2018 White Paper on Recent Issues in Bioanalysis: focus on immunogenicity assays by hybrid LBA/LCMS and regulatory feedback (Part 2 – PK, PD & ADA assays by hybrid LBA/LCMS & regulatory agencies' inputs on bioanalysis, biomarkers and immunogenicity)

The 2018 12th Workshop on Recent Issues in Bioanalysis took place in Philadelphia, PA, USA on April 9–13, 2018 with an attendance of over 900 representatives from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. WRIB was once again a 5-day, week-long 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 bioanalysis involving LCMS, hybrid LBA/LCMS and LBA/cell-based assays approaches. This 2018 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 2018 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 2) covers the recommendations for PK, PD and ADA assays by hybrid LBA/LCMS and regulatory agencies' input. Part 1 (LCMS for small molecules, peptides, oligonucleotides and small molecule biomarkers) and Part 3 (LBA/cell-based assays: immunogenicity, biomarkers and PK assays) are published in volume 10 of *Bioanalysis*, issues 22 and 24 (2018), respectively.

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Key input from regulatory agencies

- Inspection program updates and observations
- Small molecule bioanalytical method validation
- Immunogenicity assessments
- Biomarker qualification & validation

References

Key terms

Biomarker: A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions. Molecular, histologic, radiographic, or physiologic characteristics are types of biomarkers. Categories of biomarkers include: susceptibility/risk biomarker, diagnostic biomarker, monitoring biomarker, prognostic biomarker, predictive biomarker, pharmacodynamic/response biomarker and safety biomarker [54].

Biomarker qualification: A conclusion based on a formal regulatory process, that within the stated context of use, a medical product development tool can be relied upon to have a specific interpretation and application in medical product development and regulatory review [54].

Immunogenicity: The ability of a substance, including a biotherapeutic substance, to provoke an immune response *in vivo* which results in an induction of anti-drug antibodies, cytokines – among others. For the purposes of this article, ADA assays are equivalent to immunogenicity assays.

Cut point: The minimum level of response in the assay that defines the sample as positive for ADA.

Context of use: A statement that fully and clearly describes the way the medical product development tool is to be used and the medical product development-related purpose of the use [54].

Acronyms

ADA:	Anti-drug antibody
ADC:	Antibody drug conjugate
AMED:	Japan Agency for Medical Research and Development
BA:	Bioavailability
BAV:	Biomarker assay validation
BE:	Bioequivalence
BIMO:	Bioresearch monitoring
BLA:	Biologics license application
BMV:	Bioanalytical method validation
CDx:	Companion diagnostics
COU:	Context of use
CRO:	Contract research organization
DBE:	Division of Biopharmaceutics Evaluation
DMSO:	Dimethyl sulfoxide
ELISA:	Enzyme-linked immunosorbent assay
FQP:	Full qualification package
GCP:	Good Clinical Practices
HC:	Health Canada
HRMS:	High resolution mass spectrometry
IA:	Immunoaffinity
IIRM:	Innate immune response modifiers
IIRMI:	Innate immune response modifier impurity
IIS:	innate immune system
IND:	Investigational new drug

ISR:	Incurred sample reproducibility
IVDR:	<i>In vitro</i> diagnostic medical device
LBA:	Ligand binding assay
LCMS:	Liquid chromatography mass spectrometry
LLOQ:	Lower limit of quantitation
LM:	Large molecule
LOI:	Letter of Intent
LTSS:	Long term stability
mAb:	Monoclonal antibody
MRD:	Minimum required dilution
MS:	Mass spectrometry
Nab:	Neutralizing antibody
OSIS:	Office of Study Integrity and Surveillance
PBS:	Phosphate-buffered saline
PC:	Positive control, used in an immunogenicity assay
PD:	Pharmacodynamics
PK:	Pharmacokinetics
PTM:	Post-translational modification
QC:	Quality control
QP:	Qualification plan
R2D2:	Regulatory Review of Drugs and Devices
RLD:	Reference listed drug
S/N:	Signal to noise
SIL-IS:	Stable isotope label internal standard
ULOQ:	Upper limit of quantitation
WRIB:	Workshop on Recent Issues in Bioanalysis
XIC:	Extracted ion chromatogram

The 12th edition of the workshop on recent issues in bioanalysis (12th WRIB) was held in Philadelphia, PA, USA on 9–13 April 2018 with an exceptionally high attendance of over 900 representatives (likely facilitated by the Philadelphia location) from pharmaceutical/biopharmaceutical companies, biotechnology companies, contract research organizations and regulatory agencies worldwide. The workshop included three sequential main workshop days, two additional advanced specialized sessions and four full-day training sessions that together spanned an entire week in order to allow exhaustive and thorough coverage of all major issues in bioanalysis, biomarkers and immunogenicity.

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.

The active contributing chairs included Dr Chris Beaver (Syneos Health), Dr Seongeun (Julia) Cho (US FDA), Dr John Kamerud (Pfizer), Dr Steve Keller (AbbVie), Dr Fabio Garofolo (Angelini Pharma), Dr Joe Marini (Janssen), Dr Adrien Musuku (Pharmascience), Dr Hendrik Neubert (Pfizer), Dr Timothy Olah (Bristol-Myers Squibb), Dr Renuka Pillutla (Bristol-Myers Squibb), Dr Sue Richards (Sanofi), Dr Lauren Stevenson (Biogen), Dr Albert Torri (Regeneron), Mr Stephen Vinter (UK MHRA), Dr Jan Welink (EU EMA), Dr Yuanxin Xu (Alnylam) and Dr Eric Yang (GlaxoSmithKline).

The participation of regulatory agency representatives continued to grow at WRIB [1–18], covering topics and discussions in bioanalysis, biomarkers and immunogenicity:

- Regulated bioanalysis: Dr Sean Kassim (US FDA), Dr Sam Haidar (US FDA), Dr Seongeun (Julia) Cho (US FDA), Dr Sarah Rogstad (US FDA), Dr Jan Welink (EU EMA), Mr Stephen Vinter (UK MHRA), Ms Emma Whale (UK MHRA), Mr Gustavo Mendes Lima Santos (Brazil ANVISA), Dr Anna Edmison (Health Canada);
- Biomarkers: Dr Christopher Leptak (US FDA), Dr Shashi Amur (US FDA), Dr Shirley Hopper (UK MHRA), Dr Yoshiro Saito (Japan MHLW-NIHS);
- Immunogenicity: Dr Daniela Verthelyi (US FDA), Dr Yow-Ming Wang (US FDA), Dr João Pedras-Vasconcelos (US FDA), Dr Brian Janelins (US FDA), Dr Haoheng Yan (US FDA), Dr Meenu Wadhwa (UK MHRA-NIBSC), Dr Isabelle Cludts (UK MHRA-NIBSC), Dr Pekka Kurki (Finland Fimea), Dr Elana Cherry (Health Canada), Dr Akiko Ishii-Watabe (Japan MHLW-NIHS).

As usual, the 12th WRIB was designed to cover a wide range of topics in bioanalysis, biomarkers and immunogenicity suggested by members of the community and included daily working dinners and lectures from both industry experts and regulatory representatives, which culminated in open panel discussions amongst the presenters, regulators and attendees in order to reach consensus on items presented in this White Paper.

At this year's WRIB, 39 recent issues ('hot' topics) were addressed and distilled into a series of relevant recommendations. Presented in the current White Paper is the background on each issue, exchanges, consensus and resulting recommendations on these 39 topics, which are separated into the following areas:

LCMS:

- Small molecule bioanalysis and peptides by LCMS (five topics);
- Small molecule biomarkers by LCMS (four topics);
- Oligonucleotides (five topics).

Hybrid LBA/LCMS:

- Intact biotherapeutic quantification and PK assays (three topics)
- Immunogenicity assays (four topics);
- Biomarker assays (three topics).

LBA and cell-based assays:

- Gene therapy and critical reagents (two topics);
- Biomarker assay validation (three topics);
- Flow cytometry (four topics);
- ADA assays and PK, PD, ADA data correlation (four topics);
- Cut points (two topics).

Key inputs from regulatory agencies:

- In addition to the recommendations on the aforementioned topics, there is a section in the White Paper that focuses specifically on several key inputs from regulatory agencies.

Due to its length, the 2018 edition of this comprehensive White Paper has been divided into three parts for editorial reasons. This publication (Part 2) covers the recommendations for PK, PD and ADA Assays by Hybrid LBA/LCMS and Regulatory Agencies' Inputs. Part 1 (LCMS for small molecules, peptides, oligonucleotides and small molecule biomarkers) and Part 3 (LBA/cell-based assays: immunogenicity, biomarkers and PK assays) are published in volume 10 of *Bioanalysis*, issues 22 and 24 (2018), respectively.

Discussion topics

The topics detailed below were considered as the most relevant 'hot topics' based on feedback collected from the 11th WRIB attendees. They were reviewed and consolidated by globally recognized opinion leaders before being submitted for discussion during the 12th 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.

PK assays by hybrid LBA/LCMS

Quantitation of intact biotherapeutics by hybrid LBA/LCMS: current state-of-the-art workflows, successes, failures & future perspectives

What are the pros and cons of using HRMS for quantitation of intact proteins and/or subunits? Why and how should intact protein quantification be performed? What are the current industry standards in 'top-down' protein bioanalysis? Is 'lost in digestion' fact or myth? Does use of the 'bottom-up' (signature peptide) approach impact bioanalysis of biotherapeutics? Are the risks of providing insufficient information on the biotherapeutic bioanalysis by hybrid LBA/LCMS signature peptide approach confirmed by actual data? What are the most common applications for intact protein quantification: identification of intact biotherapeutics and their catabolites; quantitation of intact biotherapeutics and their catabolites; studies for better understanding of the various circulating biotherapeutic forms; biotransformation studies; glycoforms quantitation; post-translational modifications evaluations? What are the current industry strategies for overcoming sensitivity issues in intact protein quantification: maximizing enrichment by IA by using best capturing antibody for improving mass spectrometry S/N; deglycosylation; subunit quantification; summing isotope signals – that is, charge state and isotope effects on S/N or charge state coalescence with DMSO and gain in signal intensity; optimizing the extraction window (XIC) for quantitation; declustering potential and accumulation time? When optimizing chromatographic conditions for intact proteins, what are the best stationary phases for intact protein quantification? Is the use of a stable isotope-labeled internal standard version of the protein crucial? Is the industry moving towards an integrated workflow for PK measurements of complex biotherapeutics? In order to understand the data comparison and interpretation with LBA and LCMS or hybrid LBA/LCMS (intact, subunit and peptide level), when is it important that the results are comparable and can we accept that being different is not necessarily being wrong? How can concentration data between different assay platforms be interpreted better? If different platforms give different results, how can the data be used? How can workflows be designed that generate comparable or better data than current standards for LBA, LCMS and hybrid LBA/LCMS?

Application of hybrid LBA/LCMS assays for clinical studies: robustness & reliability of hybrid LBA/LCMS assays in regulated bioanalysis for biotherapeutics

PK assays using hybrid LBA/LCMS (IA-LC-MS/MS) for biotherapeutics have been used by the industry for more than 7 years with numerous published articles and White Papers from discovery to clinical applications. Is this approach now well-refined and well-established for at least PK assays? After significant initial growth, hybrid LBA/LCMS methods seem to have reached maturity for PK assays but are still evolving for biomarker assays and innovative applications like ADA assays. Is this technology for PK assays mature enough for establishing a regulatory framework (guidance/guideline)? Are regulators still skeptical about this technology and how can the industry help provide actual data to address their main concerns regarding: risk for over/underestimation of results of hybrid LBA/LCMS versus traditional LBA; selectivity and exclusivity of the signature peptide; ensuring the peak measured is the analyte; use of incurred samples for stability testing of intact biotherapeutic versus signature peptide; impact of completion of enzymatic digestion versus recovery; internal standard tracking for hybrid LBA/LCMS methods; justification for why hybrid LBA/LCMS or LCMS is used rather than LBA; robustness of hybrid LBA/LCMS assays (high throughput analysis for large clinical studies; ability to perform well over time; pharma/CRO method transfer)? What is the recent industry experience with hybrid LBA/LCMS supporting biotherapeutics in regulated clinical studies? With the technological advancement and well-established use, are we now willing to use tightened criteria for validation of hybrid LBA/LCMS? Enrichment of the target peptide from extremely complex mixtures by various LCs is critical for LM bioanalysis. How can multi-dimensional separation techniques used in proteomics be leveraged for use in protein bioanalysis? What are the critical aspects of 2D-LC method development and validation? What are the industry experience and future perspectives for reagent-free LCMS assays for biotherapeutics?

Novel ADC & bioanalytical challenges: how to cope with ADCs' increased complexity & diversity

ADC bioanalysis has historically employed four assays: total antibody, ADC, unconjugated payload and immunogenicity. Is this assay package still appropriate? If yes, how can we use investigational assays to augment our understanding of ADC design principles? What clinical data is needed to run fewer than these four assays? For

more complex ADCs (multispecifics or probodies), are these four assays still appropriate? Are there any new trends in immunogenicity testing for ADCs?

Biomarker assays by hybrid LBA/LCMS

Parallelism evaluation in protein biomarker assays by hybrid LBA/LCMS & antibody reagent-free LCMS: differences & similarities with LBA biomarker assays & with the C-Path White Paper

What are the differences and similarities between hybrid LBA/LCMS and LBA biomarker assays in regard to the C-Path White Paper concerning parallelism? What are the key points to consider for evaluating hybrid LBA/LCMS parallelism for protein biomarker assays? What is the best way to perform parallelism and matrix effect evaluations between the surrogate matrix and authentic biological matrix in hybrid LBA/LCMS? After protein digestion, the signature peptide is the same in the endogenous biomarker protein and in the recombinant protein, is it possible to have actual reference standards for this peptide? How can we use this advantage in LCMS reference material versus LBA? Are reagent-free LCMS protein biomarker assays able to get accuracy in this case (i.e., actual reference material for signature peptide), like for small molecule biomarkers? Are there cases in hybrid LBA/LCMS biomarker assays where the concentration increases if samples are diluted, like in LBA? Should hybrid LBA/LCMS method use the MRD approach, as in LBA? When would hybrid LBA/LCMS assays be considered as a 'last resort' or 'first choice' platform for protein/peptide quantitation (compared to LBA)? In cases where endogenous analyte and therapeutic protein drug are to be measured, is it practical to develop and validate a single multiplexed hybrid LBA/LCMS bioanalytical assay to quantitate both drug and endogenous analyte at the same time?

Recent advancements on discriminating biomarker proteoforms by hybrid LBA/LCMS assays: confirming the importance of quantifying the correct proteoform for clinical significance

Building on the 2017 White Paper Part 2 recommendations on the use of LCMS and Hybrid LBA/LCMS for proteoform analysis, why is LCMS the ideal technology for discriminating proteoforms (isoforms, phosphoforms, glycoforms, point mutations and other PTMs)? What are the advantages in having hybrid LBA/LCMS assays which are able to provide results on both total protein biomarkers and isoform-specific biomarkers? When dealing with proteoform analysis, what are the right questions to ask and what are the challenges to overcome? Why may LBA results be biased by proteoform variations in different subjects and between healthy and disease states (biological variability)? Is it possible to compare hybrid LBA/LCMS with LBA data when proteoform variations are present? What if there is a bias? How are under/overestimations handled? Are there examples of isoform/PTM specific assays? If not, what is preventing these? Are LCMS based assays being used to inform immunoassay design on isoforms/PTMs? What is the best approach for calibration standards for isoform/PTM specific assays? What methods are being used to characterize standards for isoform/PTM specific LBA or LCMS and hybrid LBA/LCMS methods?

Expressed protein product quantification by hybrid LBA/LCMS for gene therapy

What are the novel method development strategies for the evaluation of expression of the protein product in gene therapy? What are the innovative workflows enabling new measurements? What are the challenges in quantification of protein expression from transfected cells? What are the merits of using hybrid LBA/LCMS to support target protein quantification for antisense oligonucleotide or emerging gene therapy modalities? Hybrid LBA/LCMS is already a very sensitive technique. In addition to the current technology, what workflow or technical improvements can be made to further increase detection sensitivity? What new applications and assay formats for hybrid LBA/LCMS are emerging?

Immunogenicity assays by hybrid LBA/LCMS

Applications of hybrid LBA/LCMS in combination with traditional LBA for immunogenicity assessment

Are hybrid LBA/LCMS assays able to overcome drug tolerance in ADA assays versus LBA alone? What is the current understanding on data comparison and interpretation when these two techniques are used for immunogenicity assessments? How is the industry progressing based on the 2017 White Paper Part 2 recommendations for performing future ADA measurements (i.e., LBA for screening/confirmation and hybrid LBA/LCMS for quantification/isotyping)? After 4 years of progress and recommendations [8,11,14,17], from proof of concept to real data generation, is the industry now able to demonstrate that ADA assays by hybrid LBA/LCMS are feasible, reliable and generate data which are comparable to and, in some cases even better than, LBA data? What are the ongoing investigations to fully determine that hybrid LBA/LCMS could be a more informative and beneficial

platform over conventional LBA approaches? How should data coming from hybrid LBA/LCMS be compared with traditional LBA for immunogenicity assessments? How can we interpret the concentration data against the titers, building on the recommendations on this topic from the 2017 White Paper Part 2? Is there any clinical relevance of titer versus concentration? What is the impact of the positive control (PC) and drug tolerance on both ADA assays (hybrid vs LBA)? Is there any progress from what was discussed and agreed upon in the 2017 White Paper Part 2? What are the methodology advances and strategies in ADA assessment by hybrid LBA/LCMS for: noise reduction approaches; reducing non-specific binding to the capture phase; ADA concentration versus drug binding capacity; selecting universal isotyping peptide panels; and measurement of pre-existing ADA? How are hybrid LBA/LCMS ADA assays validated? Is it a reasonable approach to establish a calibration curve using commercial Ig standards spiked into immunocapture-processed blank matrix samples? A PC might be used as a calibration standard but it is not representative of all ADAs and also has to be species-specific. As such, is a PC better than commercial Ig for calibration? What about QC samples? Should previous ADA+/- samples or a PC (from either same species or not) be used as QCs? When looking at run acceptance for hybrid LBA/LCMS ADA assays, is it necessary to verify recovery at each batch? What recovery based on positive controls would be considered acceptable? What kind of precision would hybrid LBA/LCMS ADA assays strive for? Should LLOQ or cut points be determined from pre-dose samples for each animal or determined from control group samples for all animals? How are POS/NEG status using hybrid LBA/LCMS dealt with (LLOQ threshold or cut-point approach using statistical analysis of blank plasma)? What is next in immunogenicity assessments by hybrid LBA/LCMS?

Discussions, consensus & conclusions

PK assays by hybrid LBA/LCMS

Quantitation of intact biotherapeutics by hybrid LBA/LCMS: current state-of-the-art workflows, successes, failures & future perspectives

Protein based biotherapeutics comprise a large and promising global market [19]. Immuno-oncology research of complex protein biotherapeutics for the treatment of cancer has successfully altered the industry notion of a therapeutic modality as well as how to administer these safely and efficaciously. In order to benefit from these biotherapeutics, the current focus is the development of fast and accurate screening analytical techniques in order to rapidly select optimal biotherapeutic candidates for the studies needed to get the drugs to market.

In a recent study [19], 80 electronically submitted monoclonal antibody and protein biotherapeutic BLAs approved by the FDA between 2000 and 2015 were examined to determine the extent that mass spectrometry was used. All but one application used the MS platform for the characterization of the biotherapeutics or impurities. Several trends were investigated, such as type of biotherapeutic assayed and number of quality attributes, utilization over time, ionization type and workflows used (peptide mapping, intact mass analysis and cleaved glycan analysis).

The top eight quality attributes monitored by MS were amino acid sequence, molecular mass, disulfide bonds, glycosylation, C-terminal sequence variants, N-terminal sequence variants, deamidation and oxidation. The methods applied for the analysis of these targets were largely based on intact mass and/or peptide mapping experiments. As the complexity of the drug being analyzed for quality increases, the greater the need for higher resolution and sensitivity in the techniques used to measure its critical quality attributes. Hence, the utility of modern MS approaches is predicted to increase the number of BLAs submitted to the agency. Furthermore, the MS-based characterization assays conducted within these BLAs have been increasing in complexity and sophistication as the technology in the field has improved to include high resolution and high mass accuracy instrumentation. This trend implies that the complexity of MS assays within these applications will continue to progress as the MS field advances. These advances may also allow for increased use of MS for quality control and clinical applications.

Although significant progress has been made in the use of LCMS for the quantitative analysis of therapeutic proteins, the complexity of the current bioanalytical workflows has hampered both the speed of analysis and a wider application of the technology, especially in the regulated bioanalytical environment. Because protein biotherapeutics are significantly more complex structures than small molecules, gaining a better understanding of their different forms and their effect on potency, toxicity and overall *in vivo* characterization is a challenge.

There are a variety of bioanalytical strategies that are being developed and implemented to rapidly assess the wide variety of forms of biotherapeutics as they progress from preclinical discovery into clinical development. These strategies focus on innovative isolation techniques, including immunocapture/affinity enrichment, specific separation and detection by LCMS, as well as data processing strategies of various forms of a biotherapeutic

protein including intact (top-down analysis), reduced/enzymatically cleaved to subunits (middle-down analysis), or digested into enzymatically derived peptides (bottom-up analysis).

The most common applications for intact protein quantification include the investigation of metabolites, circulating biotherapeutic forms, biotransformation studies, glycoforms and PTM analysis including oxidation, deamidation – among others. In addition, there is a lot of interest in extending intact analysis of protein biotherapeutics into the regulated bioanalytical field as top-down protein level analysis provides benefits including: reduced method development time, quantitation of the whole biotherapeutic and not just a small portion of the molecule and the ability to perform simultaneous quantitative and qualitative analysis. A more complete picture of a protein's biotransformations can also be obtained from intact analysis. However, challenges of the intact analysis of protein biotherapeutics include protein solubility difficulties, chromatographic challenges and inadequate software workflows for quantitation (deconvolution [20,21], single charge state, or summing multiple charge states). It may help to sum the isotope signals; it was recommended to set the window and identify which charge states to monitor consistently. Optimizing the extraction window (XIC) can help, but selectivity can be affected, so care should be taken when attempting this. Also, intact protein quantification suffers from sensitivity issues due to poor ionization efficiency and the MS signal for the protein being spread over multiple charge states (charge envelope). Several strategies have been used to increase assay sensitivity including immuno purification with 2D chromatography, microflow LC and increasing the sample volume.

The quantification of protein subunits has been performed in some labs in the industry but the technology for this is still novel and it is not a routine platform for quantification. The middle down approach provides some benefits as these protein subunits still preserve some of the structural information of the original protein and suitable levels of detection and measurements can be achieved due to the increased assay sensitivity.

Still the most commonly used approach in the pharmaceutical industry for biotherapeutic quantitation is to enzymatically digest the protein (typically using trypsin) into small peptide fragments (approximately 8–18 amino acids). Then, one or more of these enzymatically derived peptides can be quantified using LCMS and used to represent the entire protein therapeutic. Some benefits of using a bottom up approach include increased sensitivity compared to top down or middle down quantitation, improved chromatography of these smaller peptides as well as the relatively large knowledge base in this area. While this is the most routine bioanalytical procedure for quantitation, challenges of using peptide analysis reside with the complexity of the assay design with multiple steps requiring multiple biological reagents (enzymes, immunocapture reagents, etc.) that often are performed over multiple days. This complexity can introduce inconsistencies in the assay that need to be properly controlled by introducing the proper quality control samples and internal standards. Also, the need to introduce an enzymatic digestion step leads to the loss of structural information associated with the intact molecule.

Given the choice between the three approaches discussed above, the consensus was to use intact proteins rather than peptide fragments. Therefore, it is recommended to use the top down approach when appropriate. This is especially useful for qualitative analysis and to understand biotransformations of the biotherapeutic at an intact level. It can also be used further for PK quantitation. If a bottom up approach is used, it is recommended to perform quantitation of multiple peptides but, it should be noted, that discrepancies in quantitation between two or more peptides often can be difficult to interpret. However, the analytical interpretation should be performed to properly assess the validity of each peptide to explain the discrepancy followed by biological interpretation of the protein. These two components should always be taken into account when designing a LCMS based quantitative assay to ensure that the assay developed provides valid and reliable results.

Finally, if different assays (immunoassay and/or LCMS) are used throughout the drug discovery and development process there should be bridging studies to allow the proper interpretation of all the data generated. It is less important that two methods provide different results than understanding why they are different and what it means overall to the drug development program. Differences in later phase studies must be properly explained to ensure proper interpretation of the data.

Application of hybrid LBA/LCMS assays for clinical studies: robustness & reliability of hybrid LBA/LCMS assays in regulated bioanalysis for biotherapeutics

The hybrid LBA/LCMS assay platform complements the traditional immunoassay platform and has become an important tool for bioanalysis of biologics. The hybrid LBA/LCMS platform offers high selectivity and increased sensitivity by taking advantage of the high specificity of the IA reagent and the resolution power of LCMS. Despite the fact that PK assays using hybrid LBA/LCMS for biotherapeutics have been in use by the industry for more than

7 years with numerous articles and White Papers published [11,14,17,22–24], there have only been IND submissions using hybrid assays and very limited BLA submissions made including data generated by these hybrid assay types for clinical studies to date.

Regulators have indicated that they do not expect any differences from an audit perspective when a hybrid LBA/LCMS assay is used, but they expect transparency. It was unanimously agreed that guidance/guidelines are needed on this topic.

It was confirmed that acceptance criteria should be based on the validation data. Wider criteria are not necessary if the validation confirms precision and accuracy within 15% (20% for LLOQ). However, due to the limited duration of data acquired over the course of a validation, the hybrid nature of these assays for large protein analysis and the use of ligand binding techniques, wider acceptance criteria may also be considered appropriate at this time [24]. As these assays are used over multiple years in support of longer clinical studies for biotherapeutic development across the industry and submitted as a part of BLAs the acceptance criteria used for validation can and should be revisited. A tighter validation can be obtained by using a high purity, well characterized SIL-peptide IS and by demonstrating that the enzymatic digestion is reproducible. Finally, the chances for successful stability determinations increase when using multiple peptides from different parts of the biotherapeutic for determining biotherapeutic protein stability. Approach the decision holistically using the drug product stability when making those assessments. Sponsors should be prepared for additional questions from regulators doing their due diligence when new technologies are used. Differences between results from hybrid LBA/LCMS and LBA assays are expected and will depend on what each assay is measuring. PK and PD results should be paired and evaluated in conjunction to improve interpretation.

Industry members have indicated some positive experience with using reagent-free multi-dimensional LCMS assays for biotherapeutics for total assays. Multi-dimensional LCMS techniques for peptide enrichment have been widely used in the field of proteomics to improve protein identification and sensitivity. A variety of different approaches to orthogonal peptide separation have been reported, the simplest being high pH and low pH combinations. Critical aspects for a successful multi-dimensional LCMS assay include the selection of different columns and mobile phases to achieve the true orthogonal peptide separations and the reproducible retention and efficient enrichment of the target peptide by the first column [25,26].

Novel ADC & bioanalytical challenges: how to cope with ADCs' increased complexity & diversity

There has been a substantial amount of progress made on the design of ADCs, with the field having gone through several generations of drugs, linkers and antibodies. The approach to bioanalysis for each of these generations is in principle the same, in that assays to measure the amount of each moiety – unconjugated drug, antibody and the ADC are all used to assess ADC pharmacokinetics [27]. In practice, the specific choice of drug, linker and antibody can significantly impact assay design [28]. The ADC assay can be especially difficult to design, because it should provide the concentration of ADC that is capable of exerting its intended pharmacologic effect, which would be influenced by antibody, linker and/or drug catabolism. ADC heterogeneity can also influence assay design: antibody and ADC assays may give variable responses to ADCs of different drug-load levels, while homogeneous ADCs are more straightforward analytically since there are fewer drug-load species to potentially confound measurements. Highly engineered ADCs constructed with stable drugs and linkers conjugated to specific sites may actually require fewer assays since the measure of the antibody and ADC should give identical results [29]. Bispecific antibodies and probodies may increase the number of assays required to characterize ADC pharmacokinetics because of the additional requirements for pharmacologic effect [30–32].

ADC bioanalysis has historically employed four assays: total antibody, ADC, unconjugated payload and immunogenicity; regulators still expect to review all these results as part of drug submissions.

More clinical data is still required to determine the best approach to ADC bioanalysis, as these are very complex molecules that often require as much data as possible to make informed decisions. Immunogenicity assays are always independent methods but sometimes total antibody, ADC and free payload can be multiplexed.

Biomarker assays by hybrid LBA/LCMS

Parallelism evaluation in protein biomarker assays by hybrid LBA/LCMS & antibody reagent-free LCMS: differences & similarities with LBA biomarker assays & with the C-Path White Paper

The quantitation of endogenous circulating peptide and protein biomarkers by LCMS is challenging but increasingly necessary as an alternative to traditional LBA. Improvements in the inherent specificity of detection afforded by the

mass analyzer can improve the quality of measurements and clarify the interpretation of results. A major application for hybrid LBA/LCMS assays is the quantification of the target of therapeutic antibody. Furthermore, hybrid LBA/LCMS assays provide a distinct advantage over traditional LBA when quantifying biotherapeutic proteins that are structurally similar to endogenous proteins. LBA may suffer from lack of specificity due to antibody reagent cross-reactivity issues and their inability to distinguish endogenous analyte from exogenously administered biotherapeutic protein [33].

Similar to LBA, hybrid LBA/LCMS biomarker assays can show matrix specific concentration increases upon dilution (non-specific binding or binding inhibition) or overloaded columns. There are also instances, though not as frequent, when increasing the dilution causes a concentration reduction, possibly due to insufficient matrix in the sample leading to analyte loss owing to some non-specific binding. In these cases, adding some proteinaceous matrix may help recovery. It is recommended that hybrid LBA/LCMS methods use the MRD approach, as with LBA.

Evaluation of parallelism is also a requirement to determine “the extent to which the dose-response relationship between two materials (i.e., calibrator versus unknown specimens) is constant for the examined range of concentrations [34].” A key consideration of hybrid LBA/LCMS parallelism for protein biomarker assays is that they are fundamentally LCMS assays and should be driven by LCMS guidelines. This includes:

- Internal standard tracking/compensation;
- Sample preparation: immunoaffinity extraction and digestion;
- Chromatography;
- MS matrix effect and ion suppression/enhancement.

Hybrid LCMS parallelism should be evaluated in the same manner as LCMS parallelism. Furthermore, the MRD should be determined to overcome matrix effects.

Because of the difficulty in securing sufficient quantities of pure endogenous proteins for use as standards, recombinant proteins are used as calibrators for hybrid LBA/LCMS assays. It is essential to evaluate their performance in different steps in the assay (i.e., immunocapture recovery from biological matrix and peptide generation following enzymatic digestion). Confirming that the recombinant calibrator behavior is consistent over the range of the assay with endogenous analytes is the first step towards implementation. It was generally agreed upon that it is not possible to guarantee equivalency with endogenous analytes, therefore, when using a recombinant protein reference material the assay should still be considered to have relative accuracy.

It is recommended that a hybrid LBA/LCMS assay be used as the initial platform for protein/peptide quantitation when only one antibody reagent is available, issues are known or new workflows are enabled using the hybrid approach that would otherwise not be possible. Currently the majority of attendees indicated that hybrid LBA/LCMS biomarker or target assays are requested when the LBA assay is deemed insufficient to generate data required for a specific study. Relatively few pharmaceutical labs initiate assay development using a hybrid LBA/LCMS platform. In cases where the endogenous analyte and therapeutic protein drug are to be measured, it was proposed to assess the feasibility to develop a single multiplexed hybrid LBA/LCMS assay to simultaneously quantitate both, with the understanding that it might be difficult to validate.

Recent advancements on discriminating biomarker proteoforms by hybrid LBA/LCMS assays: confirming the importance of quantifying the correct proteoform for clinical significance

Quantitation of protein biomarker pharmacodynamics for a given biotherapeutic is paramount to drug development. A single proteoform, rather than the bulk protein population, can provide a more stratifying PD biomarker of target engagement or clinical efficacy. LBA platforms have traditionally been used, but are reliant on highly selective antibody reagents to develop an assay with sufficient sensitivity and specificity. Developing LBAs for quantitation of specific protein isoforms and post-translational modifications often requires the development of additional, more selective reagents. LCMS provides a platform that is less dependent on reagent selectivity for enrichment of proteins of interest because it utilizes the selectivity of the mass spectrometer to differentiate analytes of interest from other interfering substances. Because most proteoforms can be resolved by MS detection, there is less need for new reagent generation. The use of LCMS platforms in proteoform specific assays or in clarifying what proteoforms are quantified in LBAs will be crucial for future biomarker assay development, thus providing

a deeper understanding of disease biology. There are more examples of isoform/PTM specific assays appearing in the literature [35].

Building on the published 2017 White Paper Part 2 recommendations detailing the use of LCMS and hybrid LBA/LCMS for proteoform analysis, it was agreed that there are advantages in using this platform because it provides more comprehensive data on both target protein and isoform-specific biomarkers which can improve our understanding of the sample.

There are challenges with proteoform analysis including securing quality calibrators, selecting the appropriate proteoform to analyze (the right sequence) and confirming that the antibody reagent is capturing all proteoforms equivalently. For example, the capture reagent may have a higher affinity for a phosphorylated proteoform versus a non-phosphorylated proteoform. Furthermore, a proteoform specific assay will require higher sensitivity than a total assay because the analyte concentration is expected to be much lower. Finally, proteoform stability is another potential challenge.

Because LBAs measure proteoforms based on antibody reagent binding interactions, results may be biased by proteoform variations in different subjects and between healthy and disease states (i.e., biological variability). Additionally, assay results can vary if reagent binding characteristics of the proteoforms are different. It is always theoretically possible to compare hybrid LBA/LCMS and LBA results but if there is no way to confirm which proteoforms are binding, the results cannot be translated with clinical outcomes. If there is a bias, then both methods may still be equivalent but the numerical data will not be identical. If it is not known which results are more accurate, then over/under estimation of the measurement becomes impossible to assess.

Currently, LCMS based assays are not typically used in immunoassay design for isoforms/PTMs. However, LCMS can be used to assess assay specificity, thus building confidence in immunoassay performance and results.

The best approach for selecting calibrators for isoform/PTM specific assays is to use the specific protein isoform as the calibrator. If one is not available, it will not be possible to generate the specific isoform signature peptide, so only relative concentrations will be generated. Synthetic peptide standards that are proteoform specific can be used, but in this case differences in digestion efficiency of different sequences cannot be accounted for. If a standard is available, it should be characterized in both bridging and stability studies to maintain consistency across lots. If the standard contains a mixture of isoforms then the extent of target isoform purity should be determined.

Expressed protein product quantification by hybrid LBA/LCMS for gene therapy

Novel therapeutic approaches in gene therapy are gaining significant momentum, particularly in addressing unmet medical needs in a range of rare diseases. Gene therapy promises only a single dose of treatment to result in lifelong causal improvement for disorders that may have no other significant treatment options. However, as a new therapeutic modality, gene therapy presents several technical and regulatory challenges. As one of the technical challenges, an urgent need has surfaced for advanced bioanalytical tools that can measure the expression of the transgene to enable the correlation with clinical outcome. Current gene therapy focuses on diseases caused by single-gene defects, requiring the transgene expression analysis of a single protein typically in selected tissues. As gene therapy targets protein classes including structural proteins, enzymes and membrane proteins that are not typically measured using traditional bioanalytical techniques, new bioanalytical strategies for their measurement are required.

To this end, the gene therapy field can take advantage of hybrid LBA/LCMS that are increasingly employed for the quantification of protein targets in translational and early clinical research of biologics. These assays can be very sensitive and selective. It was discussed whether novel workflows could be employed to further increase sensitivity and while high sensitivity can be accomplished through adequate sample preparation it was felt that disruptive technologies with significantly increased sensitivity are instrument limited at this time.

A recently emerging workflow consisting of protein precipitation, pellet digestion and anti-peptide antibody enrichment prior to LCMS based quantification of a surrogate peptide provides a new bioanalytical strategy for tissue proteins [36].

Such a strategy can be adapted for the development of a transgene expression assay for tissue analysis via the creation of anti-peptide antibodies specific for selected proteotypic peptides. If the protein expressed is different than the endogenous protein, then a true blank matrix may be available. These new assays have the potential to pave the way for quantitative transgene expression analysis catering to an unmet need in the emerging area of gene therapy.

Immunogenicity assays by hybrid LBA/LCMS

Applications of hybrid LBA/LCMS in combination with traditional LBA for immunogenicity assessment

In the biopharmaceutical field, the development of therapeutic mAbs to address new targets in fields such as inflammatory or oncology diseases is on the rise. Administration of therapeutic mAbs can induce a product immunogenicity characterized by the production of ADA which may include NABs. The presence of ADA can lead to a decrease of therapeutic mAb efficacy and/or safety. From the perspective of product safety and efficacy, PK and PD parameters of mAbs need to be accurately measured. ADA detection and quantification could be critical in addressing adverse events and issues with efficacy. Detection and semi-quantification of ADA against biological therapeutics is generally performed by LBA using a tiered approach consisting of screening, confirmation and titration steps. This assessment is widely based on a cut-off approach which does not allow absolute quantification of ADAs due to the absence of suitable standards and the diversity and heterogeneity of individual responses. Moreover, these immunological methods require the availability of biological reagents which could take a long time to produce, require labeling and be difficult to characterize.

However, over the past few years an increasing number of reports describing detection and semi-quantification of the ADA responses using hybrid LBA/LCMS have appeared that offer the possibility of performing simultaneous semi-quantitation and isotyping of antibody responses [57]. Some practical considerations need to be addressed before hybrid LBA/LCMS can be widely used for detecting ADA, such as assay validation, quality control, regulatory acceptance – among others. Discussions and recommendations on how to address those questions and on strategies to implement this technology have been issued over the past 3 years in the White Papers in *Bioanalysis* Part 2 [11,14,17]. A consolidation and evolution of these recommendations continued this year.

It is important to distinguish between human and pre-clinical studies because the immunoglobulin sequences of humans and animal species are different and, therefore, different surrogate peptides are used for LCMS ADA analysis; in addition, depending on biotherapeutic molecule structure, different immunoaffinity sample processing methods may be used. In preclinical species, there is the opportunity to employ alternative LBA methods other than the traditional tiered drug–drug bridging approach that are efficient, specific and fully drug tolerant. By capturing the human biotherapeutic mAb with an anti-human antibody that does not cross react with the preclinical species IgG and detecting preclinical ADA with an anti-preclinical antibody that does not cross react with the human biotherapeutic, preclinical study samples can be analyzed in a single assay with and without the addition of sequential concentrations of the human biotherapeutic mAb to measure both drug specific circulating immune complex and total ADA in a fully drug tolerant format [37–39]. When there is an advantage to invest in MS (e.g., isotyping), a hybrid LBA/LCMS ADA assay can be an option [40]. The case and need will be specific to the individual molecule and/or program. There was consensus that the most useful area for hybrid LBA/LCMS ADA assays to be developed further is the potential future application for clinical ADA assessments.

This approach for immunogenicity assays is still in its early stages of development and acceptance and could show great utility for specific applications. Examples of these include detection of ADAs in unusual biological matrices such as mucosal secretions, detection of therapeutic antibodies with specific point mutations; and as an orthogonal method for ADA confirmation, if the traditional LBA has drug tolerance issues. In LBA, the acid-dissociation step (utilized to improve drug tolerance) could denature the ADA, resulting in potential under-detection of ADA positive samples. The hybrid LBA/LCMS method holds the promise to be more drug tolerant as only one ‘arm’ of the ADA needs to be free to be able to be captured, albeit more work needs to be done to demonstrate the improved drug tolerance of all ADA LCMS assays. High drug tolerance has been demonstrated for some hybrid LBA/LCMS formats [41]. In certain cases, such as human Fc monoclonal antibody drugs, one may not be able to distinguish between native ADA and the mAb drug, so a universal assay may not be possible. For drug specific assays it is possible to distinguish between drug and ADA with unique peptides to drug and unique peptides to ADAs and have a more drug tolerant assay than with LBA alone [41]. Covalent coupling of drug to beads can also be explored as only ADA and not the immobilized drug will be eluted out from beads. For regulatory submissions, regulators initially may require drug tolerance data for both LBA and hybrid LBA/LCMS to cross-compare assay platforms. The initial requirement for cross-comparison may be reduced when sufficient hybrid LBA/LCMS data is available and when hybrid LBA/LCMS is used as a sole method in immunogenicity assessment in a study. Even in such a case, it is important to know the drug tolerance limit of the hybrid LBA/LCMS assay for evaluating the validity of the assay.

Approaches to ADA semi-quantification require an understanding of the extraction efficiency of the ligand-capture step. Hybrid LBA/LCMS assay validation should include a recovery assessment. Non-specific binding to beads must also be controlled in development, validation and analysis, so as not to confound ADA positivity with background binding to the beads. Beads from different manufacturers often show different levels of background binding. Consideration should be given to checking beads from multiple manufacturers with numerous blocking buffers. In addition, other solid supports such as ELISA plates [42] can also be investigated for minimizing non-specific binding.

Data from the hybrid LBA/LCMS assay, while not fully quantitative, may enable relative semi-quantitation across isotypes and provide additional information compared to traditional LBA titer measurements. Summing the concentrations of individual isotypes in hybrid LBA/LCMS assays may be required to get an assessment of total antibodies. For regulatory submissions, comparisons across assay platforms should include orthogonal assessment of total ADA levels as well as specific ADA isotypes by each method.

In LBA bridging assays, the positive control source species can vary. Since the sequence of the positive control is critical in hybrid LBA/LCMS assays, allowance needs to be made for non-human positive controls. Use of a positive control to semi-quantify and show consistency of extractions is very valuable. Stability of the positive control should be monitored as part of routine assay performance.

During the discussions, the status of ADA assays by hybrid LBA/LCMS across the industry was re-evaluated and it was confirmed that several companies were developing hybrid LBA/LCMS immunogenicity assays. Given the novelty of the methodology, the first companies to submit hybrid LBA/LCMS-based assays will likely face greater regulatory scrutiny, but regulators are widely open to submissions using hybrid LBA/LCMS for ADA assessment. Regulators repeated a recommendation [14,17] to present these novel technologies early to regulatory agencies through scientific forums to allow for better understanding of the methods and impact on ADA data interpretation. Furthermore, discussions with the regulators will need to address custom method validation approaches for hybrid LBA/LCMS and how cross validation between platforms will be performed. Ultimately regulators will need to be assured that novel hybrid LBA/LCMS-based ADA assays are suitable for their intended purpose through satisfactory validation exercises.

Recommendations

Below is a summary of the recommendations made during the 12th WRIB.

PK assays by hybrid LBA/LCMS

1. Given the choice, it is preferred to use intact proteins rather than peptide fragments for the analysis of biotherapeutics. Robust **deconvolution software** is needed;
2. Strategies to overcome **sensitivity issues** when assaying intact proteins include IA purification with 2D chromatography microflow and increasing the assay volume. Deglycosylation is not typically used. Summing the isotope signals is an option by setting the window and identifying which charge states to monitor consistently. Optimizing the extraction window (XIC) can help, but selectivity can be affected, so care should be taken when attempting this;
3. There should be **bridging studies** for biotherapeutics analyzed by hybrid LBA/LCMS between discovery and development in order to align all approaches to ensure the results can be properly interpreted as the drug candidate moves further down the development program;
4. If different assays (immunoassay and/or LCMS) are used throughout the drug discovery and development process there should be bridging studies to allow the proper interpretation of all the data generated. It is less important that two methods provide different answers than understanding why they are different and what it means overall. Differences in later phase studies must be properly explained to ensure proper interpretation of the data:
 - Differences between results from hybrid LBA/LCMS and LBA assays are expected, depending on what each assay is measuring. PK and PD results should be paired to improve interpretation;
 - Guidance/guidelines for hybrid LBA/LCMS is an urgent need.
5. No differences are expected from a **regulatory audit** perspective when a hybrid LBA/LCMS assay is used, but regulators expect transparency;
6. **Acceptance criteria** for hybrid LBA/LCMS assays should be based on the validation. Wider criteria are not necessary if the validation confirms precision and accuracy within 15% (20% for LLOQ). Wider criteria may

be considered initially but as more clinical data is obtained and large molecule hybrid LBA/LCMS assays in production for longer periods continue to perform as during validation, the criteria can and should be revisited. A tighter validation can be obtained by using a high purity, well characterized SIL-peptide IS and by demonstrating enzymatic digestion is reproducible;

7. **ADC bioanalysis** has historically employed four evaluations: total antibody, ADC, unconjugated drug and immunogenicity and regulators still expect to review all these results as part of drug submissions:
 - Immunogenicity assays are always independent methods but sometimes total antibody, ADC and unconjugated drug can be multiplexed.

Biomarker assays by hybrid LBA/LCMS

8. The evaluation of **parallelism** is a requirement for hybrid LBA/LCMS assays and should be driven by LCMS guidelines;
9. The **MRD** should be determined to assess and, if needed, to overcome matrix effects;
10. Hybrid LBA/LCMS assay should still be considered to have relative accuracy since the same issue of differences in standard vs. endogenous materials remain in these types of assays;
11. It was recommended to use a hybrid LBA/LCMS assay as a first choice for protein/peptide quantitation when only one antibody is available, or when selectivity is important; new workflows are needed for emerging bioanalytical questions that cannot be easily addressed with other techniques;
12. Hybrid LBA/LCMS that provide results on both total protein biomarkers and isoform-specific biomarkers could be helpful in getting a better understanding of biology/disease;
13. The best approach for **calibration standards for isoform/PTM** specific assays is to use the isoform as the standard. If one is not available, then synthetic versions of the specific isoform signature peptide should be used and only relative concentration will be measured;
14. If a standard is available, it should be characterized in both bridging and stability studies to maintain consistency across lots. If the standard contains a mixture of isoforms then the extent of target isoform purity should be determined;
15. One strategy for development of a transgene expression assay for **gene therapy** is the creation of an anti-peptide antibody specific for the selected proteotypic peptide, particularly if the target matrix is a solid tissue. If the protein expressed is different as compared with the endogenous protein, then a true blank matrix is available.

Immunogenicity assays by hybrid LBA/LCMS

16. For **regulatory submissions**, regulators will require data supporting drug tolerance and drug tolerance limits for both LBA and hybrid LBA/LCMS to cross-compare methods;
17. Extraction efficiency should be maximized and kept constant. Validation should include a recovery assessment;
18. **Non-specific binding** to beads must be controlled and consistent in development, validation and analysis. It is recommended that sponsors check beads from multiple manufacturers with numerous blocking buffers during the method development;
19. Summing of isotypes in hybrid LBA/LCMS assays may be required to get an assessment of total ADAs. For regulatory submissions, comparisons across assay platforms should include orthogonal assessment of total ADA levels as well as specific ADA isotypes by each method;
20. Use of a positive control to semi-quantify and show consistency of extractions is very valuable. Stability of the positive control should be monitored;
21. Companies contemplating the use of hybrid LBA/LCMS for ADA assays should consider presenting these novel technologies early to regulatory agencies through scientific forums to allow regulators to better understand the methods proposed, including their limitations and advantages over traditional LBA and impact on the ADA data interpretation;
22. Regulators need to be assured that validation efforts show that the chosen ADA assays, regardless of the format (LBA or hybrid LBA/LCMS-based), are fit for their intended purpose.

Key input from regulatory agencies

The 12th WRIB continued with its long-standing contributions from several international regulatory agencies, including the US FDA, EU EMA, Health Canada, UK MHRA, Brazil ANVISA, Japan MHLW and Finland Fimea.

These agencies shared their views on multiple topics of interest for the global bioanalytical community attending this event, to provide clarification on unresolved issues or expectations. Regulators provided input on a variety of topics across all three core days, including:

- Inspection program updates and observations;
- Small molecule bioanalytical method validation;
- Immunogenicity assessments;
- Biomarker qualification and validation.

Inspection program updates and observations

BIMO at the **US FDA** has a long tradition of ensuring clinical and non-clinical research conducted in support of FDA regulated products supports the protection of human subjects, is adequate to support regulatory decisions and is performed per regulations. The OSIS in FDA/CDER/OTS strives to uphold this mission through inspections and review of sites conducting clinical and bioanalytical studies supporting drugs and therapeutic biologics around the world. Recent findings detected during these inspections, focusing on the challenges seen in bioanalytical and analytical laboratories, were presented.

Between 2014 and 2016, FDA domestic BA/BE site visits represented 46% of the total visits performed. The remaining visits were conducted in India (38%) and other international sites (18%). The number of visits has steadily increased between 2011 (790) and 2017 (1302). In 2017, 68% of the visits concluded with a No Action Indicated classification, 27% Voluntary Action Indicated and only 5% Official Action Andicated.

The six most common deficiency categories observed during analytical BA/BE inspections in FY17 were validation (27%), record keeping (17%), stability (16%), reserve samples (9%), chromatograms (6%) and QC samples (6%). Regarding validation, some of the missing evaluations, or those not properly addressed were matrix effect, specificity and dilution integrity covering study conduct of sample analysis. Methods were not always fully validated. Finally, not all precision and accuracy runs (i.e., failed runs) were included in the intra- and inter-run precision and accuracy assessment. Record keeping issues included inadequate documentation of sample receipt, storage, handling, processing or correspondence. Inadequately assessed stability involved insufficient source documentation often related to e-data and different storage conditions between validation and sample analysis. Reserve samples of test and reference drug products were not randomly selected and retained or not adequately secured. Deficiencies with chromatograms ranged from unjustified manual integrations, no documentation or justification for exclusion of chromatograms or no clear procedures for priming/equilibration runs. Finally, some deficiencies were due to QC sample concentrations not being representative of subject samples, IS response of QCs not being representative of subject samples and inappropriate acceptance criteria.

Health Canada embarked on an initiative entitled the R2D2 [43], which was born out of the Federal Minister of Health's 2015 Mandate Commitment and was confirmed at the 2016 Health Ministers' meeting as well as in the 2017 budget 2017 in which Health Canada received \$71 million to support the initiative. The project seeks to improve access, affordability and appropriate use of prescription medications in partnership with provincial and territorial governments.

Canada recognizes there is a growing need to modernize their regulatory system. The current system was designed for a different time, with limited consideration of the needs of the healthcare system. Like their international counterparts, Health Canada must become more flexible and adaptable, by reducing lag times between new product reviews and funding decisions, prioritizing the review of therapeutic products that meet healthcare system needs and increasingly using post-market data to inform regulatory decisions across the product lifecycle.

Health Canada's plan allows 5 years for transformation, involving 15 projects focused on expanded collaboration with health partners (includes use of foreign reviews/decisions and aligning CADTH review with the HC review), more timely access to drugs and devices (e.g., expansion of priority review pathways, improving access to biosimilars/generics), enhanced use of real-world evidence by leveraging data for assessing drug safety and effectiveness and modern and flexible operations by using updated system infrastructure and an appropriate cost recovery structure.

Some of the benefits of this new initiative include greater alignment and collaboration with regulatory agencies throughout the product lifecycle, more timely and transparent review of drugs that meet healthcare system needs, increased engagement and information sharing in the review of post-market drug safety and effectiveness and improved availability of new and innovative medical devices for Canadians.

Small molecule bioanalytical method validation

Health Canada presented their views on regulatory matters pertinent to the bioanalysis of small molecules. The DBE is responsible for the review of BE studies and pivotal comparative bioavailability studies using the May 2012 HC guidance document *Conduct and Analysis of Comparative Bioavailability Studies* [44] as well as the EMA BMV guideline [45]. It is Health Canada's expectation that BA/BE studies are to be conducted in accordance with GCP [46].

The view of DBE on bench top stability is that cumulative time on the bench in between freezing cycles is not the same as consecutive hours on the bench. Therefore, adding the time that QCs were on the bench during the freeze-thaw investigation (2 hours + 1 hour + 2 hours) is not the same as leaving QCs on the bench for 5 hours.

Samples above the ULOQ were discussed. Section 5.3 of the EMA guideline [45] indicates that the calibration curve should adequately reflect the concentrations of the study samples and should be adjusted or QC samples added if a large number of the analyte concentrations of the study samples appear to be above the ULOQ. The determination of what constitutes a large number is subjective, but dilution of samples should be the exception. DBE acknowledged that dilution of samples in these cases has been validated, however considers the following two scenarios acceptable. The first option requires that the calibration curve be extended, new QC levels added as necessary and stability demonstrated at the new high QC concentration. The second option is not to extend the curve range, but to include dilution QCs at elevated concentrations in all runs containing study samples and to perform stability assessments with a new high QC (frequently the dilution QC).

If anomalous results are seen as part of the ISR evaluation, an investigation should be conducted, as required by all regulatory agencies. Health Canada confirmed that this is not needed for single instances of anomalous results, but when trends are observed such as all samples from one subject or one run fail.

Finally, matrix based stabilities were discussed in order to expand on prior discussions of the associated Notice and Addendum [17,47,48]. They reiterated that the purpose of stability studies is to evaluate the effect of storage conditions on the accuracy and variability of the drug concentrations in biological samples. As it is a general scientific principle that the effect of an independent variable on a given outcome requires appropriate replication to determine a precise estimate of the effect, stability is to be evaluated using sets of low and high QC samples, consisting of multiple replicates (minimum of three samples) at each concentration, that are subjected independently to the stability conditions being evaluated.

US FDA is conducting research using model drugs with and without stability concerns as the use of one versus three tubes as sources of QC aliquots for stability testing is currently a topic of interest among various regulatory agencies and industry alike [17,49]. Some of their preliminary results were presented. The main objective is to determine if there is a practical impact in the use of one vs. three tubes as sources of QC aliquots for stability testing. LTSS (1 month, 3 months and 12 months), in addition to bench top and freeze-thaw testing are being conducted with teriflunomide (considered a stable compound) and other drugs considered unstable: acetylsalicylic acid and salicylic acid, tenofovir alafenamide, simvastatin and valganciclovir. Experiments were designed to test different variables (e.g., location in freezer, sample size, different thawing times, etc.). Results of the 12-month LTSS experiments with teriflunomide across all the variables did not demonstrate differences in %accuracy when one tube or three tubes were used. The effect of the impact of location in the freezer on accuracy and variability of drug concentrations for unstable drugs was assessed using three tubes stored in various locations in the freezer; after 30 days no effect on precision and accuracy was seen. Additionally, currently available data under usual assay conditions did not demonstrate any differences in %accuracy for less stable compounds such as acetylsalicylic acid, tenofovir alafenamide and simvastatin. Evaluations with valganciclovir and longer storage durations are ongoing.

Immunogenicity assessments

US FDA discussed current thinking about assay development, validation and immunogenicity risk assessments for biosimilars. As of March 2018, 62 programs were enrolled in the Biosimilar Product Development Program. CDER has received meeting requests to discuss the development of biosimilars for 30 different reference products. Since the inception of the program, 12 companies have publicly announced submission of 23 351(k) BLAs to FDA; nine of these have been approved.

For biosimilars, structural, functional and animal data are generally not adequate to predict immunogenicity in humans. At least one clinical study that includes a comparison of the immunogenicity of the proposed product to that of the reference product is expected. The extent and timing of the clinical immunogenicity assessment will

vary depending on a range of factors including the extent of analytical similarity between the proposed and the reference product as well as the known incidence and clinical consequences of immune responses for the reference product.

Three FDA guidance documents provide information on immunogenicity risk and assessment [50–52], with one focusing specifically on biosimilarity [51]. This guidance stipulates that immunogenicity studies are part of the totality of evidence required to establish ‘biosimilarity’ between a 351(a) reference product and a 351(k) biosimilar applicant. For clinical testing of product immunogenicity for biosimilar candidates, a parallel design study with multiple sampling time is recommended. Sample testing strategy and assay requirements for biosimilar products are similar to those of new molecular entity products: a multi-tiered approach is recommended for sample testing and assays must be capable of sensitively detecting immune responses in the presence of the expected concentration of circulating drug product at the time of sampling. The guidance allows for the possibility of a single assay based on the biosimilar product used to test patient samples from both biosimilar and reference product treated subjects. For the Nab assay, if a bioassay is used, comparable cellular responsiveness to biosimilar and reference product should be demonstrated. Validation parameters are similar for 351(a) and 351(k) biologics. The design of any study to assess the immunogenicity between a biosimilar and a reference product and acceptable differences in ADA incidence and other parameters of immune response should be discussed with FDA before initiating the study.

US FDA also discussed immunogenicity risk related to impurities that act as IIRMs. Triggering of the IIS leads to inflammation and facilitates the initiation of cellular and humoral immune responses. Products that trigger the IIS receptors are called IIRMs and are investigated in applications for cancer, vaccines, allergies and asthma. Conversely, impurities in therapeutic products that act as IIS activators (innate immune response modifying impurities or IIRMI) can foster unwanted immune responses to self-proteins leading to increased risk of immunogenicity for therapeutic proteins, which could reduce their life-saving potential. Only a fraction of possible IIRMI has been identified and different combinations may synergize in their adjuvant activity. Cell based assays are available for measuring IIRMI, although the product or impurities in the product may interfere with their sensitivity. Ongoing research aims to develop new *in vitro* methods and *in vivo* models to improve the detection of a broad range of IIRMI and understand the role of specific product and process related impurities on product immunogenicity. Demonstrating the absence of impurities or contaminants that produce a greater or distinct stimulation of innate immune activity as compared to the RLD can inform the immunogenicity risk assessment and help determine the type of clinical studies needed for selected products.

According to **Japan MHLW**, to date, more than 100 therapeutic recombinant protein products including insulins, somatropins, erythropoietins, enzymes and monoclonal antibodies have been approved in Japan. In addition, ten biosimilar products (Somatropin, Epoetin alfa BS1, Filgrastim BS1, 2, 3, Insulin Glargine BS1, 2 and Infliximab BS1, 2, Rituximab BS1) were approved from 2009 to 2017. Immunogenicity is thought as one of the critical issues for ensuring the quality, safety and efficacy of therapeutic proteins, however, currently there is no specific guideline for the immunogenicity assessment in Japan. Therefore, immunogenicity is assessed on a case-by-case basis in industry as well as in the regulatory agency during the evaluation of marketing authorization application dossiers.

Recently, AMED research group summarized the immunogenicity discussion and published a special report [53]. This report includes the sections: 1) immunogenicity assessment of therapeutic protein products approved in Japan; 2) current view and recommendations for immunogenicity assessments (positive control, validation, cut-point determination for ADA assays, drug tolerance, sampling in clinical trials, immunogenicity assessments in nonclinical study and biosimilars) and 3) future perspectives in Japan.

In summary, AMED research group recommends a tiered approach for ADA assays; consisting of screening and confirmatory assays and characterization, which is well accepted in Japan. Appropriate cut point settings for screening and confirmatory assays are important. Finally, the influence of the residual drug in the study sample on detecting ADA should be evaluated and a strategy for avoiding false-negative results is to be considered.

Biomarker qualification & validation

In December 2016, the United States Congress passed the 21st Century Cures Act. This Act introduced significant changes to the **US FDA** Biomarker Qualification Program in an effort to increase the number and quality of qualified biomarkers that have the potential to assist and/or expedite drug development. As part of biomarker qualification, it is imperative that the analytical measurement method be sufficiently validated to ensure the biomarker data is

both reproducible and interpretable. An overview of the Biomarker Qualification Program was presented along with specifics of how analytical validation is key to a biomarker development effort. The BEST resource document was discussed. This online, dynamic resource is a glossary of terminology and uses of biomarkers and endpoints in basic biomedical research, medical product development and clinical care [54]. It harmonizes terms and definitions and addresses nuances of usage and interpretation among various stakeholders, including biomedical scientists, translational and clinical researchers and patient/disease advocacy groups.

The US FDA regulatory approach to biomarkers is broadly defined, with multiple biomarker types including molecular, histologic, radiographic and physiologic. The specific COU for a biomarker drives the extent of evidence needed for quantification. Analytical validation establishes performance and acceptance characteristics of the biomarker assay, while clinical validation establishes that the biomarker acceptably identifies, measures or predicts the concept of interest. The first step in the process is a feasibility assessment of the proposal including the proposed COU, the drug development need for the biomarker and information to support that measure of the biomarker is possible through submission of a LOI. The next step is the project development plan, termed the QP to support the biomarker's COU. It is important to ensure that the analytical validation has been mostly completed and the information submitted prior to submission of or concomitantly with the QP. Finally, the last step is the review of the FQP that contains data that supports the clinical validation of the biomarker for the COU.

Japan MHLW described the current situation on biomarker assay validation (BAV) in Japan. A research group funded by Japan AMED sent a survey on this issue to pharmaceutical companies, in cooperation with the Japan Pharmaceutical Manufacturers Association. Answers were received from 37 companies investigating 82 biomarkers for 52 test drugs in clinical trials from January 2012 to July 2017. Approximately one third of the companies had an SOP on biomarker measurements. Assay methods were newly developed in 35 out of the 82 biomarker cases, while most of the other cases used preexisting (including commercial) kits. In most chromatography assays, validation was performed which included selectivity, calibration curve, LLOQ and ULOQ, accuracy, precision and stability in matrix. In the majority of cases using an LBA platform, calibration curve, LLOQ and ULOQ, accuracy, precision, stability in matrix and dilution linearity were validated. Surrogate matrices such as PBS were used in approximately one third of the cases.

The new **EU IVDR** [55] defines CDx as a device which is essential for the safe and effective use of a corresponding medicinal product. In view of the publication in 2017 of the new IVDR, it is timely to consider developing **EU EMA** guidance relating to the interface between drugs and predictive biomarker assays, including CDx.

In Europe, the legislations covering the marketing of drugs and IVDs are not directly linked. However, the new IVDR envisages cooperation between medicines and devices regulators. The proposed guidance will highlight possible options to achieve more closely linked development programs for drugs and predictive biomarker-based assays, including use of clinical drug trials to generate evidence required to support validation of the diagnostic.

An established IVD may not be available to measure potentially predictive biomarkers during drug development, particularly in the case of novel biomarkers. In this scenario, the assay used in clinical development may itself be co-developed as an eventual CDx. The potential to align technical assay validation and clinical evidence requirements for drug approval with analytical and clinical performance requirements for CDx conformity assessments will be discussed in the guidance.

The technical performance requirements of assays used to measure predictive biomarkers will vary in a stepwise fashion depending on the stage of drug development (early explorative study versus pivotal study) and whether the biomarker status affects clinical study entry, subject eligibility and/or treatment allocation.

When a predictive biomarker test is recommended for the safe and effective use of an approved drug, a continuing positive-benefit risk will depend in part on the availability of a suitably validated assay. The guideline will discuss concordance testing and bridging studies, including testing of stored patient samples. This will facilitate the development of suitable tests for use in the clinic.

A concept paper on predictive biomarker-based assay development in the context of drug development and lifecycle was published in July 2017 [56]. The publication of draft guidance for public consultation is planned for Q1 2019.

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References

1. Savoie N, Booth BP, Bradley T *et al.* 2008 White Paper: the 2nd calibration and validation group workshop on recent issues in Good Laboratory Practice Bioanalysis. *Bioanalysis* 1(1), 19–30 (2009).
2. Savoie N, Garofolo F, van Amsterdam P *et al.* 2009 White Paper on recent issues in regulated bioanalysis from the 3rd calibration and validation group workshop. *Bioanalysis* 2(1), 53–68 (2010).
3. Savoie N, Garofolo F, van Amsterdam P *et al.* 2010 White Paper on Recent issues in regulated bioanalysis and global harmonization of bioanalytical guidance. *Bioanalysis* 2(12), 1945–1960 (2010).
4. Garofolo F, Rocci M, Dumont I *et al.* 2011 White Paper on recent issues in bioanalysis and regulatory findings from audits and inspections. *Bioanalysis* 3(18), 2081–2096 (2011).
5. DeSilva B, Garofolo F, Rocci M *et al.* 2012 White Paper on recent issues in bioanalysis and alignment of multiple guidelines. *Bioanalysis* 4(18), 2213–2226 (2012).
6. Stevenson L, Rocci M, Garofolo F *et al.* 2013 White Paper on recent issues in bioanalysis: ‘hybrid’ – the best of LBA & LC/MS. *Bioanalysis* 5(23), 2903–2918 (2013).
7. Fluhler E, Hayes R, Garofolo F *et al.* 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 1 – small molecules by LCMS). *Bioanalysis* 6(22), 3039–3049 (2014).
8. Dufeld D, Neubert H, Garofolo F *et al.* 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 2 – hybrid LBA/LCMS, ELN & regulatory agencies’ input). *Bioanalysis* 6(23), 3237–3249 (2014).
9. Stevenson L, Amaravadi L, Myler H *et al.* 2014 White Paper on recent issues in bioanalysis: a full immersion in bioanalysis (Part 3 – LBA and immunogenicity). *Bioanalysis* 6(24), 3355–3368 (2014).
10. Welink J, Fluhler E, Hughes N *et al.* 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 1 – small molecules by LCMS). *Bioanalysis* 7(22), 2913–2925 (2015).
11. Ackermann B, Neubert H, Hughes N *et al.* 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 2 – hybrid LBA/LCMS and input from regulatory agencies). *Bioanalysis* 7(23), 3019–3034 (2015).
12. Amaravadi L, Song A, Myler H *et al.* 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 3 – LBA, biomarkers and immunogenicity). *Bioanalysis* 7(24), 3107–3124 (2015).
13. Yang E, Welink J, Cape S *et al.* 2016 White Paper on recent issues in bioanalysis: focus on biomarker assay validation (BAV) (Part 1 – small molecules, peptides and small molecule biomarkers by LCMS). *Bioanalysis* 8(22), 2363–2378 (2016).
14. Song A, Lee A, Garofolo F *et al.* 2016 White Paper on recent issues in bioanalysis: focus on biomarker assay validation (BAV): (Part 2 – hybrid LBA/LCMS and input from regulatory agencies). *Bioanalysis* 8(23), 2457–2474 (2016).
15. Richards S, Amaravadi L, Pillutla R *et al.* 2016 White Paper on recent issues in bioanalysis: focus on biomarker assay validation (BAV): (Part 3 – LBA, biomarkers and immunogenicity). *Bioanalysis* 8(23), 2475–2496 (2016).
16. Welink J, Yang E, Hughes N *et al.* 2017 White Paper on recent issues in bioanalysis: aren’t BMV guidance/guidelines ‘scientific’? (Part 1 – LCMS: small molecules, peptides and small molecule biomarkers). *Bioanalysis* 9(22), 1807–1825 (2017).
17. Neubert H, Song A, Lee A *et al.* 2017 White Paper on recent issues in bioanalysis: rise of hybrid LBA/LCMS immunogenicity assays (Part 2: hybrid LBA/LCMS biotherapeutics, biomarkers & immunogenicity assays and regulatory agencies’ inputs). *Bioanalysis* 9(23), 1895–1912 (2017).

18. Gupta S, Richards S, Amaravadi L *et al.* 2017 White Paper on recent issues in bioanalysis: a global perspective on immunogenicity guidelines & biomarker assay performance (Part 3 – LBA: immunogenicity, biomarkers and PK assays). *Bioanalysis* 9(24), 1967–1996 (2017).
19. Rogstad S, Faustino A, Ruth A *et al.* A retrospective evaluation of the use of mass spectrometry in FDA biologics license applications. *J. Am. Soc. Mass Spectrom.* 28, 786–794 (2017).
20. Xu K, Liu L, Saad OM *et al.* Characterization of intact antibody–drug conjugates from plasma/serum *in vivo* by affinity capture capillary LC–MS. *Anal. Biochem.* 412, 56–66 (2011).
21. Jian W, Kang L, Burton L, Weng N. A workflow for absolute quantitation of large therapeutic proteins in biological samples at intact level using LC–HRMS. *Bioanalysis* 8(16), 1679–1691 (2016).
22. Kaur S, Liu L, Cortes DF *et al.* Validation of a biotherapeutic immunoaffinity–LC–MS/MS assay in monkey serum: ‘plug-and-play’ across seven molecules. *Bioanalysis* 8(15), 1565–1577 (2016).
23. Xu K, Liu L, Maia M *et al.* A multiplexed hybrid LC–MS/MS pharmacokinetic assay to measure two co-administered monoclonal antibodies in a clinical study. *Bioanalysis* 6(13), 1781–1794 (2014).
24. Jenkins R, Duggan JX, Aubry AF *et al.* Recommendations for validation of LC–MS/MS bioanalytical methods for protein biotherapeutics. *AAPS J.* 17(1), 1–16 (2015).
25. Zhang G, Sexton DJ, Faucette RR, Qiu Y, Wu J. 2D–LC–MS/MS to measure cleaved highmolecular-weight kininogen in human plasma as a biomarker for C1-INH–HAE. *Bioanalysis* 9(19), 1477–1491 (2017).
26. Shen Y, Zhang G, Yang J *et al.* Online 2D–LC–MS/MS assay to quantify therapeutic protein in human serum in the presence of pre-existing antidrug antibodies. *Anal. Chem.* 87, 8555–8563 (2015).
27. Younes A, Bartlett NL, Leonard JP *et al.* Brentuximab vedotin (SGN-35) for relapsed CD30-positive lymphomas. *N. Engl. J. Med.* 363(19), 1812–21 (2010).
28. Gorovits B, Alley SC, Bilic S *et al.* Bioanalysis of antibody–drug conjugates: American Association of Pharmaceutical Scientists Antibody–Drug Conjugate Working Group position paper. *Bioanalysis* 5(9), (2013).
29. Sussman D, Torrey L, Westendorf L *et al.* Abstract 4634: Engineered cysteine drug conjugates show potency and improved safety. *AACR 103rd Annual Meeting 2012*. Chicago, IL, USA, 31 March–4 April 2012.
30. Uchida N, Sambe T, Yoneyama K *et al.* A first-in-human Phase 1 study of ACE910, a novel factor VIII-mimetic bispecific antibody, in healthy subjects. *Blood* 127(13), 1633–1641 (2016).
31. Yoneyama K, Schmitt C, Kotani N *et al.* A pharmacometric approach to substitute for a conventional dose-finding study in rare diseases: example of Phase III dose selection for emicizumab in hemophilia A. *Clin. Pharmacokinet.* 57(9), 1123–1134 (2018).
32. Desnoyers LR, Vasiljeva O, Richardson JH *et al.* Tumor-specific activation of an EGFR-targeting probody enhances therapeutic index. *Sci Transl Med.* 5(207), 207ra144 (2013).
33. Stevenson LF, Purushothama S. Parallelism: considerations for the development, validation and implementation of PK and biomarker ligand-binding assays. *Bioanalysis* 6(2), 185–98 (2014).
34. Piccoli SP, Sauer J-M; Critical Path Institute. Points to consider document: scientific and regulatory considerations for the analytical validation of assays used in the qualification of biomarkers in biological matrices. https://healthpolicy.duke.edu/sites/default/files/atoms/files/cpath_ptc_biomarker_qualification_assays_20170623.pdf
35. Huang HW, Chen CH, Lin CH *et al.* B-cell maturation antigen is modified by a single N-glycan chain that modulates ligand binding and surface retention. *Proc. Natl Acad. Sci. USA* 110(27), 10928–33 (2013).
36. Fan YY, Neubert H. Quantitative analysis of human neonatal Fc receptor (FcRn) tissue expression in transgenic mice by online peptide immuno-affinity LC–HRMS. *Anal. Chem.* 88(8), 4239–4247 (2016).
37. Kronenberg S, Husar E, Schubert C *et al.* Comparative assessment of immune complex-mediated hypersensitivity reactions with biotherapeutics in the non-human primate: critical parameters, safety and lessons for future studies. *Regul. Toxicol. Pharmacol.* 88, 125–137 (2017).
38. Pierog P, Krishna M, Yamniuk A *et al.* Detection of drug specific circulating immune complexes from *in vivo* cynomolgus monkey serum samples. *J. Immunol. Methods* 416, 124–136 (2015).
39. Stubenrauch K, Wessels U, Essig U, Vogel R, Schleyen J. Evaluation of a generic immunoassay with drug tolerance to detect immune complexes in serum samples from cynomolgus monkeys after administration of human antibodies. *J. Pharm. Biomed. Anal.* 52(2), 249–254 (2010).
40. Roos D, Chen L, Vesapogu R *et al.* Detection of cynomolgus monkey anti-protein XYZ antibody using immunocapture–LC/MS. *J. Appl. Bioanal.* 2(4), 117–128 (2016).
41. Neubert H, Grace C, Rumpel K, James I. Assessing immunogenicity in the presence of excess protein therapeutic using immunoprecipitation and quantitative mass spectrometry. *Anal. Chem.* 80(18), 6907–14 (2008).
42. Chen L, Liu S, Pagels S, Quatrano C, Roos D, Philip E, Zhao H, Yu H. Development of an ELISA–LC/MS hybrid assay for quantification of biotherapeutics. *Bioanalysis* 10(17), 1427–1438 (2018).

43. Improving the regulatory review of drugs and devices. <https://www.canada.ca/en/health-canada/corporate/transparency/regulatory-transparency-and-openness/improving-review-drugs-devices.html>
44. Health Canada, Bureau of Pharmaceutical Sciences Therapeutic Products Directorate. Conduct and Analysis of Comparative Bioavailability Studies (2012). <https://www.canada.ca/content/dam/hc-sc/documents/services/drugs-health-products/drug-products/applications-submissions/guidance-documents/bioavailability-bioequivalence/conduct-analysis-comparative.pdf>
45. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). Guideline on Bioanalytical Method Validation. London, UK (2011). EMEA/CHMP/EWP/192217/2009.
46. Health Canada, Bureau of Pharmaceutical Sciences Therapeutic Products Directorate. Notice: Submissions Containing Clinical Data from Studies Conducted Outside of Canada (2009).
47. Health Canada, Bureau of Pharmaceutical Sciences Therapeutic Products Directorate. Notice: Clarification of bioanalytical method validation procedures (2015).
48. Health Canada, Bureau of Pharmaceutical Sciences Therapeutic Products Directorate. Addendum to Notice: Clarification of bioanalytical method validation procedures (2016).
49. Cape S, Islam R, Nehls C *et al.* The 10th GCC Closed Forum: rejected data, GCP in bioanalysis, extract stability, BAV, processed batch acceptance, matrix stability, critical reagents, ELN and data integrity and counteracting fraud. *Bioanalysis* 9(7), 505–516 (2017).
50. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research (CDER), Biologics Evaluation and Research (CBER). Guidance for Industry, Immunogenicity Assessment for Therapeutic Protein Products. Rockville, MD, USA (2014).
51. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research (CDER), Biologics Evaluation and Research (CBER). Guidance for Industry, Scientific Considerations in Demonstrating Biosimilarity to a Reference Product. Rockville, MD, USA (2015).
52. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Biologics Evaluation and Research, Center for Devices and Radiological Health (CDRH). Draft Guidance for Industry, Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products. Rockville, MD, USA (2016).
53. Ishii-Watabe A *et al.* Immunogenicity of therapeutic protein products: current considerations for anti-drug antibody assay in Japan. *Bioanalysis* 10(2), 95–105 (2018).
54. FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource. BEST (Biomarkers, EndpointS, and other Tools) Resource. US FDA, Silver Spring, NIH, Bethesda, MD, USA (2016).
55. Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on *in vitro* diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU. https://ec.europa.eu/growth/sectors/medical-devices/regulatory-framework_en
56. EMA Reflection paper on methodological issues associated with pharmacogenomic biomarkers in relation to clinical development and patient selection (EMA/446337/2011). http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/07/WC500108672.pdf
57. Chen L. Current status of anti-drug antibody analysis using immunocapture-liquid chromatography/mass spectrometry. *J. Appl. Bioanal.* 4(3), 74–80 (2018).

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