2017 White Paper on recent issues in bioanalysis: aren't BMV guidance/guidelines 'Scientific'? (Part 1 – LCMS: small molecules, peptides and small molecule biomarkers)

The 2017 11th Workshop on Recent Issues in Bioanalysis (11th WRIB) took place in Los Angeles/Universal City, California from 3 April 2017 to 7 April 2017 with participation of close to 750 professionals from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. WRIB was once again a 5-day, weeklong event – A Full Immersion Week of Bioanalysis, Biomarkers and Immunogenicity. As usual, it was specifically designed to facilitate sharing, reviewing, discussing and agreeing on approaches to address the most current issues of interest including both small and large molecule analysis involving LCMS, hybrid LBA/LCMS and ligand-binding assay (LBA) approaches. This 2017 White Paper encompasses recommendations emerging from the extensive discussions held during the workshop, and is aimed to provide the 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 2017 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 1) covers the recommendations for Small Molecules, Peptides and Small Molecule Biomarkers using LCMS. Part 2 (Biotherapeutics, Biomarkers and Immunogenicity Assays using Hybrid LBA/LCMS and Regulatory Agencies' Inputs) and Part 3 (LBA: Immunogenicity, Biomarkers and PK Assays) are published in volume 9 of *Bioanalysis*, issues 23 and 24 (2017), respectively.

First draft submitted: 11 September 2017; Accepted for publication: 14 September 2017; Published online: 16 November 2017

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The 11th edition of the Workshop on Recent Issues in Bioanalysis (11th WRIB) was held in Los Angeles/Universal City, California from 3 April 2017 to 7 April 2017 with attendance of over 750 professionals from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. The workshop included three sequential core workshop days, two additional advanced specialized sessions and four training courses that together spanned an entire week to allow exhaustive and thorough coverage of the current major issues in bioanalysis, biomarkers and immunogenicity.

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Abbreviations	
ADME:	Absorption, distribution, metabolism, and excretion
AUC:	Area under the curve
BAV:	Biomarker assay validation
BE:	Bioequivalence
BMV:	Bioanalytical method validation
CID:	Collision induced dissociation
CGMP:	Current Good Manufacturing Practices
CMS:	Capillary microsampling
CRO:	Contract research organization
CSF:	Cerebrospinal fluid
DBS:	Dried blood spots
DMS:	Differential mobility spectrometry
DMSO:	Dimethyl sulfoxide
ELN:	Electronic laboratory notebook
ESI:	Electrospray ionization
EU:	European Union
FFP:	Fit for purpose
GCP:	Good Clinical Practice
HRMS:	High resolution mass spectrometry
IMS:	Ion mobility spectrometry
IS:	Internal standard
ISR:	Incurred sample reanalysis
IVIVC:	<i>In vitro/in vivo</i> correlations
LBA:	Ligand binding assay
LCMS:	Liquid chromatography mass spectrometry
LIMS:	Laboratory Information Management System
LLOQ:	Lower limit of quantitation
m-NBA:	m-nitrobenzyl alcohol
MIM:	Multiple ion monitoring
MOA:	Mechanism of action
MRM:	Multiple reaction monitoring
MS:	Mass spectrometer
PD:	Pharmacodynamic
PK:	Pharmacokinetic
QA:	Quality assurance
QC:	Quality control
QTOF:	Quadrupole Time-of-flight
SIM:	Single ion monitoring
SRM:	Selected-reaction monitoring
SOP:	Standard operating procedure
TK:	Toxicokinetic
VAMS:	Volumetric absorptive microsampling
WRIB:	Workshop on Recent Issues in Bioanalysis

As in previous years, the 11th WRIB gathered a wide diversity of international industry opinion leaders and regulatory authority representatives working in the field of small and large molecule bioanalysis, to facilitate the sharing and discussions focused on improving quality, increasing regulatory compliance and achieving scientific excellence on bioanalytical issues.

The active contributing chairs included Dr Eric Yang (GlaxoSmithKline), Dr Jan Welink (EMA), Dr An Song (Genentech), Dr Hendrik Neubert (Pfizer), Dr Fabio Garofolo (Angelini Pharma), Dr Shalini Gupta (Amgen), Dr Binodh DeSilva (Bristol-Myers Squibb) and Dr Lakshmi Amaravadi (Sanofi).

The participation of regulatory agency representatives continued to grow at WRIB [1–15], covering topics and discussions in bioanalysis, biomarkers and immunogenicity:

- Dr Sean Kassim (US FDA), Dr Sam Haidar (US FDA), Dr Seongeun (Julia) Cho (US FDA), Dr Nilufer Tampal (US FDA), Dr Jan Welink (EU EMA), Dr Olivier Le Blaye (France ANSM), Mr Stephen Vinter (UK MHRA), Ms Emma Whale (UK MHRA), Dr Isabella Berger (Austria AGES), Mr Gustavo Mendes Lima Santos (Brazil ANVISA) and Dr Mark Bustard (Health Canada) – Regulated Bioanalysis
- Dr Shashi Amur (US FDA), Dr John Kadavil (US FDA) and Dr Yoshiro Saito (Japan MHLW-NIHS) – Biomarkers
- Dr Kara Scheibner (US FDA), Dr João Pedras-Vasconcelos (US FDA), Dr Pekka Kurki (Finland Fimea), Dr Isabelle Cludts (UK MHRA-NIBSC), Dr Laurent Cocea (Health Canada) and Dr Akiko Ishii-Watabe (Japan MHLW-NIHS) – Immunogenicity

The 11th WRIB was designed to cover a wide range of topics, in bioanalysis, biomarkers and immunogenicity, suggested by members of the community. Industry experts and regulatory representatives participated in daily working dinners and delivered lectures with each themed session culminating in an open panel discussion between the presenters, regulators and conference attendees in order to finalize the consensus items to be included in this White Paper.

At this year's WRIB, 40 recent issues ('hot' topics) were addressed and distilled into a series of relevant recommendations. Presented here are the exchanges, consensus and resulting recommendations on these 40 topics, which are separated into the following areas:

- LCMS for small molecules:
 - Biomarkers and peptide bioanalysis (five topics);
 - Bioanalytical regulatory challenges (seven topics);
- Hybrid LBA/LCMS for biotherapeutics:
 - PK assays and biotransformations (two topics);
 - Immunogenicity assays (one topic);
 - Biomarker assays (five topics);
- LBA for biotherapeutics:
 - Immunogenicity assays (11 topics);
 - PK assays (four topics);
 - Biomarker assays (five topics).

In addition to the recommendations on the aforementioned topics, there is also an additional section that focuses specifically on several key inputs from regulatory agencies.

Due to its length, the 2017 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 1) covers the recommendations for small molecules, peptides and small molecule biomarkers using LCMS. Part 2 (biotherapeutics, biomarkers and immunogenicity assays using hybrid LBA/LCMS and regulatory agencies' inputs) and Part 3 (LBA: immunogenicity, biomarkers and

PK assays) are published in volume 9 of *Bioanalysis*, issues 23 and 24 (2017), respectively.

Discussion topics

The topics detailed below were considered as the most relevant ‘hot topics’ based on feedback collected from the 10th WRIB attendees. They were reviewed and consolidated by globally recognized opinion leaders before being submitted for discussion during the 11th WRIB. The 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.

Biomarkers & peptide bioanalysis

Small molecule biomarker assay validation: surrogate analyte versus surrogate matrix approach

What are the current industry standards that are used to determine criteria for the selection of the surrogate analyte versus the surrogate matrix approach? Can a consensus be reached between industry and regulators on tests needed during biomarker assay validation (BAV) to evaluate the ionization efficiency of different stable isotope-labelled standards (Surrogate Analyte approach)? When selecting the Surrogate Matrix approach, what criteria can be used and what evaluation needs to be performed to select the best matrix: Selection of blanks with low endogenous levels, or synthetically prepared surrogate matrices (e.g., artificial CSF as a surrogate matrix)? During 4 β -Hydroxycholesterol (4 β HC) quantification (used as small molecule biomarker for CYP3A activity), should 4 α HC be quantified too? If yes, why? How should the target analyte concentration(s) be assigned for the endogenous matrix pool used for category 2 BAV; choices include: replicate analysis prevalidation; first accepted run; mean of three validation runs; other? Should matrix factor and extraction recovery be required for category 2 BAV? Can best practices be defined for estimating matrix effects associated with BAV? When, if ever, should spiked recovery be allowed as a surrogate for accuracy with category 2 BAV? Should parallelism, testing authentic versus surrogate matrix, always be included in fit-for-purpose (FFP) BAV using the surrogate matrix approach?

Why bioanalysis & metabolism of peptides is not easy: overwhelming complexity and unusual metabolic pathways

What is the best approach for the identification of potentially active peptide metabolites, therapeutic peptides and alternative metabolic pathways? What are the recommended workflows? What software solutions are presently the most efficient for the analysis of HRMS peptide metabolism data? How should we best characterize the soft spots which may impact peptide stability and PK?

Novel advancements in using HRMS to overcome peptide sensitivity & selectivity issues: is HRMS the ‘way’?

What are the advantages, if any, to using full scan mode versus MS/MS to achieve maximum selectivity in peptide quantification? Is this choice platform dependent (i.e., QTOF vs Orbitrap)? How can pharma help CROs successfully implement HRMS in bioanalysis with a focus on complex peptides? Why is it necessary to have HRMS in both pharma and CRO laboratories to solve tough bioanalytical issues (e.g., quantification of very potent peptides)? What are the main pharma concerns regarding: transferring HRMS peptide methods to CROs using different platforms/modes; transferring methods between different QTOF brands/models; transferring methods between Orbitrap and QTOF or vice versa; transferring methods from Orbitrap full scan to QTOF SRM or ion mobility spectrometry (IMS)-QTOF? How can CROs demonstrate successful implementation of HRMS and the actual capacity to successfully execute HRMS outsourced work? Is it possible to design a successful cross-validation between different HRMS methods (different platforms and different modes) to facilitate peptide method transfer?

Potent cyclic peptide method development: approaches to overcome their additional & unique bioanalytical challenges

Numerous potent cyclic peptides are under development as a new generation of therapeutic treatments. What is the most difficult challenge to overcome for potent cyclic peptides versus peptides (e.g., complex structure; cross-linked; no suitable CID; low fragmentation efficiency = low sensitivity; very low LLOQ required; others) and why? HRMS instruments are being used routinely in pharma labs. What types of molecules are being analyzed? What MS resolution is needed for routine use? Are there other MS options outside of HRMS that can be used to improve assay selectivity (i.e., ion mobility, etc.)? How often is IMS-QTOF actually used in the industry beyond commercial advertising?

Key terms

Audit trail: An audit trail is a process that captures details such as additions, deletions, or alterations of information in an electronic record without obliterating the original record. An audit trail facilitates the reconstruction of the course of such details relating to the electronic record.

Enantiomer: Stereoisomers that are mirror images of each other that are nonsuperimposable.

All you need to know to successfully use peptide supercharging: how to achieve adequate sensitivity for peptides requiring extremely low LLOQ

How can supercharging reagents be efficiently coupled to selectivity enhancing methods to realize sensitivity gains? How and when do supercharging reagents work? Is it possible to use supercharging reagents with very potent peptides to reach fg/ml LLOQ? What is the best strategy? What are the possible short and long-term effects of supercharging reagents on LCMS?

Bioanalytical regulatory challenges

'Scientific validation' versus fit-for-purpose validation versus bioanalytical method validation guidance/guidelines

What is the difference between FFP validation and 'scientific validation'? The concept of FFP validation has been accepted by regulators. However, it requires a strong scientific justification when used in support of submitted data and preliminary discussions between industry and regulators before submission. What about '*Scientific Validation*'? What is the regulators' perspective on the '*Scientific Validation*' concept as a predetermined validation approach versus FFP and bioanalytical method validation (BMV)? Are regulatory agencies presently accepting methods validated using the '*Scientific Validation*' approach/criteria? How frequently are studies using methods validated at variance to the BMV guidance documents [16–18] rejected by regulatory agencies? Can '*Scientific Validation*' criteria be used to validate methods of early clinical and preclinical studies rather than BMV?

Matrix-based stability experiments in bioanalytical method validation

Since the Health Canada Notice, industry has conformed to conducting stability testing in three individual tubes but how are labs ensuring that only one (or two) aliquots per tube is analyzed? How is this documented? Does conducting stability testing with only one aliquot per tube (e.g., 150 µl aliquot for a 100 µl assay volume) really reflect the study situation, where, for example, the aliquot volume may be 1 ml and the assay volume may be 100 µl? Three independent samples versus a bulk procedure may be impacted differently by parameters such as plasma aliquot volumes, tube types, spatial positioning, head space and other variables that can happen inadvertently. For stability testing, how far do we really need to go to show potential differences?

Inconsistent internal standard responses: focus on the need for appropriate evaluation to determine the impact on data accuracy

Is inconsistent IS response still an issue? If yes, how often are IS problems observed? What is the industry standard to correctly diagnose IS problems/impact? Are the causes of inconsistent IS always identified? If yes, what actions are usually taken? Are these 'actions' clearly specified in a SOP?

Chiral or achiral methods: regulators & industry input on what to quantify, what method to use & their impact on bioequivalence

When are chiral methods really needed for bioequivalence (BE)? Does EU EMA require chiral methods more often than US FDA? If yes, why? Is the sum of R and S enantiomers equivalent to the results obtained with an achiral method? What about ion suppression with co-eluting enantiomers? Are there any ionization differences between R and S enantiomers?

Industry & regulators' perspective on electronic data & audit trail management

How should **audit trail** review be documented? How do we enable efficient inspections in the age of electronic laboratory notebooks? Is an understanding of the science/analytical technique necessary for an informed view of the data and associated audit trail(s)? With respect to the new draft guidance "Data Integrity and Compliance With CGMP" [19], do all requirements in this document also apply to regulated bioanalysis? How should electronic data be archived (e.g., logical or physical separation, within the system versus outside of the system)?

Key terms

Microsampling: Sampling of very small sample volumes (typically $\leq 50 \mu\text{l}$) from animals and humans to assess drug and chemical exposure in biological matrix.

Challenges with method development & validation of drugs in ocular fluids/tissue

Should noninvasive techniques in clinical studies be prioritized over invasive ones (e.g., collection of tears using strips/filter paper vs capillaries), perhaps compromising the quality of the sample? Is it common practice to wash the collected part of the eye after collection, prior to storage (e.g., perfusion to remove blood)? Can a unique surrogate matrix be used or are there regulatory concerns? What kind of surrogate matrix is preferred to replace ocular fluids/tissue? How can the determination of recovery from tissues be resolved? True recovery from tissue is only possible with the use of radioactive material (to be administered to the animal). Do we need the full package, LCMS quantitative tissue analysis, and imaging?

Head-to-head comparison of the most used & successful microsampling techniques: VAMS & capillary microsampling

If a switch is made from traditional sampling to microsampling within a program but the matrix remains the same, is there a need to perform a bridging study? In capillary microsampling (CMS), does stability have to be tested in capillaries? What are the remaining hurdles to implementing microsampling within the regulated environment: Requirement to perform bridging studies, need for a separate sample to perform ISR, additional validation experiments for a VAMS or CMS assay compared with traditional sampling? Is a liquid matrix (CMS) preferred over a dried matrix (VAMS)?

Discussions, consensus & conclusions**Biomarkers & peptide bioanalysis****Small molecule biomarker assay validation: surrogate analyte versus surrogate matrix approach**

This topic was discussed in Part 1 of the 2016 White Paper, specifically through preliminary recommendations regarding the surrogate analyte versus the surrogate matrix approach [13]. The aim of the 2017 discussion was to re-evaluate the challenge of the analyte of interest being endogenous, which needs to be taken into account for accurate analysis of study samples, and reformulate more in-depth recommendations. In order to circumvent this issue, the bioanalyst can choose a surrogate analyte to prepare the standard curve and QC samples, or select a surrogate matrix. In the 2016 recommendations it was concluded that the surrogate analyte approach was often preferred, since it eliminates risks of matrix effects from the surrogate matrix, and because it depended largely on the biology of the analyte and the availability of reference materials [13,20].

At the 2017 WRIB, a case study was discussed to thoroughly evaluate the benefit of the surrogate matrix approach, in which a LCMS method was developed and validated for a subset of four key neurotransmitter metabolites in human CSF for the purpose of clinical translation [21]. Artificial CSF containing 0.2% (w/w) bovine serum albumin was qualified as a surrogate matrix for the quantitative analysis of all four analytes using a combination of parallelism approaches: standard addition, spike recovery and dilutional linearity, as described in the literature [22]. In addition, the concentration of each endogenous analyte in a pool of human CSF was determined to allow for the preparation of QC samples at known theoretical concentrations by over-spiking, as well as by dilution with surrogate matrix.

It was agreed that parallelism is the most important factor when qualifying assays using either a surrogate analyte or surrogate matrix, and for deciding on what to use as the surrogate matrix (e.g., synthetic matrix, analyte depleted matrix, stripped plasma). As mentioned above, there are three methods to assess parallelism: standard addition, spike recovery and dilutional linearity. Standard addition involves spiking the reference standard into biological matrix. A linear regression is then applied without taking into account the analyte levels already present, and the line is extrapolated through to the negative x-intercept to provide the estimation of the endogenous concentration of the analyte. This technique provides an assessment of the endogenous pool without being subjected to possible matrix effects with the surrogate matrix. Furthermore, a comparison of the endogenous matrix concentration obtained by extrapolation versus interpolation provides a convenient assessment of parallelism. However, this method assumes that the spiked analyte resembles the endogenous form, and any errors in extrapolation are influenced by experimental design and can be significant.

The spike recovery method requires that both the biological and surrogate matrices are spiked with known analyte concentrations. The signals are compared and the %recovery is calculated as:

$$[(\text{spiked} - \text{blank})_{\text{matrix}} / \text{spiked}_{\text{surrogate}}] * 100 \quad \text{or} \quad [\text{spiked}_{\text{matrix}} / (\text{blank}_{\text{matrix}} + \text{spiked}_{\text{surrogate}})] * 100$$

This experiment is simple, allowing for the evaluation of several lots of matrix and useful for a rapid prevalidation assessment of surrogate matrix viability. However, as with the standard addition method, it assumes that the spiked analyte resembles the endogenous form. Furthermore, there is no consistent method or formula used within the industry; however, the second method which requires estimating the concentration of the endogenous pool is recommended since it more closely reflects the equation used for accuracy determination.

The final method is dilutional linearity. An endogenous sample of high concentration is subjected to serial dilution with surrogate matrix to determine if the linear response is preserved. This is the only method that does not require spiking with the analyte, however, high concentration endogenous samples must be available. This method also does not provide an estimate of the endogenous pool concentration.

Given the limitations of each method as described above, it is likely that evidence supporting the use of surrogate matrix in bioanalysis should come from a combination of several approaches.

In order to evaluate the surrogate analyte method, a case study regarding the analysis of 4βHC was presented. 4βHC has been proposed as a potential endogenous biomarker for CYP3A activity in multiple species, including human [23–25]. For several reasons, developing bioanalytical assays for 4βHC is challenging. Such reasons include: 4βHC being present in free and ester forms in circulation, the inherent lack of mass spectrometry sensitivity for the free form, endogenous background levels of 4βHC in plasma and the presence of multiple positional isomers in plasma. To address these challenges, bioanalytical assays for 4βHC in plasma from mice, rats, dogs and humans were developed using alkaline de-esterification, picolinic derivatization to increase analytical sensitivity, a surrogate analyte (d7–4βHC) in authentic matrices as standards and quality control samples, and chromatographic conditions that showed good separation of 4βHC from several isobaric, positional isomers (4α-, 7α-, 7β-, 24S-, 25- and 27-HC).

Attendees were asked if, during 4βHC quantification, 4αHC should be quantified as well since 4βHC levels can be very variable between subjects.

For the case study, the presenter concluded that 4αHC or cholesterol levels could be measured to normalize for inter-subject variability. However, this may not be necessary as CYP3A activity is determined from the percentage of change in 4βHC levels within a subject pre- and postdosing of the drug of interest.

If the surrogate analyte method is selected, ¹³C or ¹⁵N labeling may be preferred, as deuterated analytes may have issues depending on the site of the label or stability of the label [22]. The ionization efficiency of different stable isotope labeled standards should be verified during development by assessing the potential response differences between the isotope labeled analyte and the endogenous analyte (e.g., fragmentation, purity). If differences are notable or significant, then the MS response can be equalized by tuning the MS parameters or applying a correction factor.

In order to establish the target analyte concentration(s) to be assigned for the endogenous matrix pool used for BAV, three options are used by industry: Replicate analysis prevalidation; first accepted run; mean of three validation runs. Regulators have predominantly reviewed data using prevalidation replicate analysis, which is useful if multiple lots of endogenous matrix will be used (bridging experiments will be needed between lots). It was recommended that the mean of three validation runs was the preferred approach and concerns were raised over using only one run, since analyst/instrument variability can have a serious impact on the results.

Why bioanalysis & metabolism of peptides is not easy: overwhelming complexity & unusual metabolic pathways

Peptides offer unique opportunities for therapeutics despite their high cost and challenging synthesis. Advantages include broad disease targets, high binding affinities, well understood MOA and low risk of drug–drug interactions [26]. With the rapid growth of protein and peptide therapeutics, there has been an increased need for

Key terms

Clearance: The rate at which the active drug is removed from the body.

understanding the impact of their biotransformation to support discovery and structure optimization in order to allow for improving their ADME properties (e.g., metabolic stability, permeability, oral bioavailability, half-life). Strategies for increasing metabolic stability include modification of the N- and C-terminal, as well as cyclization or the incorporation of D-amino acids. The PK can be improved by increasing the plasma protein binding of the therapeutic peptide through lipidation, or reducing the renal clearance using fusion proteins or pegylation. Finally, permeability can be improved with N-methylation or with the use of permeability enhancers.

Current workflows for small molecule metabolite identification (Met ID) are analogous but not directly applicable for large peptides and proteins because of their overwhelming complexity and alternative metabolic pathways. To better comprehend the trafficking and metabolism of peptide therapeutics from site of administration to target engagement, different tools such as HRMS can be utilized in combination with innovative sample preparation, low flow ionization techniques and novel software applications to analyze complex *in vivo* metabolism profiles and assess *in vitro* / *in vivo* correlations (IVIVC) of clearance and metabolic profiles. In order to build IVIVC, proper *in vitro* stability assays need to be developed for each compartment that a therapeutic peptide may encounter. Current proteomic profiles should be consulted and on-line informational databases utilized as resources for understanding various compartment catabolizing enzymes and peptidases.

It was agreed that there is no singular analytical approach for the identification of potentially active peptide metabolites, or alternative metabolic pathways beyond amide hydrolysis, such as oxidation or deamidation. Control samples should be compared with dosed samples using HRMS and differential MS data processing software to help detect and identify metabolites of therapeutic peptides. Commercial software packages are available to assist in these workflows.

Metabolic soft spots which may impact peptide stability and PK are characterized using optimized HRMS workflows and software tools to assess *in vitro* and *in vivo* metabolism, typically starting with *in vivo* and then working backward to *in vitro* to facilitate IVIVC. Sample handling can also have a significant impact on the analytical outcome (e.g., adsorptive loss, *ex vivo* degradation) and proper handling techniques are critical. Once metabolites are properly identified, small molecule LCMS workflows may be followed for future analyses.

Novel advancements in using HRMS to overcome peptide sensitivity & selectivity issues: is HRMS the 'way'?

Quantitation of peptide therapeutics to provide an understanding of the PK or measurement of peptide biomarkers to give PD information is paramount for the development of therapeutic molecules. LBAs have traditionally been used for quantitation of therapeutic peptides and peptide biomarkers, but it has almost been replaced by LCMS since LBA suffers from lack of selectivity at the lower limits of quantification needed for study support. However, the overall LCMS sensitivity has limited its utility in a few cases [7–8,10–11,13–14].

Case studies were discussed where HRMS has significantly improved the overall sensitivity and dynamic range leading to an increased use in the bioanalytical laboratory specifically in the analysis of peptides. Modern HRMS instruments provide additional selectivity without compromising sensitivity for the analysis of peptides. While the trend has been toward HRMS, due to its simpler assay development and increased selectivity, platform selection (Triple Quad vs HRMS) is still compound dependent and driven by peptide size (intact analysis), the desired LLOQ and the level of sample clean up required. Hence, it is highly recommended to have both instrument types available for use.

It has been challenging to transfer HRMS peptide methods from pharma companies to CROs as not many CROs have HRMS and/or the advanced skill set needed to use this instrument platform, though in recent years there has been an increase as pharma is adopting more HRMS in their bioanalytical laboratories. The key for pharma to help CROs increase HRMS availability and the skill level required is to create a demand for HRMS as a broadly applicable tool and to provide training as required. It is in the best interest for pharma and biotech companies to partner with CROs and vendors, due to their limited internal capacity. It could also be possible to successfully cross-validate between different HRMS methods (different platforms and different modes) to facilitate peptide method transfer. The rules for cross-validation are independent of platform and are most successful when pharma and CRO partner together [27–28].

Potent cyclic peptide method development: approaches to overcome their additional & unique bioanalytical challenges

Cyclic peptides are a class of molecules that are currently being studied for various therapeutic and diagnostic applications. In LCMS, these molecules are typically observed as singly or multiply charged precursor species

with poor signal to noise ratio in Q1 scan. Moreover, they are difficult to fragment in the gas-phase which may produce poor CID efficiency in Q2, resulting in low intensity product ions of interest in Q3 for MRM detection, and hence low assay sensitivity. An alternative approach to managing this issue is to apply multiple ion monitoring (MIM) or single ion monitoring (SIM) methodology using HRMS in the 'Survivor SIM' mode [12,29]. However, the MIM or SIM is often accompanied by high background noise.

Differential mobility spectrometry (DMS), a subset of ion mobility spectrometry (IMS), has previously been employed for reducing the chemical background noise in many LCMS bioanalysis applications [30] and is an emerging technology. DMS efficiency in separating analytes of interest from interfering components can be significantly enhanced with the incorporation of modifiers. Accordingly, addition of a DMS device to a conventional triple quadrupole or QTRAP mass spectrometer can be the method of choice in LCMS with MIM for quantitative analysis of cyclic peptides with poor CID efficiency.

In a case study evaluated during the discussion, a DMS-MIM method was developed and validated for quantitative LCMS bioanalysis of pasireotide (SOM230), a cyclic peptide that exhibits poor CID efficiency during MRM detection. By selecting the most abundant doubly charged precursor ion in both the Q1 and Q3 of the mass analyzer in MIM and combining the DMS capabilities, the background noise was significantly reduced. The performance of the LC-DMS-MIM assay for quantitative analysis of pasireotide in human plasma was evaluated and compared with those obtained via LC-MRM and LC-MIM without DMS. Overall, the assay sensitivity with DMS-MIM was about five-fold better than that observed in MRM or MIM mode without DMS.

It was concluded that DMS-MIM might be used to overcome CID fragmentation issues with cyclic peptides. However, DMS-MIM instrumentation is not common in bioanalytical laboratories. Hence, it was recommended to utilize HRMS as another potential alternative to mitigate the CID fragmentation issue. Recommendations on preferred HRMS instrument resolving power was also discussed; when using an Orbitrap one should acquire data at 70K resolving power, and for most, QTOF's 20–40K resolving power is sufficient. Overall, optimum HRMS performance is only achievable when mass resolving power, mass accuracy, scan speed and mass extraction window are balanced based on requirements for each assay [31].

All you need to know to successfully use peptide supercharging: how to reach adequate sensitivity for peptides requiring extremely low LLOQ

As discussed in the previous paragraphs, LCMS is a powerful tool in the quantification of peptides and proteins offering unparalleled levels of selectivity. However, limits in sensitivity remain a major drawback of this technique when applied to the analysis of peptides. ESI of peptides produces multiple precursor ions distributed over a range (or envelope) of charge states for peptides in the MS source. This signal dilution effect often results in significant sensitivity loss, as usually only a single charge state is selected for quantitation. Modifying the ionization characteristics of peptides in the ion source is difficult. Changing the level of normal mobile phase modifiers usually has minimal effects; however, the addition of certain supercharging reagents can significantly shift the charge states of peptides and proteins present in the ESI source [32]. This often enables the bioanalyst to select a more intense and favorable precursor ion for quantitative purposes, with potential sensitivity gains.

Supercharging reagents include DMSO, *m*-NBA and sulfolane. Case studies were discussed using *m*-NBA [33–35]. Results demonstrated at least two- to three-fold increase in sensitivity for some analytes, however, benefits are compound dependent; supercharging reagents appear to work better with large peptides. Current industry experience indicates that low pg/ml LLOQ levels can be reached. Hence, the use of this approach is highly recommended to enhance sensitivity of peptides.

It should be taken into account that this methodology, although potentially increasing the signal, will typically also increase the matrix noise, so a net improvement to sensitivity may not always be realized. Therefore, using supercharging reagents often requires more sample clean up in order to reduce the noise level, and it may also require other injection techniques to keep the instrument clean (e.g., 2D chromatography, divert valves). With the use of supercharging reagents, the MS inlet tends to get contaminated quickly, requiring more frequent cleaning and maintenance.

Bioanalytical regulatory challenges

'Scientific' validation versus fit-for-purpose validation versus BMV guidance/guidelines

In recent years, a variety of confusing and potentially conflicting terms have been applied to 'method validation' that do not, or not fully, follow the BMV Guidance/Guidelines [16–18], beyond the traditional and well-

established FFP validation. Terms, including regulatory, scientific, partial, tiered, full validation and qualified method, have all been used in connection with some intended form of BMV. Even 'GLP' Validation has frequently been heard, without considering that this term is meaningless. Different validation paradigms have been used in an effort to streamline validation activities, and, to some degree, align validation activities with the probability that a developmental drug candidate will ultimately succeed to become a marketed product. While many of these terms are in widespread use, their meanings are ill-defined and the situations under which these different types of validation approaches are applicable are not universally understood. This was confirmed by the many questions and concerns received through the WRIB website (a small sample is in section 2 – Discussion Topics) which prompted this discussion and the need for clarification.

'Scientific' validation is the latest of these terms and probably the most confusing of all, because it can unfortunately create or increase the incorrect perception within the bioanalytical industry that BMV guidance/guidelines are not science-based and are just an unnecessary regulatory burden put together by the 'regulators' without apparent reasons. It was agreed not to use the inappropriate term 'Scientific' validation within the bioanalytical community for the above reasons and also because the term 'Scientific' validation is just an alternative term for FFP validation, which is already a term recognized by regulators. FFP validation may involve a reduced number of experiments (compared with a 'full' BMV) or deviation from existing guideline requirements, but should still be scientifically sound and appropriate for the objectives of the assay and study in question. For any doubt on the use of FFP validation for a specific study, it is recommended to directly contact the regulatory agencies for scientific advice.

The use of FFP validation for early clinical and preclinical studies should be thoroughly justified and to alleviate any doubt as to future acceptability in a submission, it is recommended to contact the regulatory agencies in advance for scientific advice. All phases of clinical studies, including early clinical, fall within the scope of the EMA guideline [17]. Traditionally, with justification, regulators are accepting FFP validation approaches, although it was emphasized that for BE studies, a full validation is required.

There was unanimous consensus that bioanalytical methods should always be fit for their intended purpose. Indeed, the BMV guidance/guidelines define what regulators consider to be a demonstration of fitness for the purpose of submitting pivotal data in an application. These provide a general framework, and specific items may need to be assessed case by case, requiring a robust scientific approach in users' interpretation and application. When it has been decided not to follow the BMV guidance/guidelines, it is crucial to have a formally predefined experimental plan (protocol, study plan, validation plan) with transparency and appropriate scientific justification (purpose and risk evaluation) for ease of review by regulators and to facilitate the acceptance of the FFP validation.

Matrix-based stability experiments in bioanalytical method validation

On October the 8th, 2015, Health Canada issued a clarification on their expectations for the performance of matrix-based stability experiments (i.e., bench-top, freeze-thaw and long term) [36,37]. They had discovered that some companies had employed a procedure of subjecting one 'bulk' QC sample at each low and high QC concentration to the stability conditions and then analyzing the impact by repeated measurements from a single tube after the stability stressing (Procedure 1). In the 2016 White Paper [14], the agency's concerns related to the practice were discussed. Health Canada and several other regulators stated in 2016 [14] that they did not consider this a scientifically sound approach; any variability in the repeated measurements would be a reflection of the variability of the analytical method and not as a result of the stability assessment. Method validations supporting BE studies that followed this approach were therefore considered 'deficient' and all BE submissions filed after October 8th, 2015, were expected to follow the procedure in which sets of low and high QC samples should be aliquoted prior to the stability experiments and a minimum of three tubes should be subjected to the stability condition and then analyzed (Procedure 2).

Numerous discussions between industry and regulators followed, as many in industry believed there was no scientific reason for differences in the results obtained by either approach, but agreed that testing was required to generate data for evaluation. The CRO bioanalytical industry came together to collate actual data on how bioanalytical laboratories perform matrix-based stability evaluations. The survey conducted by GCC revealed that while there are some procedural differences in conducting stability testing, an overwhelming majority of CROs were using a single tube from one bulk preparation that undergoes stability conditions (Procedure 1) and have never seen any significant differences in subsequent retesting using different tubes.

Furthermore, due to differing views on this issue and to stimulate further discussion, the FDA's Office of Study Integrity and Surveillance and Office of Testing and Research is conducting research using five selected

drugs with and without known stability concerns. The main objective of these studies is to determine if there is an impact in the stability results between one versus three tubes of stability challenged samples. Long term stability (1 month, 3 months), in addition to bench-top and freeze–thaw testing, is being conducted with teriflunomide and other drugs. While the data presented at the conference were limited, it does not show any scientifically significant differences in stability data between one tube versus multiple tubes. In a related case study that was presented at the meeting, a drug with known light sensitivity was tested and differences were detected between bulk and individual tubes, supporting the position that conducting stability testing in individual tubes may be critical for light-sensitive analytes.

Despite the differences of opinion between industry and regulators, bioanalytical laboratories have conformed to conducting storage stability testing in individual tubes. It is highly recommended to adjust SOPs to specify the procedure used, and ensure that validation studies contain traceable documentation of the number of tubes removed from the freezer. Ideally, this tracking would be done electronically. Furthermore, the volume within the tubes should reflect, as closely as possible, the volume of the stored study samples, ensuring the difference in surface area does not have an impact. It is also understood that from study to study, the volume of sample stored may differ and revalidation based on a different aliquot volume may be excessive. In addition, in cases using rare matrices and animal matrices, storage of excess volume may not be possible or be considered wasteful/unethical.

Inconsistent internal standard responses: focus on the need for appropriate evaluation to determine the impact on data accuracy

For typical LCMS-based bioanalysis, a constant amount of an appropriate IS is added to all samples at the beginning of the analytical process, with the intent to account for sample-to-sample procedural variations and to increase accuracy of data during sample analysis. When the pattern of IS responses of study samples is similar to those of spiked samples (i.e., calibration standards and QC samples), it may indicate that there are no significant differences in matrices among these samples that may affect analytical responses and the accuracy of the measurements. However, differing IS variability between study samples and calibration standards/QC samples can occur. These variabilities may come from different reasons/root causes (e.g., different postpreparative time elapsed prior to injection and greater evaporation from earlier processed calibration standards, or differential extraction), and proper monitoring during the study could provide investigational insights to the analytical site as well as inspectional focus to regulatory investigators. When differences (variations/fluctuations) in IS responses in study samples compared with calibration standards and QC samples are observed, it is the responsibility of the bioanalytical investigator to follow up the observation and to ensure there is no impact on the accuracy of the determined analyte concentrations in study samples. This may range from a simple assessment to a full-blown scientific investigation to determine root cause for the differences, and corrective action may be required.

Many bioanalytical laboratories apply outlier tests with acceptance criteria or a maximum/minimum acceptable response approach for the analysis of IS variability. Generally, it has served its purpose in detecting experimental/instrumental errors. While there are no specific criteria or new requirements that were recommended [4,7,10], it was emphasized that thorough and consistent approaches implemented by SOPs with predefined criteria are important steps to ensure data accuracy. Discussions also led to an agreement that these criteria should not impede the scientific evaluation of the results. For example, additional investigations with proper documentation may be warranted (e.g., use of the predose samples from the same subject to evaluate the difference in response between calibration standards and incurred samples) when reviewing IS data from a batch if unusual responses (even when *a priori* criteria are met) are observed.

Chiral or achiral methods: regulators & industry input on what to quantify, what method to use & their impact on bioequivalence

Recently, the expectations of the chiral separation deployed for the analysis of chiral drugs, especially when a racemic mixture is administered, are increasingly set by the regulatory agencies. There have been inconsistencies observed between the global regulatory agencies' expectations both in the necessity of chiral assays and the expectations for performance of those assays. Chiral assays are expensive and often challenging because they may require long development times and lack ruggedness in use.

The EMA adopted the guideline on the investigation of bioequivalence in 2010 [38]. As indicated in this guideline, different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an active substance

are considered to be the same active substance, unless they differ significantly in properties with regard to safety and/or efficacy. In general, bioequivalence must be proven for the parent drug between a Test and a Reference formulation. The use of achiral bioanalytical methods is generally acceptable except in cases where the parent drug consists of enantiomers. Individual enantiomers should be measured when all the following conditions are met or unknown:

- The enantiomers exhibit different pharmacokinetics;
- The enantiomers exhibit pronounced difference in pharmacodynamics;
- The exposure (AUC) ratio of enantiomers is modified by a difference in the rate of absorption.

Furthermore, if one enantiomer is pharmacologically active and the other is inactive or has a low contribution to activity, it is sufficient to demonstrate bioequivalence for the active enantiomer. As would be expected since all three of the above conditions have to be fulfilled before a chiral method must be employed, achiral methods have been utilized in most regulatory submissions. Therefore, the regulatory burden related to this issue seems to be limited.

Although there may be cases where the use of chiral methods may be applicable, even in these cases it triggers discussions among EU regulators on the need for it. Several case studies were presented to illustrate how all the conditions listed above need to be met [39–41]. Typically, it is the third criteria regarding the difference in the rate of absorption that is the unknown in the equation, although this is normally investigated during drug development. If the T_{max} between the Test and the Reference products is similar, this effect is considered small, and an enantiomer nonspecific method (i.e., achiral) may be used.

Industry & regulators' perspective on electronic data & audit trail management

Changing from paper-based to electronic data recording requires an interdisciplinary input from various experts on GXP, as well as IT. The use of computerized systems (from simple commercial off-the-shelf software packages to complex site-specific systems) by companies all over the world is increasing. By now, electronic laboratory notebooks have been well established in many regulated and nonregulated bioanalytical laboratories. However, even for those using paper-based data recording, software systems control LCMS systems and acquire and process chromatographic data. Sample management is typically governed by a LIMS system and all data processing is software driven. Therefore, it is important to keep in mind that there is a data life cycle within the computerized system life cycle. Ensuring data integrity throughout the complete lifecycle of a computerized system is paramount.

Electronic data should be defined for each system, and include not only the electronic record but also the associated metadata. Metadata is data that explains the record and is typically present in an audit trail or elsewhere in the software. Critical information in metadata can be who generated or modified the record (i.e., username), when it was generated or modified (date/time), reasons for any modification and the ability to still see the original data. Data review must include a review of the data audit trail prior to the acceptance of the data in order to ensure that all data are complete, consistent and accurate throughout the data lifecycle. In the same way that paper records must adequately document corrections and dated signatures, these elements must also be present in electronic records. The heightened scrutiny of electronic data integrity, however, raises the question how ELN or other electronic data systems are validated and integrated into the overall systems strategy of regulated laboratories. The shift toward technical control versus procedural control through electronic systems enables a significant increase in quality through reduction of human error but it is crucial that data integrity starts with the design and requirements of the system. Requirements need to clearly define elements like roles and security, LIMS synchronization interface, audit trail and log, electronic signatures, error prevention and archiving. Systematic deficiencies of electronic data capturing and storage systems may raise questions about the reliability of reported data. Two guidelines have been recently released [19,42], discussing data integrity in the scope of the CGMP space. To some extent, the principles outlined within these documents are relevant and easily transferrable to GCP and/or GLP laboratories, demonstrated by the fact that the MHRA has since released a draft revision of the document widening the scope to GxP studies [43].

MHRA and AGES representatives presented some findings related to data integrity. The MHRA found issues where the audit trails were not always available, where data had become corrupt when transferred to DVD for archival, and where limitations in the software resulted in data manipulation to satisfy the software require-

ments that were not clearly documented or reported. These findings confirmed that audit trail review should be mandatory, and that all data processing manipulation needs to be transparent and documented. Furthermore, proper checks of archived data must be done to ensure studies can be subsequently reconstructed. Similar AGES findings demonstrated further that a noncompliant process will always be noncompliant regardless of its support by a state of the art, validated IT system. Therefore, all expected data should be included in the records (e.g., time samples are in transit and time of storage to reconstruct stability conditions). Additionally, the use of prefilled values (often designed into the process for efficiency reasons) should be balanced with good documentation practices so that analysts do not just enter what is expected of them, but what they actually did.

In conclusion, it was recommended that review of audit trails should be built into the quality system. A quality control review should take place for each run, followed by an independent review as part of quality assurance audits of the study. Regulatory inspectors should have read-only access with the ability to reconstruct activities when inspecting ELN systems, with assistance from subject matter experts as necessary. Finally, electronic records (including the associated audit trails and metadata) should follow the same archival principles as paper records. It should be ensured that there are appropriate controls so that no changes to the data can be applied once the study has been archived.

Challenges with method development & validation of drugs in ocular fluids/tissue

Globally, 80% of all visual impairment, estimated to affect 285 million people worldwide (WHO, August 2014), can be prevented or cured. Many of the debilitating visual impairments are classified as high unmet medical needs as there are either no approved treatments or the approved treatments are inadequate, limited or associated with serious complications. As a result, more pharmaceutical companies are developing ophthalmic drugs, creating an increasing need for measuring drugs in ocular fluids and tissues.

The quantification of the drug concentrations in the eye compartments is important as it enables the determination of the exposure of the drug in the target organ and may help to understand its mechanism of action, the interpretation of the pharmacological effect or elucidate toxicity effects. Typically, even after topical administration of the drug into the eye, a portion of the drug is systemically adsorbed either directly from the conjunctival sac or through the nasal cavity. This peripheral exposure of the drug is often the cause of side effects. The concentrations of the drug in the eye compartments are usually evaluated in combination with plasma concentrations to build PK/PD models which allow for the prediction of the effect. The drug ocular bioavailability is usually low (5–10%) as the corneal epithelium limits the drug absorption, thus in order to improve ocular bioavailability more lipophilic drugs are being developed. The invasive administration route (e.g., intravitreal injection) of some of the ocular medicines, in addition to the noncompliance dosage, creates the need for drugs with longer duration of action in order to reduce the frequency of administration to weekly or even monthly.

Studies on drugs for ocular applications present many bioanalytical challenges, from the accurate collection of the entire eye, the eye compartment or secreted liquid (which requires highly trained scientists), to the correct storage conditions and preparation of the samples [44]. These factors all determine the quality of the final bioanalytical result. The limited sample volume, the provision of the matrix, together with the need to develop highly sensitive assays present additional bioanalytical challenges. Due to the intrinsic physicochemical properties of the ocular drugs, nonspecific adsorption of the drug to the container is also often a challenge that needs to be overcome during method development.

In general, noninvasive techniques for sample collection are preferred, both practically and ethically. However, the collection approach must be scientifically sound for the purpose of the data. If the collection of eye compartments is required, no washing of the tissue is required prior to storage. As with any tissue bioanalytical method, the use of a surrogate matrix may be required, resulting in a FFP approach to method validation.

Head-to-head comparison of the most used & successful microsampling techniques: VAMS & CMS

CMS is a useful technique due to the reduced sample volumes (4–10 µl). In the preclinical space, by implementing microsampling, study animals undergo less stress while being serially bled, which is perfectly in line with the 3Rs principles. In the clinical environment, microsampling facilitates sampling in pediatric subjects and can accelerate enrollment. Microsampling techniques have been discussed in previous White Papers [5–7,10,13].

When using CMS, stability testing should mimic the study situation and stability should be tested in capillaries to cover the period that the matrix/analyte is stored in capillaries.

Some discussions focussed on the ability to switch from traditional sampling to microsampling within a program. It was suggested that if the matrix remains the same and only the sampling volumes differ, no bridging

studies may be required. There was no preference in using a liquid matrix (CMS) over a dried matrix (VAMS). The method that best serves the need of the study should be selected. However, dried matrix may require robust method validation and the possibility of bridging studies.

Several regulatory hurdles still exist. For example, it is difficult to perform the ISR evaluation in order to meet the objectives of the evaluation. A separate sample would need to be collected, resulting in a time difference between the original and retested samples and, obviously, the same sample is not being retested. The collection of multiple samples misses the scope of microsampling. However, for ISR where 10% of sampling time points are reassayed, an additional sample may be considered. Finally, additional validation experiments for a VAMS or CMS assay compared with traditional sampling may be required.

Recommendations

Below is a summary of the recommendations made during the 11th WRIB.

Biomarkers & peptide bioanalysis

Small molecule BAV

- Both surrogate analyte and surrogate matrix approaches are currently industry standard practices. Parallelism is the most important factor when qualifying assays using either a surrogate analyte or surrogate matrix, and for deciding on what to use as the surrogate matrix.
- During 4βHC quantification as a marker for CYP3A activity, 4αHC or cholesterol levels may be measured to normalize inter-subject variability as 4βHC levels can be highly variable between subjects.
- If the surrogate analyte method is selected, ¹³C or ¹⁵N labeling may be preferred over deuterated analytes. The ionization efficiency of different stable labeled standards should be verified during development by assessing the potential response differences between the SIL-analyte and the endogenous analyte.
- When establishing the target analyte concentration(s) assigned to the endogenous matrix pool used for BAV, the mean of three validation runs was the most common practice. Bridging experiments will be needed when using new matrix lots.

Peptides metabolic pathways

- Current workflows for small molecule metabolite identification (MetID) are analogous but not directly applicable for large peptides and proteins because of their overwhelming complexity and alternative metabolic pathways.
- There is no singular analytical approach for the identification of potentially active peptide metabolites or alternative metabolic pathways beyond amide hydrolysis, such as oxidation or deamidation.
- Control samples should be compared with dosed samples using HRMS and differential MS data processing software to detect and identify metabolites of therapeutic peptides.

HRMS for peptides

- HRMS provides extra selectivity, generally without compromising sensitivity for peptide analysis.
- It is important to strike a balance between mass resolving power, mass accuracy, scan speed and mass extraction window for optimum HRMS performance/sensitivity.
- Platform selection between HRMS and triple quadrupole still depends on the size of the peptide, the desired LLOQ and the level of sample clean up required. It is recommended to have both instrument types available for use.
- Currently, not many CROs have HRMS and/or the advanced skill set to use it. A key incentive for pharma is to help CROs increase HRMS availability and skill level in order to create a demand for this instrumentation as a broadly applicable tool and to help provide training as required.

Cyclic peptide fragmentation issues

- Use of survivor-SIM or MIM, preferably using HRMS, is the method of choice for assays of cyclic peptides that exhibit poor CID efficiency during MRM detection. DMS, a subset of IMS, is an emerging technology that may also mitigate cyclic peptide sensitivity issues.

Peptide supercharging

- Benefits of using supercharging reagents are compound dependent, but can lead to at least two- to three-fold increase in sensitivity, typically best for large peptides.
- Current industry experience indicates that low pg/ml LLOQ levels can be reached.
- Using supercharging reagents requires more sample clean up in order to reduce the noise level, as well as other injection techniques to keep the instrument system clean (e.g., 2D chromatography, divert valves). The MS inlet tends to get contaminated, requiring more frequent cleaning and maintenance.

Bioanalytical regulatory challenges

'Scientific' validation?

- The recently introduced '*Scientific*' validation terminology is confusing, inadequate and can be misinterpreted. This term should not be used in the bioanalytical community but replaced with the well-known and established FFP validation which is already a term recognized by regulators/industry. For any doubt on the use of FFP validation for a specific study, it is recommended to contact the regulatory agencies for scientific advice.
- 'GLP' validation is meaningless and should not be used in the bioanalytical community.
- Bioanalytical methods should always be fit for their intended purpose. FFP validation for preclinical and early clinical studies should be thoroughly justified considering subject safety. Again, it is recommended to contact the regulatory agencies in advance for scientific advice.
- For BE studies, a full validation as per BMV Guidance/Guidelines is required. When it has been decided not to fully follow the BMV Guidance/Guidelines, it is crucial to have a formally predefined experimental validation plan with transparency and proper justifications.

Matrix stability

- To meet Health Canada requirements, SOPs should be updated to reflect the use of at least three tubes for matrix stability experiments.
- Validation studies should contain traceable documentation of the number of tubes removed from the freezer. Ideally, this tracking would be done electronically.
- The volume within the tubes should reflect, where possible and practical, the volume of the stored study samples to ensure differences in surface area do not impact stability determinations.

IS variability

- During study sample analysis, thorough and consistent approaches with predefined acceptance criteria for IS responses are important to ensure accuracy of results.
- *A priori* criteria should consider scientific merits. Additional investigations and proper documentation may be warranted when unusual IS responses are observed.

Chiral or achiral methods

- No differences were noticed between US FDA and EU EMA as far as the request for chiral methods is concerned.

- In most cases, achiral methods are sufficient.
- Where the parent drug consists of enantiomers, individual enantiomers should be measured only when all the following conditions are met or unknown:
 - The enantiomers exhibit different pharmacokinetics;
 - The enantiomers exhibit pronounced difference in pharmacodynamics;
 - The exposure (AUC) ratio of enantiomers is modified by a difference in the rate of absorption.
 - If the T_{max} between the Test and the Reference products is similar, this effect is considered small, and an enantiomer nonspecific (i.e., achiral) method may be used.

Electronic data & audit trail management

- Review of audit trails should be built into the quality system. A quality control review should take place for each run, followed by an independent review as part of quality assurance audits of the study.
- Regulatory inspectors should have read-only access with the ability to reconstruct activities when inspecting ELN systems, with assistance from subject matter experts as necessary.
- Electronic records should follow the same archival principles as paper records. It should be ensured that no changes to the data can be applied once the study has been archived.

Ocular fluids/tissue

- Noninvasive techniques for sample collection are preferred, both practically and ethically. However, the collection approach must be scientifically sound for the purpose of the data.
- If the collection of eye compartments is required, no washing of the tissue is required prior to storage.
- The use of a surrogate matrix may be required, resulting in a FFP approach to method validation.

Microsampling techniques

- CMS and VAMS are presently the most used microsampling techniques. Previous recommendations on microsampling remain valid.
- When using CMS, stability should be tested in capillaries to cover the period that the matrix/analyte is stored in capillaries.
- If transitioning from traditional sampling to microsampling during a course of drug development program, no bridging studies may be required provided the only difference is the sample volume. There was no preference in using a liquid matrix (CMS) over a dried matrix (VAMS). The method that best serves the need of the study should be selected. However, dried matrix may require robust method validation and the possibility of bridging studies.

Acknowledgements

The authors would like to acknowledge the US FDA, Europe EMA, UK MHRA, Austria AGES, France ANSM, Brazil ANVISA, Health Canada, Japan MHLW and Finland Fimea for supporting this workshop. S Gupta (Amgen), B DeSilva (Bristol-Myers Squibb), L Amaravadi (Sanofi), E Yang (GlaxoSmithKline), J Welink (EU EMA), A Song (Genentech), H Neubert (Pfizer), F Garofolo (Angelini Pharma) for chairing the workshop and/or the white paper discussions. All the workshop attendees and members of the bioanalytical community who have sent comments and suggestions to complete this White Paper. Future Science Group as a trusted partner.

Financial & competing interests disclosure

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

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

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