from the authentic matrix is from -13.0% to $+17.6\%$. Slope has a reciprocal relationship with concentration % bias and is not a systematic criterion. The qualification of the surrogate matrix lots should be conducted when changing to a new lot of standards and/or QCs.

Since there are currently no slope criteria in BMV Guidances/Guidelines [32,33], there was reluctance to add additional criteria when other orthogonal procedures can be used. However, it was decided to further test the slope approach within the global bioanalytical community and re-discuss the need for slope criteria in a future WRIB meeting.

Abnormal Internal Standard Response: 2018 FDA Guidance and ICH M10 Draft Guideline

An IS should have physicochemical properties very close or identical to the analyte of interest to normalize signal tracking in LCMS assays. Thus, SIL-IS is preferred whenever possible. When a SIL-IS is not available, a structural analog or other molecule may be used as IS as long as it tracks the analyte of interest. Different approaches for monitoring IS drift can be used (e.g., 2 curves – front and back of the run; single curve with QC interspersed), but the overall recommendation was that the use of SIL IS can make IS drift less of a concern, which is the rationale for using SIL IS in the first place.

Incurred samples can be more complex in their composition than the pre-screened blanks used for the preparation of standards and QCs. The presence of drug related metabolite(s), co-medication(s) and metabolite(s), dosing vehicle or subject specific endogenous components may have an impact on the IS response (e.g., signal enhancement or suppression). In some cases, excess variability in IS response can be encountered due to human error(s) or instrument malfunction.

Due to the complexity of biological samples and LCMS processes, a certain level of IS response variability across a run or a study is expected. However, the acceptable amount of IS response variability has been a point of discussion for many years; discussions are summarized in the Decennial Index [22]. The FDA also embarked on a collaborative project between scientists from OSIS (Office of Study Integrity and Surveillance) and OTR (Office of Testing and Research) in CDER (Center for Drug Evaluation and Research). The project was focused on systematic evaluation of IS variation and its effect on the accuracy of measured analyte concentrations and the associated mitigation strategies using the Rapid-Fire MS-MS platform. Refer to the 2019 White Paper in Bioanalysis [23] and Part 2 of this White Paper [80] for further information on this project.

A common approach in the bioanalytical community to evaluate IS response variability and identify abnormal IS response is to compare the IS response of interest vs the mean of (1) all the known samples (i.e., standards and QCs) and unknown samples; (2) unknown samples only; or (3) known samples only. The acceptance window for the IS response variability varies from one organization to the other, ranging from for example, 25–175%, 30–170%, 50–150%, 50–175%, to 50–200%. These arbitrary acceptance criteria can help identify sporadic IS response outlier(s) for immediate repeat analysis of the respective samples to confirm the reportable values. While these acceptance criteria may be more meaningful if an analog IS is used, they may not always be rooted in a solid scientific and/or regulatory rationale. IS response(s) of interest meeting the pre-defined acceptance criteria may not guarantee the accuracy of the measured analyte concentrations. The approach used to identify abnormal IS response should be consistent as this is more important than the actual number used as the acceptance criteria. Criteria should result in an unbiased evaluation of the data, be simple to implement for the identification of systemic variability, if any, which is of greater concern compared to individual anomalies that can be addressed on a case-by-case basis.

Therefore, the evaluation of IS response patterns and trends of unknown samples vs. known samples should be made for any given LCMS run to identify systematic IS response variability that may have an impact on the accuracy of the respective results. This is followed by an investigation of a root cause and assessment of the possible impact on the results due to the abnormal IS response variability. These exercises should lead to either (1) reporting the original results or (2) repeat the analysis after resolving the problem. In practice, whatever the criteria and/or approaches are employed, it is important to be consistent and be able to explain the rationale for their use (e.g., following SOPs) for an unbiased, scientific interpretation.

In 2019, the FDA issued a guidance document on ‘Evaluation of Internal Standard Responses During Chromatographic Bioanalysis: Questions and Answers’ [71], which has provided some clarity for industry on FDA regulatory expectations and reporting requirements. It was discussed if some of the key points addressed in this document should be added to the ICH M10 Draft Guideline. The conclusion was that no extra key points are needed.

Microsampling in Regulated Bioanalytical Juvenile & Pediatric Studies

Children (0 to 16 years old) are a vulnerable population, as they have not reached the legal age to ethically and legally consent to take part in clinical investigations. It is acknowledged that historically, many marketed drugs were not studied adequately and/or authorized for pediatric use. This caused difficulties for pediatricians who did not administer drugs with a potential benefit to pediatric patients (as they were not registered for the intended use) or treated them off-label with a drug that had only been investigated in the adult population. In more recent years, the EMA, FDA and ICH introduced new regulations for pediatric medicines, which also included financial incentives to companies that voluntarily conducted pediatric studies [81–83]. Pediatric patients should have access to drugs that have been investigated for their intended use and drug development programs should include pediatric studies when pediatric use is anticipated. Because of the new regulations that were introduced in order to better protect the health of children and support the development of medicines for children, progress was made over the past decade that resulted in an increase in therapies available for childhood diseases.

Children are not small adults, in fact physiological and biochemical changes occur in childhood that result in differences compared with adults in the PKPD of a drug. Age-related differences in drug ADME (absorption, distribution, metabolism and elimination) should be considered during dose determination and adjustments to optimize drug efficacy and to avoid toxicity. Plasma protein binding (PPB) in children can differ from adults, leading to a change in free drug fraction. A decrease in the free fraction with increasing age is common particularly for highly bound drugs [84,85]. Changes in PPB can also occur based on the disease state. Therefore, accurate estimates of the free plasma fraction at different ages are essential for relevant predictions of the pharmacological efficacy and safety in humans. Constraints in the blood volumes allowed to be collected from children should be considered when planning any PPB sampling, in addition to PK.

Dose range finding juvenile non-clinical studies are typically included in pediatric drug development programs to support clinical trials in children. Traditional PK sampling volumes (up to 500 μ L per time point, depending on the species) can be challenging or impossible to obtain in juvenile species due to the reduced total blood volume. To overcome this problem, the microsampling technique is often used. It allows lower blood volume collection (down to 10–20 μ L per time point) via capillary sampling, which is usually less stressful than traditional PK sampling. Furthermore, multiple samples can be collected from individual animals so that an entire PK profile can be obtained from the same animal and evaluated together with the drug safety.

For pediatric patients, blood loss should not exceed 3% of total blood volume over four weeks, and it should not exceed 1% of total blood volume at any single time [86]. The frequency of blood sampling and volume can be minimized by using micro-volume drug assays and sparse sampling. Microsampling approaches include either sampling and analyzing whole blood (e.g., dried blood spots, Volumetric Absorptive Microsampling (VAMS)) or sampling whole blood into a capillary or microtainer/minivette, and then harvesting and analyzing plasma (capillary microsampling). The planning and conduct of pediatric studies need special attention and ethical, scientific and operational considerations, such as recruitment, the need for specialized clinics, formulation adaptations and so on [87].

Microsampling has clear advantages in terms of remote patient access, smaller sampling volumes, enhancing data collection, capturing PK at home during episodic events, ability to ship without dry ice and serial sampling in smaller species in preclinical studies. It does come with some challenges to overcome related to the small sample volume, stability in the dried state, sample handling and storage, mature automated workflows in support of the analysis to name a few. That said, when used thoughtfully, microsampling can enable collections otherwise not easily attainable and can add value to studies.

Strategies for how to approach microsampling including case studies with successful health authority acceptance has been published [88]. Bridging PK data when switching from a liquid to a dried matrix (e.g., VAMS) is thought to be required and an important consideration in the overall strategy. Industry experience has demonstrated that an equivalency study is typically being performed.

One concern with the limited sample volume might be the ability to conduct ISR. In cases where ISR are required on a study, suggestions to overcome single low volume sample collections include “washing out” the capillary and saving both the capillary and the sample to increase the sample volume. Overall there seems to be enough value in microsampling to further advance the science and overcome some of the logistical challenges. When using microsampling as a sample collection technique, an assessment of the comparability of the exposure measurements between microsampling and the conventional method may be necessary, particularly when sampling site and matrix are substantially different from conventional sampling (e.g., dried vs liquid sample) [72] or making a change in matrix from plasma via venous sampling to blood from a capillary finger sample. However, if the matrix remains the same and only the sampling volumes differ, no bridging studies may be required [16]. Finally, consensus was reached that no new recommendations on microsampling are required compared to those already published in the 2019 White Paper in Bioanalysis [23].

RECOMMENDATIONS

Below is a summary of the recommendations made during the 14th WRIB:

Data Integrity and Regulatory Factors to Consider when Using Cloud Computing

- All certifications are voluntary and tend to lump all requirements under the GxP umbrella and do not focus on regulatory nuances specific to GLP (i.e., archive) and GCP (e.g., identity protection and sponsor monitoring responsibilities)
- The sponsor needs to verify that a cloud provider is compliant with applicable regulations (GCP, GLP, 21 Part 11, General Data Protection Regulation, etc.)
- Existing regulatory guidance [74–79] does apply to cloud computing, requiring that:
 - The application of predicate rule requirements regarding qualification of staff and training records, system validation and data integrity; and
 - Records must be retained and available for inspection for as long as the records are required by applicable regulations
- The application should have:
 - Archiving capabilities
 - Access controls and authorization checks for users’ actions
 - Secure, computer-generated, time-stamped audit trails of users’ actions and changes to data
 - Encryption of data at rest and in transit
 - Electronic signature controls
 - Performance records of the electronic service vendor and the electronic service provided; and

- The ability to monitor the electronic service vendor's compliance with electronic service security and the data integrity controls
- Risk assessments should be conducted and documented by the sponsor
- A service level agreement is required between the sponsor and the cloud service provider
- Sponsors should also be aware and conduct appropriate assessments of cloud use by bioanalytical CROs that support their studies
- The ongoing ability to review and analyze archived data should be considered by ensuring that the software and computing environment are still available when needed
- Share the compression server co-location with data storage for performance, and to split up extremely large data sets for portability reasons
- There is a regulatory expectation that sponsors know the exact geographic location(s) where their data are stored, both primary location and secondary location for business continuity
- The cloud provider does not currently take on any fiduciary responsibility, however they should be aware that regulated data is being stored there, and this should be taken into consideration prior to decommissioning the infrastructure

Impact of Excipients on Bioanalytical Methods

- To achieve acceptable sensitivity and selectivity to characterise C10 excipient exposure, two solutions were presented:
 - Use a deuterated form of the excipient (C10-D¹⁹) in the formulation during preclinical testing in order to allow for the management of environmental contamination of C10 in the assay which enabled characterisation of C10 exposure; and
 - Use GCMS to increase sensitivity and a FFP validation approach for safety study support. Use a surrogate matrix and standard addition method to manage the ubiquitous C10 in the laboratory, reagents and matrices
- GCMS headspace analysis:
 - Validation using this technique is FFP
 - Calibrants and QC samples are prepared using serially diluted neat gas transferred to Tedlar[®] gas sampling bags
 - Suitable volumes of the gas spiking standard are added to the headspace analyser vials using gas tight glass syringes piercing through the gas tight septum of the vial to fortify whole blood QC samples and produce instrument calibration standards
 - Head space gas stability data indicates that samples cannot be frozen as the vial seal can be impacted. Samples must be analyzed within 48 hours of sampling to minimize gas loss
 - Staggered dosing regimens should be used to mitigate sampling time vs stability constraints and the in-life facility and bioanalytical facility should be in close proximity

Parallelism in Small Molecule Endogenous Compounds - New Considerations on ICH M10

Draft Guideline

- There are similarities between biomarkers and endogenous compounds. However, endogenous compounds are regulated by BMV Guidance for PK assays whereas there is no guidance for biomarkers
- It was agreed that no additional recommendations are needed at this time since the 2018 White Paper in Bioanalysis [20] recommendation on small molecule biomarkers for LCMS can also be applied to small molecule endogenous compounds by LCMS
- It was confirmed that surrogate matrices should be demonstrated to be adequate for use
- Regulators want to see that the use of the surrogate matrix is justified (e.g., lack of authentic matrix, limited quantities, etc.), and that the surrogate matrix is "similar" to the authentic matrix
- The LCMS parallelism section for endogenous compounds should be separated from the LBA section in the ICH M10 Draft Guideline

- ICH M10 Draft Guideline can follow the recommendation of the 2018 White Paper in Bioanalysis [20] for the small molecule endogenous compound parallelism
- It was not recommended to include calibration curve slope criteria in any guidance at this time

Abnormal Internal Standard Response: 2018 FDA Guidance and ICH M10 Draft Guideline

- Different approaches for monitoring IS response drift can be used (e.g., 2 curves – front and back of the run; single curve with QC interspersed), but the overall recommendation was that the use of SIL-IS can make IS drift less of a concern as it generally tracks the analyte of interest well. It was however acknowledged that this may not always be possible and an analogue IS also has value to allow meaningful criteria to be applied
- Whatever acceptance criteria are used to identify abnormal IS response, the approach should be consistent and defined by an SOP, be an unbiased and scientific evaluation, be simple to implement and focus on the interpretation and remediation of systemic variability
- Evaluation of IS response patterns and trends of unknowns vs. knowns should be made for any given LCMS run to identify systematic IS response variability that may have an impact on the accuracy of the respective results. This is followed by an investigation of a root cause and assessment of the possible impact due to the abnormal IS response variability
- No extra key points need to be added to ICH M10 Draft Guideline

Microsampling in Regulated Bioanalytical Juvenile & Pediatric Studies

- When using microsampling as a sample collection technique, an assessment of the comparability of the exposure measurements between microsampling and the conventional method may be necessary, particularly when toxicokinetic samples are substantially different from conventional sampling (e.g., dried vs liquid sample). Regulators have requested equivalence between different sampling techniques
- Bridging PK data when switching from a liquid to a dried matrix (e.g., VAMS) might be required
- Bridging studies can be built into the study design to avoid the need for independent bridging studies
- Bioanalysts might need:
 - To plan for frequent PK measurement with rapid turnaround times for dose adjustments and deal with the associated challenges with samples and data reconciliation
 - To perform protein binding measurements to exclude potential changes of free drug fraction with age. Microsample collection might not be possible for this purpose
- As with any blood sampling, ISR is not required for every study, but when it is required, for liquid capillary sampling the washout diluent can result in sufficient sample to perform ISR, on the diluted sample alternatively multiple samples may be taken
- Strategies for a potential Therapeutic Drug Monitoring post-approval may need to be considered
- No new recommendation on microsampling are required since those published in the 2019 White Paper in Bioanalysis [23]

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