‘Large Meets Small’: connecting the bioanalytical community around peptide and protein bioanalysis with LC–MS(/MS)

The European Bioanalysis Forum 2011 Open Meeting

20–21 June 2011, Brussels, Belgium

This conference report provides an overview of the discussions at the 2nd European Bioanalysis Forum (EBF) Focus Meeting ‘Large Meets Small’ held on 20 and 21 June 2011 in Brussels. The meeting discussed scientific progress in the bioanalysis of peptides and proteins with MS-based techniques. Bioanalytical experts in ligand-binding assays (LBAs) and MS from industry and academia presented at the meeting or joined the discussion. The conference hosted sessions on technology developments, validation requirements, cutting edge (bio)analytical approaches for both proteins and peptides and discussions on the analytical challenge presented by the metabolism of peptides or proteins. The engagement of the scientists as well as the bioanalytical challenges identified were real: by shifting peptide or protein analysis from the LBAs laboratory into the LC–MS laboratory, the bioanalytical scientist is moving into partially uncharted territory. The conference delegates strongly shared the feeling that success in overcoming the challenges of peptide and protein bioanalysis will require further integration of the expertise of LBAs and LC–MS/MS experts.

The European Bioanalysis Forum (EBF) is a non-profit organization comprised of European Pharmaceutical companies and, since 2011, Contract Research Organizations (CRO) [101], and has created a broad European network for discussions on scientific, technological and regulatory topics of bioanalytical interest. The second EBF Focus Meeting, held in Brussels on 20 and 21 June 2011 [102], aimed at connecting strategies for the quantitative bioanalysis of large molecules, that is, peptides and proteins, with small-molecule technologies, predominantly LC–MS/MS. The meeting included a review of LC–MS platforms used in peptide and protein bioanalysis, regulatory challenges, technical challenges and solutions in sample preparation and analysis of large molecules by LC–MS in biofluids. In addition, the meeting included an introductory session on the impact of the International Conference of Harmonization (ICH) M3(R2) guideline [103] in relation to peptide and protein metabolism.

In his introduction and opening remarks, Philip Timmerman (EBF, Janssen R&D) highlighted the importance of increasing the connectivity of the ligand-binding assay (LBA) and the LC–MS bioanalytical scientist regarding the science and process of large-molecule bioanalysis, and emphasized that success in the bioanalysis of these peptides and proteins requires the two disciplines to work closely together and share their knowledge.

Session I: new technology developments for analysis of large molecules, new instrument developments

The first speaker, Lieve Dillen (Janssen R&D) gave an overview on LC–MS technology used at Janssen R&D for the quantification of peptides in the discovery phase. She compared technologies including conventional multiple reaction monitoring (MRM) on the AB Sciex QTRAP® 5500 with a Waters Xevo TQS system connected to narrow-bore LC–MS or nano-LC. Lieve also discussed the potential of running QTRAP instruments in MRM³ mode or using a QTOF instrument in high resolution mode to deliver additional selectivity in case ultimate sensitivity is not needed. Debadeep Bhattacharya (Waters Corporation) presented on how retention, peak shape, resolution and, surprisingly, sensitivity could be improved for basic drugs when running LC at pH 10, whereas common understanding and practice is to run basic compounds under acid conditions in positive ion ESI. Of course, the prerequisite for this high pH approach is to find appropriate LC columns that are stable at these elevated pHs. Kai Scheffler (Thermo Fischer Scientific) discussed the Q Exactive benchtop Orbitrap® LC–MS/MS and showed some advantages of the instrument. Examples included applications for inter- and intra-sample identification and quantification of proteins, by applying different sample pre-treatment, re-combined sample analysis and data

**Philip Timmerman^{*1},
Berthold Lausecker²,
Begona Barosso³, Peter
van Amsterdam⁴, Silke
Luedtke⁵ & Jessica
Dijksman⁶**

¹Janssen Research & Development, Belgium

²CRS Mannheim, Germany

³Astellas, The Netherlands

⁴Abbott Healthcare Products, The Netherlands

⁵Boehringer-Ingelheim, Germany

⁶Solusacta, The Netherlands

^{*}Author for correspondence:

E-mail: ptimmerm@its.jnj.com

**FUTURE
SCIENCE**

part of
fsg

analysis software tools. Lester Taylor (Agilent) described the new dual ion funnel on the Agilent 6490 Triple Quadrupole and illustrated improved performance using an example of quantification of a common protein on a nanosystem compared to a narrow-bore system. Further improvements with respect to specificity and sensitivity were achieved by using the 1290 UPLC together with a selective sample enrichment protocol.

Session 2: cutting edge analytical approaches with focus on peptides

The session focused on peptide quantification using MS, highlighting the (potential) differences or similarities between small molecules and peptides, and subsequent special requirements with respect to sample preparation, chromatography and mass spectrometry conditions. Lee Goodwin (Covance) pointed out some basic aspects such as increased importance of mobile phase composition when analyzing peptides. Because the principle of peptide chromatography is adsorption and not partition, small changes in mobile phase composition can lead to immediate and complete loss of chromatographic properties. A major challenge in peptide analysis is adsorption of peptides to containers, tubing and so on. Veniamin Lapko (Celerion) reported that adsorption effects may be minimized or eliminated by coating polypropylene tubes with bovine serum albumin (BSA) or by addition of 'keeper' peptides. Hermann Mascher (Pharm-Analyt Laboratory) addressed the challenge of low quantification limits often required in peptide quantification. Gerard Hopfgartner (University of Geneva, Lausanne, Switzerland) encouraged the audience to critically assess established procedures/opinions in peptide and protein analysis and to be brave enough to try other approaches. For example, his team could reduce the time needed for sample digestion from several hours to only 15–30 min by using a Thermomixer at 37/60°C or microwaves instead of the classical tryptic digest. Hopfgartner also emphasized the importance of sample preparation to include various strategies depending on molecule size, a statement which nicely introduced the next speaker. Indeed, Magnus Knutsson (Ferring A/S) described a highly selective sample preparation procedure in combination with a multiple column LC system for the quantification of peptides at low pg/ml levels. The accuracy and precision achieved was comparable to small molecule methods. Erin Chambers (Waters Corporation) presented a LC–MS/MS method for quantification of amyloid peptides 1–38, 1–40 and 1–42

in CSF samples. Usually these peptides are quantified using immunoassays. While individual immunoassays are required for each individual A β peptides, all three of them could be quantified simultaneously using LC–MS/MS.

Session 3: validation requirements for large molecule analysis with MS

Philip Timmerman (Janssen R&D, for EBF) presented an overview of EBFs current thinking on the analysis of large molecules using small molecule technology. The presentation touched on the multifaceted challenges ahead of us, starting with a short review of past and current challenges of Pharma with respect to their portfolio composition and ratio of new chemical entities versus new biological entities against a background of even more exciting changes in the field of analytical chemistry and instrumentation. As part of their current thinking, EBF focused on the analytical challenges, regulatory challenges and, last but not least, the human challenge of peptide and protein analysis using chromatographic techniques. Anecdotally, the audience's attention was drawn to the historical perception of a divide between small- and large-molecule science. Both scientific communities are already working closer together, an evolution which we hope to see increase in the future. Bioanalysis of peptides and proteins using LC–MS(/MS) technology is a challenging scientific arena. Although the experience from the bioanalytical community is growing, we need to appreciate that there is still a lot to learn. EBF emphasized that in order to see continued success in peptide and protein quantification both small- and large-molecule bioanalytical experience needs to go hand-in-hand. Last but not least, EBF reflected on the current risk of (potentially premature or inappropriate) 'carbon copying' of current chromatography practices and guidelines into LC–MS of peptides and proteins. Gary Schultz (Advion Bioservices) continued the session and presented a biomarker assay validation and challenges he faced with respect to specificity, sensitivity and regulatory requirements. Biomarker assays are currently not fully regulated and many scientists are following to a certain level the 2001 FDA guideline [1,2] or a 'fit-for-purpose' approach [3]. Schultz demonstrated that by using a surrogate matrix for calibration combined with a complex sample preparation and the NanoMate® technology, a robust, sensitive and selective method was achievable. Nico van de Merbel (PRA) reviewed the value of an internal standard (IS) in small-molecule bioanalysis and how this could translate into the field of large-molecule bioanalysis. As

evident from history in bioanalysis a stable label isotope (SIL) analogue is superior compared to a structural analogue or none. He compared four different possibilities for the use of using an IS in protein analysis, all of which have limitations or benefits in their ability to compensate for fluctuations in sample preparation (i.e., extraction and/or digestion), chromatography or detection in a mass spectrometer. Ben van Baar (QPS) closed the session on proposing validation requirements when setting-up a generic protein LC–MS/MS method. He used the example of trastuzumab. As did van de Merbel, van Baar stressed the importance of adding an IS, preferably at the start of the sample preparation process. A key message of his presentation was the importance of selectivity over sensitivity.

Session 4: cutting edge analytical approaches with focus on proteins

The session was dedicated to the latest trends in protein analysis using mass spectrometric approaches. Protein digestion, followed by LC separation and MRM quantification of specific signature peptide(s), still remains the most general employed approach. Several speakers focused on the poor sensitivity encountered when dealing with these molecules. Due to low recovery during the often complex sample preparation (reduction/alkylation/digestion), together with poor ionization of many peptides as well as multiply charged species formation, most of the methods do not reach the desired or required LOQ. Different presentations discussed strategies to increase the sensitivity. Yong-Xi Li (Medpace Bioanalytical laboratories) described the use of MRM³ with the fast scan rate of linear ion trap systems and high resolution MS to increase sensitivity and reduce interferences. Exanatide, glucagon and salmon calcitonin were practical examples. Stephanie Fischmann (Abbott) combined affinity enrichment with LC–MS/MS quantitation for the same purpose of achieving good LOQs for monoclonal antibodies. Also Fischmann highlighted the importance of a good IS in protein analysis. Ideally, this IS should be the same protein as the analyte, but incorporating stable labeled aminoacids. Olivier Heudi (Novartis Pharma) confirmed. He continued that the lack of resources to produce these types of IS leads many laboratories to use isotope (¹³C, ¹⁵N)-labeled peptides. Aspects such as the importance of tailored sample preparation and quantitation procedures were also highlighted. Ejving Mortz (Alphalyse) emphasized that this is not only protein and peptide specific but also matrix dependant. Techniques

for relative quantification such as iTRAQ, SILAC, iCAT applicable in the proteomics field are deemed suitable for absolute quantification required for PK/toxicokinetic analysis. In this case the use of well characterized and specific signature-labeled peptides is essential.

Ian Ward (Quotient bioresearch) discussed the challenges in validating protein and peptide LC–MS/MS methodologies. He gave an interesting and comprehensive overview of the technologies needed for successful protein and peptide analysis. In his conclusions, referring to a representative peptide example, he suggested that small molecule GxP LC–MS/MS procedures and guidelines can be applied to well-developed protein or peptide LC–MS/MS assays. Alberto Guenzi (Hoffmann-La Roche) compared immunoassay and LC–MS/MS for the PK analysis of a case study involving a 300 kDa fusion protein containing 50% phospholipids. One single signature isotope-labeled peptide was used as IS and both orthogonal techniques showed excellent agreement. However, Rand Jenkins (PPD) preferred to use signature peptide ratios (up to three) and two labeled peptides as IS: one for quantitation and two for control and monitoring of the assay. Using this approach, he presented a reproducible hybrid assay for somatotropin. Combining immunoaffinity capture, reduction, alkylation and rapid on-plate tryptic digestion prior to LC–MS/MS, high recoveries and specific quantitation was achieved. Regarding method validation and run acceptance criteria, he noticed a trend to extrapolate small molecule LC–MS/MS criteria to validation and analysis of peptide and protein assays using LC–MS/MS as indicated by the previous presenter. Some discussions in the session challenged this trend and suggested that such a proposed generalization may be premature. Multiple peptide or protein assays will involve a combination of chromatography and enzymatic and/or immunological technologies. In addition, issues such as nonspecific binding to labware or stability of the analyte in biological fluids are in general more critical for large molecules (protease activity). Also the quality and type of enzymatic digestion and the purity of test materials may impact in the validation. In summary, the bioanalytical community may need to acquire more data prior to understanding what is routinely possible with respect to run acceptance criteria. A general comment from this session was also that we recognized that analysis of biopharmaceuticals is still easier than protein biomarkers, because of the availability of good reference standard materials and blank matrices for biopharmaceuticals.

Session 5: analytical strategies on combining LBA and LC–MS(/MS) for large molecules

Martin Nemansky (PRA) opened the session with a stimulating and challenging presentation on consequences of changing from traditional LBA to instrumental orthogonal methods such as LC–MS/MS. Current understanding dictates that new drugs should be evaluated based on their pharmacokinetic (PK) profiles. The trend in PK analysis for biologics is moving from LBA toward LC–MS/MS methods. Martin posed several relevant questions: “Is this trend motivated by intrinsic biochemical attributes of the analytes or by the wish to emulate small molecule PK analysis? Are there alternative methods to be considered for PK analysis of biologics and if so, how should these fit into traditional small molecule paradigms? Do regulatory authorities require the use of specific test methods?” These and further questions will need to be explored in relation to novel biologics, new batches of biologics as well as development of biosimilars. In the end, technologies such as LC–MS/MS, LBA, potency assays and immunogenicity assays will be evaluated for their functionality within the development and registration process of large-molecule drugs. In his presentation, Brian Geist (Janssen R&D) talked about using LC–MS for critical reagent characterization for ligand binding assays. There is growing interest in applications of LC–MS technology for characterization and bioanalysis of large molecules. However, most LC–MS techniques do not yet achieve the reproducible levels of sensitivity in biological matrices seen in bioanalytical LBA platforms (ng–pg/ml). Advances in LC–MS technology continue to improve sensitivity, but current standard practice for the bioanalysis of protein therapeutics continues to be LBA platforms. Reagent antibody characterization represents one application where LC–MS can provide critical information on reagent quality ensuring consistent performance of LBA. Michael Adler (Chimera Biotech GmbH) presented on highly sensitive cytokine quantification using Imperacer®, an Immuno-PCR-based ligand binding assay system. In modern drug development for treatment of autoimmune diseases, the main targets of biotherapeutic drug candidates are inflammatory cytokines. Michael reported on the development of a high sensitive Imperacer assay (typically 1000-fold increase compared to standard ELISA) for the quantification of free and total amounts of a specific cytokine in the presence of a biotherapeutic lead compound to support a clinical study.

Session 6: proteins in the MIST

This session aimed at connecting the audience again with relatively uncharted territory: metabolite quantification for peptide and proteins and requirements as per ICH M3 (R2) guideline. Experience in this field is still limited, which limited the session to familiarization of the topic and interesting technology updates. Michael Przybylski (University of Konstanz) presented on ion mobility mass spectrometry as a new tool in proteins analysis. In his inspiring talk, Przybylski presented cutting edge work from some of his collaborators. Examples were shared and some perspectives were given on technology as contributors to progress, for example online identification and affinity quantification, biomarker identification and characterization or investigation into reactive intermediates and aggregation. Richard Key (Quotient Bioresearch) presented on strategies for LC–MS quantification of plasma protease-susceptible peptides. Plasma can be a hostile environment for both synthetic and endogenous peptides, where circulating proteolytic enzymes can quickly cleave unprotected N- or C-termini. This can occur both inside the body (metabolism) as well as post-sampling (degradation). As a consequence the plasma matrix may need to be stabilized by inactivating enzyme activity. Quantification of protease susceptible peptides using immunological-based methods can generate false PK results if the antibody recognizes a metabolized peptide as the intact peptide. The application of LC–MS technologies enable quantification of intact peptide and can also be used to simultaneously quantify metabolites. Two case studies of how plasma protease-derived peptide metabolites were identified were discussed, together with the strategies to stabilize the peptides (and their metabolites) upon blood collection. William van Dongen (Proxy Laboratories) presented on LC–MS of therapeutic oligonucleotides (OGNTs). Therapeutic OGNTs are important biopharmaceutical drugs for the future because of their ability to selectively reduce or knockout the expression of target genes. Synthetic OGNTs are typically 19- to 25-base pairs long and can be single- or double-stranded RNAs (6000–8000 Da per strand). Single-stranded antisense oligonucleotides (asRNAs) are currently the most successful representatives of the first generation OGNTs. The development of OGNTs requires reliable and relatively high-throughput bioanalytical methods for the quantification of OGNTs and their metabolites in biological fluids. Currently immunoaffinity methods, especially ELISA, are widely applied

for this purpose. The potential of LC–MS/MS in OGNT analysis is under investigation because of its inherent ability to monitor the individual target OGNTs as well as their metabolites. In his presentation, William reviewed the state-of-the-art of bioanalytical LC–MS of OGNTs and their metabolites in biological fluids (plasma, urine, tissue and so on). Advantages and limitations were highlighted. The final speaker of the conference, Fillip Cuyckens (Janssen R&D) shared his experience on metabolite identification for peptides. The development of a peptide as a drug requires a different strategy compared to that used for small molecule drugs. In particular, the identification of metabolites of peptides needs to be handled differently. Critically, the identification of peptide metabolites is more challenging because of the ubiquitous presence of endogenous peptides in *in vitro* and *in vivo* matrices. The antiretroviral peptide drug enfuvirtide was selected as a test compound and dosed intravenously to rats. Plasma samples were collected at several time points after dosing and analysed on two TOF instruments: a Q-TOF Synapt™ G2 (Waters) and a TripleTOF 5600 (AB Sciex). Different post-acquisition processing tools were evaluated to identify the metabolites enfuvirtide. Simple tools applied to reduce the matrix background (e.g., ion mobility, charge state filtering) were compared and combined with more comprehensive software tools generally used for large molecule analyses as well as tools designed for small molecule metabolite identification work.

Conclusion

The conference successfully connected the bioanalytical community around peptide and protein analysis with LC–MS/MS. Almost 30 interesting presentations were shared and close

to 200 scientists from a wide range of companies attended. Again at this meeting the open discussion and interactions, seen at many EBF meetings, visualized the engagement of the scientists to connect around important changes in drug development. At the same time, all agreed that the challenges are real: by shifting from LBA into LC–MS for the analysis of peptides and proteins, the bioanalytical community is moving into partially uncharted territory. A careful understanding of this new environment is required. The key element for the success of the bioanalysis of peptides and proteins will be the increased integration of the LBA and LC–MS/MS expertise. At the same time, the industry looks forward to see further developments and enhancements of the LC–MS/MS tools. A lot of progress has been made in recent years, but the scientists call for even more performance from LC–MS/MS equipment. In conclusion, collaboration, new tools and good science will help us to bring valid solutions. Again, and in line with their goals, EBF wants to provide a home base for this continued discussion.

Acknowledgements

The authors wish to thank Richard Abbott (Shire Pharmaceuticals) for the proof reading.

Financial & competing interests disclosure

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

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

References

- Guidance for Industry Bioanalytical Method Validation. US Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), May (2001).
- Viswanathan CT, Bansal S, Booth B *et al.* Workshop/Conference Report – quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. Workshop/Conference Report. *AAPS J.* 9(1), E30–E42 (2007).
- Lee JW, Devanarayan V, Barrett YC *et al.* Fit-for-purpose method development and validation for successful biomarker measurement. *Pharmaceutical Res.* 23(2), 312–328 (2006).
- European Bioanalysis Forum. www.europeanbioanalysisforum.eu
- Focus meeting – large meets small; connecting strategies on analyzing large molecules with small molecule technologies. <http://bru2011.europeanbioanalysisforum.eu/>
- ICH Topic M3 (R2). Nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002941.pdf