The 3rd EBF Focus Meeting: 'Hatching' – emerging technologies approaching the regulated bioanalysis laboratory

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One hundred and eighty scientists from industry and academia discussed the progress in emerging technologies approaching regulated bioanalysis, with a focus on what potential hurdles prevent them becoming broadly accepted by industry and regulators. The conference delegates agreed that moving innovative technologies forward can only be achieved by providing solid data to support the application. In addition, also establishing an open dialogue with health authorities is key for success. By successfully integrating new technologies in the bioanalytical laboratory we contribute to bringing more safe and efficacious therapies faster to patients.

Philip Timmerman (on behalf of the European Bioanalysis Forum [EBF]) opened the EBF Focus Meeting by elaborating on the theme of the meeting. He asked several poignant questions such as 'Is there life after LC–MS/MS and ELISA?' and 'How to move innovations into the regulated environment?' Timmerman reflected on booming opportunities in bioanalysis. In addition, he reminded the audience that bioanalysis is truly becoming global; more regions are getting involved, new guidelines are emerging across the globe and the frequency of health authorities (HA) inspections is increasing. However, at the same time, and maybe as a consequence, industry is becoming increasingly reluctant to adopt new technologies in a regulated environment. What is driving this? Are guidelines and regulators pushing back or is the industry just becoming more risk averse? Asking 'When does emerging technology become 'established technology?', Timmerman started the discussion and urged the community to come up with answers and solutions.

CT Viswanathan (CT Viswanathan & Associates Inc.), in his talk 'Bringing new technologies into regulatory space', gave his insight on the interplay between HA and sponsors when introducing innovations into regulatory space. Innovation leads to new technologies, but innovations are associated with unpredictability. HA look at the risks associated, public and health safety impact and focus on quality of the data. For the sponsor, regulatory violations, warning letters and administrative follow-up are a risk. Viswanathan stressed that quality of the data and a dialogue with HA covering the complete development and implementation of new

technologies will help reduce risks. However, he explained it is not practical to expect guidance on new technologies. Early data is used for proof-of-concept and a fit-for-purpose approach is encouraged. Viswanathan warned that it is not sufficient to be thrilled only about the advantages and not objectively weigh pros and cons. In his opinion, sponsors should educate regulators on the innovation and develop a working relationship. Time and space to address the issues raised by regulators is needed and should be considered as an opportunity and not a roadblock. He concluded his talk stating that he sees a vital and important role for the Global Bioanalysis Consortium.

Special topics & new techniques

Willem van Dongen (TNO Triskelion) presented a cost-efficient, systematic and accurate approach to human drug metabolite profiling. His talk reflected the practical consequences of ICH M3(R2). This guideline provides requirements for metabolite characterization. Typically, non-clinical characterization of a human metabolite(s) is only warranted when that metabolite(s) is observed at exposures greater than 10% of total drug-related exposure and at significantly greater levels in humans than the maximum exposure seen in the toxicity studies. Such studies should be conducted to support Phase III clinical trials [1]. Although the guideline only requires data to be available for Phase III, new metabolite findings in mass-balance studies generated too late in development can become a burden on costs and timelines. van Dongen presented the iHumite® workflow, which consists of distinct steps to optimize metabolite profiling and characterization work.

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In his talk, Martijn Hilhorst (PRA) discussed advantages and recent developments in the use of atmospheric photospray ionization for quantitative bioanalysis. After explaining the technology, he continued with some practical examples of where this ionization technique can be used to broaden the application of LC–MS/MS.

Bruce Thomson (AB Sciex) presented a talk on pushing the limits of increased selectivity, dynamic range and selectivity for bioanalytical applications. He evaluated strategies AB Sciex have employed to increase sensitivity and selectivity. These efforts delivered a sensitivity increase of four- to ten-times and an increase in dynamic range of the detector by 20–50-times compared with existing technology. In addition, he discussed how sensitivity can be combined with selectivity through MRM, MRM3 and differential mobility technology.

Alternative MS techniques for quantitative bioanalysis

Benno Ingelse (MesoScale Discovery [MSD]) discussed the application of high resolution-MS (HRMS) in regulated bioanalysis. He listed the merits of triple quadrupole-MS (LC–MS/MS) and compared them with HRMS, also discussing some challenges of the technology. He concluded that HRMS can reach sensitivity and linearity competing with conventional LC–MS/MS. Selectivity of HRMS is clearly superior. Method development is easier and more time efficient as sample preparation methods and chromatography are simplified. Laboratory throughput is increased because total injection run time and extraction time are shortened. For Ingelse, the remaining challenge is in generating manageable data files.

Jaap Wieling (QPS) presented on the application of inductively coupled plasma-MS (ICP-MS) and LC–ICP-MS in drug development. He started by explaining the principles of ICP-MS and elaborated on the multiple applications of the technique. For a variety of elements, the technique is extremely sensitive, with quantification limits in the parts per trillion range. ICP-MS is an excellent detector for HPLC in bioanalysis and provides an orthogonal technique to other detectors. It enables simple quantification in many matrices, with little to no clean up due to its inherent specificity.

Eric Baltussen (WIL Research) discussed work done in his laboratory with inorganic, metallic or specific elements with ICP-MS and/or HPLC–ICP-MS. ICP-MS was applied

to analysis in various tissues with a different kind of sample preparation. He focused on requirements for sample preparation and stressed that it is key to keep the organic load low, eliminate background interferences and support constant viscosity for a stable nebulizing spray. A prerequisite for a good measurement is that the sample extract is 100% in solution. This can be a challenge and requires strong sample preparation methods for tissue.

Steven Watt (A&M) presented on HR and accurate MS for specific and sensitive quantification of target compounds in complex matrices. The Q-Exactive® was used to test the capabilities of HR-selected ion monitoring (SIM) in quantitative bioanalytical assays. It works by utilizing a quadrupole for selection of incoming ions, followed by a C-trap to store ions and finally an orbitrap for mass detection. He concluded that HR-SIM is suitable for quantitative experiments in a complex matrix. HR-SIM can be an ideal approach to quantify proteins and peptides. Indications are that the accurate mass HR Q-Executive performance is at least comparable to high-end triple quadrupole instruments.

In his talk, Fabio Garofolo (Algorithme Pharma) asked the question: “Can HRMS be used for quantification in regulated bioanalysis?” For Garofolo, the technology has tremendous added value because it resolves chromatographic problems, reduces method development time and is considered to be a robust and reliable detector. One of the main challenges he foresees – as also put forward by Ingelse – is data handling, more specifically the generation of manageable files without affecting the processing throughput. The investment required is larger so a good return on investment evaluation is recommended. In continuation, Garofolo presented examples illustrating the potential of HRMS. Garofolo concluded that, in his opinion, HRMS is no substitute for conventional LC–MS/MS (yet), but is certainly complementary.

Diego Rodriguez Cabaleiro (Waters) presented on accurate mass MS/MS, an attractive option for the quantification of biotherapeutics in drug metabolism/PK studies. He focused on the need for fast analysis, resolution of metabolites from the parent ion and sensitivity in discovery in support of PK evaluations. Cabaleiro elaborated on accurate TOF-MS instruments to provide the necessary speed and robustness for small and large molecules, and concluded that modern qTOF instruments deliver sufficient mass and sensitivity–stability for routine operation.

Miniaturization &/or automation of sample preparation techniques

Kevin Bateman (MSD) started this session by presenting on development of quantitative nanoscale techniques for ultra-trace analysis of biological microsamples. The demand to reduce sample volumes and LLOQ is driving the need for improved bioanalytical techniques, including robust and reliable nano LC–MS. His project objectives were to develop optimized LC–MS techniques that could support regulated bioanalysis of a microdose study using microsample volumes (100 μ l or less). Bateman concluded that micro and nano techniques for sample collection, processing and analysis are becoming more robust. The need for analysis of smaller samples while improving LLOQs is driving the science forward. Lessons learned from protein and peptide analysis are being translated to small-molecule bioanalysis and vice versa.

Lieve Dillen (Janssen R&D) presented on blood microsampling and bioanalysis in preclinical species. Dillen discussed the merits of using microcapillaries for blood sampling and evaluated practical and validation aspects. The initial conclusions were that plastic capillaries should be avoided, and the inclusion of a strong extraction solvent (e.g., DMSO) was recommended. Dillen also discussed preparation strategies for calibration standards and QCs and pipetting techniques pivotal to generating good quality data. Dillen concluded that capillary microsampling (CMS) is a simple, fast and ethical blood sampling method.

Ove Johnson (AstraZeneca R&D) shared his broad experience on CMS. Ove confirmed that CMS is applicable to blood, plasma, serum and other biofluids. Validation of CMS methodologies is similar to conventional methodologies. The exact sample volume and the liquid matrix enables stabilization within 5–10 s in blood and can facilitate analysis of rare matrices. Automated liquid handling tools are already available. For toxicokinetics, CMS can remove the need for satellite animals.

Brigitte Buscher (TNO Triskelion) presented on microdialysis as a tool in drug R&D. She concluded that microdialysis has evolved into a mature technique. It is one of the few techniques for studying *in vivo* tissue concentrations. By capitalizing on current developments in LC (resolving power and speed) and MS (selectivity and sensitivity), microdialysis has the potential to further grow or be applied in other areas, such as metabolite identification, metabolomics or biomarkers.

Sheelen Ahmad (GlaxoSmithKline) presented on the application of solid-phase micro-extraction (SPME) and microsampling. After explaining the technology, Ahmad showed some examples. All of the datasets demonstrated good correlation of reported drug concentrations between SPME probes and conventional blood draws (i.e., profiles reported correlated within experimental error). The SPME-based method was faster than conventional approaches, interfered minimally with the investigated system, minimized errors associated with sample preparation and limited personnel exposure to hazardous biological samples.

Tissue analysis

Markus Stoeckli (Novartis) presented ‘We can see mass makes the difference’, elaborating on MS imaging with MALDI applied in distribution studies. He showed examples of imaging of drug targets such as amyloid- β peptides and discussed limitations. Current limiting factors are variation caused by difficulty with extraction of the analyte, selectivity, co-crystallization of matrix and analyte, delocalization, crystal size, resolution and sensitivity. Matrix disposition variations and errors can cause deposition artifacts, resulting in different end conclusions. Quantification can be carried out by using an internal standard. Stoeckli emphasized that definition and evaluation of tissue suppression factors are pivotal, when comparing a response of an external standard under experimental conditions with those of the compound.

Jonathan Strauber (Imabiotech) presented on a qualitative and quantitative MS imaging technique for benzalkonium distribution in the eye. He saw the same advantages and limitations of MS imaging as Stoeckli. Strauber gave examples of quantification of olanzapine, propranolol and a lead compound and concluded that often semi-quantitative information is obtained instead of quantitative information. He considers MS imaging a technique of interest especially in small organs (difficult to dissect) or for whole body distribution and concluded MS imaging is even more powerful in combination with other techniques. Strauber feels the future of MS imaging is to improve sensitivity and reproducibility (for quantitation) and throughput (four- to eight-images/day/instrument).

Andrew McEwen (Quotient Bioresearch) presented on ‘MALDI tissue imaging: more than just a pretty picture?’ In his talk, he emphasized the promise of MALDI, a technique that aligns with ELISA, LC–MS

with microsampling and quantitative whole body autoradiography and/or staining to provide tissue distribution information. According to McEwen, the speed of analysis, as well as more information from one experiment and additional information compared with LC-MS/MS, are clear advantages for MALDI. The technique is developing rapidly due to the availability of new separation technologies and improvements in mass spectrometers.

In his talk on tissue analysis, Hermann Mascher (pharm-analyt) illustrated the sometimes daunting challenge the bioanalyst faces when confronted with (large) tissue samples. The contrast between the destruction technique required and the delicacy of trace analysis can be impressive. He presented different sampling and homogenization techniques and evaluated the problem of sample constitution, size and extraction efficiency. He suggested that four different challenges must be faced: first, taking a representative sample, then good homogenization, followed by recovery and last, dealing with the matrix effect.

Finally, Nathalie Mokrzycki presented on behalf of the EBF topic team on tissue analysis. This new team is gathering data on current best practice for tissue analysis among EBF member companies: the extent of method establishment done, work performed under GLP and how to include potential non-GLP data in a GLP study. The final aim of this team is to come forward with an EBF recommendation on the topic.

Emerging platform in support of the ligand-binding assay

During this session, new technologies based on the ligand-binding assay (LBA) format for assessment of large molecules were discussed.

John Allinson (ICON Development Solutions) gave an overview on new and automated technology platforms. During this keynote presentation he discussed potential benefits and limitations of integrating new platform technologies. The main message was that automated systems can bring many advantages in performance (i.e., lower CVs and improved total error), but that a company needs several types of platform to cover all their bioanalytical requirements (i.e., some machines are good at some assays, and some are better than others). Resistance to accept new assay platforms in industry is, however, apparent. Regulatory requirements and fear of change are two denominators slowing down the integration of new platforms in industry. Regulators should,

therefore, encourage the use of these new platforms.

Karen Dowers (Charles River Laboratories) discussed recent developments of (ProSeek®) LBA-qPCR technologies. ELISA data were compared with the use of qPCR in this LBA format. ProSeek kits from Olink Bioscience utilize oligonucleotide-labeled detection antibodies to detect an antigen in a biological matrix. This format is based on a proximity extension assay in which two antibodies are labeled with a pair of oligonucleotides. Once the two oligonucleotides anneal, a signal is emitted, which is detected by a specialized reader. Matrix effects are limited, making this technology suitable for quantification of proteins in the presence of high serum matrix concentrations.

Denise Sickert (Novartis) discussed the AlphaLISA®, a relatively new assay platform. The throughput of this platform is high because no wash steps are necessary. PK, PD and immunogenicity assessments have been performed using this new technology. Sensitivity was not improved when compared with common ELISA, and hook effect was evident at 8–10 µg/ml. It was recommended that measurements should, therefore, be done at two dilutions to ensure the correct concentration level is determined. The AlphaLISA does improve immunogenicity assessment. Sensitivity was below the required level (≤150 ng/ml) and acid dissociation tremendously improved drug tolerance. In conclusion, AlphaLISA may not improve the PK and PD assessment, but it is an interesting alternative for the assessment of immune responses to the drug product.

Craig Stovold (Quotient Bioresearch) gave some refreshing insights into an 'old' new technology: the Gyrolab™ system. This technology is still developing and more data have become available, outlining the possibilities and limitations of this system. The system often requires low dilutions and small sample volumes compared with other platforms, enabling determination of complexed proteins with minimal disruption to the sample. Its sensitivity for certain factors in the serum (for instance lipemic serum) and hook effect is minimal. Furthermore, the Gyrolab compares very well with MSD as it appears to be better at detecting certain disease factors. Sometimes overall sensitivity is somewhat less. Finally, carryover is a point of attention when using Gyrolab.

Bryan Krastin (ThermoFisher) presented 'The use of MS as a technology platform for immunoassays (MSIA)'. This technique uses

a solid phase in a tip used for the isolation of sample. The solid phase consists of a coupled matrix (e.g., prot-G). Antigen is subsequently captured by the bound ligand and digested prior to MS detection. During a comparison for IGF-1 determinations, a positive correlation between ELISA and MSIA was observed. Since it uses a more direct detection of the antigen, this technology may be interesting as an alternative to common ELISA.

Miniaturization & diversification in LC-MS interfacing

The keynote presentation was by Sabeth Verpoorte (University of Groningen, The Netherlands) on incorporating multifunctionality into microchip separations. Its potential application is in chemical analysis, synthesis, biochemistry and biology. The use of very low volumes enhances performance, facilitates automation, matches dimensions in the biological regime and makes implementation of multiple sequential or parallel operations less troublesome. Current examples include integrated electrodes for electrochemical detection and integrating capillary electrophoresis onto the chip. In her opinion, further developments will rely on adjacent developments in the areas of stationary phases (e.g., monolithic phases), new chip materials, integrated detection and so on.

Marc De Meulder (Janssen R&D) presented on 'Hatching sensitivity in bioanalysis: the promise of chip-based micro-LC-MS using a NanoAquity-TQS'. He suggested that micro-LC in bioanalysis supplies the answer to a growing demand for sensitivity in bioanalysis. This sensitivity boost is needed to answer questions posed by new concepts such as microdosing or microsampling or for introducing green chromatography. De Meulder showed a few case examples focusing on the potential of the technology, as well as some hurdles related to carryover and sensitivity.

In his presentation on 'Capillary-scale LC-MS/MS', Paul Rainville (Waters) listed the drivers and advantages of moving towards nanoscale LC-MS/MS. The Trizaic system he presented is mainly in use as a proteomics platform and not yet established as a (regulated) bioanalysis platform. He presented data on important aspects of the nanoscale separations: increase of signal intensity, excellent peak peptide separation and data showing retention time reproducibility below 1%, plus an evaluation of the pressure behavior as well as a loading study result. He concluded that reduction of

chromatography is beneficial in order to increase sensitivity (small sample volumes) and believes peptide analysis might even benefit more than small-molecule measurement.

Tina Settineri (Eksigent) presented on 'Miniaturizing LC for LC-MS'. She listed similar advantages for miniaturization of LC-MS/MS. In her experience, miniaturization to microflow UHPLC shows improved throughput. The very low dead volume of the system enables ultrafast gradients for LC-MS without compromising chromatography. Sample volumes are small (1 µl). Similarly, solvent flows are low in volume, leading to significant savings (>95%) in solvent costs. The system provides improved sensitivity for LC-MS because an ionization efficiency and chromatographic peak capacity are increased, which translates into improved sensitivity and signal to noise. Excellent separations are achieved within 0.5–1 min.

Applications & validation approaches for emerging platforms in support of LBA

Jenny Hendriks (Crucell) gave a keynote presentation on 'Challenges in development and validation of cytokine staining assays'. She recommended the use of an adapted EMA guidance in the absence of a T-cell/technology-specific guidance [2]. Calibration of equipment can be done according to existing guidelines. In the case of intracellular cytokine staining, Hendriks aimed for standardization and developed a validation strategy. Development of ICS (cytokine staining assay) involves the following points: acquire material and establish separate cytokine staining first, then combine and test for interference. Next, optimize staining, stimulation and thawing protocol. Record the number of stimulated cells, amount of 'acquired events', but also stability of stained samples. Evaluate interference with multicytokine staining, and standardization of gating. Development is then followed by validation assessing linearity, specificity, LLOQ, precision and defining acceptance criteria for the assay run. Trending charts for internal controls are helpful and training is of major importance. Hendriks finds major challenges are standardization of assay execution and data analysis. A comprehensive validation approach should address inclusion of assay controls, as well as a comprehensive definition of raw data and sufficient data retention.

Carsten Lindeman (EUFETS GmbH) talked on validation of a PCR for the quantification

of residual plasmid DNA in samples in a biodistribution study for a therapeutic RNA vaccine. In his talk, he described the project of quantitative determination of plasmid DNA from mouse organs by real-time PCR. The plasmid serves as a template for production of an RNA vaccine. The plasmid remains as an impurity in the end product. Residual plasmid therefore needs to be investigated and quantified as part of a biodistribution study. Method development, validation and sample analysis using regulated bioanalysis standards are part of the project.

Jan Detmers (Chimera Biotech GmbH) presented case studies on ultra-sensitive immunoanalytics based on Imperacer® (Immuno-PCR) in clinical settings. Chimera has completed 287 preclinical and clinical projects using this technology. The application of the technique has been reviewed by the US FDA and EMA.

Jason Cook (AstraZeneca) presented a talk entitled 'Validation of a multiplexed cytokine panel in human serum using a Randox evidence analyzer and comparison to other platforms (Luminex and MSD)'. Multiplex application potentially saves costs, reduces complexity in the clinic, reduces analysis time, gives improved performance and gives more information from limited samples. However, it also means increased complexities in handling validation and quality control. This, in turn, can potentially increase cost, raise questions on reliability and regulatory study suitability. He compared three platforms: Randox Evidence EV 180, MSD SI60000 and Bioplex 100. Historically, within Cook's former department, multiplexed instruments have been underutilized. However, an opportunity presented itself through a request for a multiple cytokine measurement. A project investigated a suitable multiplexed analysis on the Randox to assess its cost-benefit, demonstrate suitability for regulatory studies and then compare this

with other platforms. Cook emphasized the importance of having realistic expectations and of making acceptable compromises on quality. All platforms had issues with reliability. A partial validation was successfully performed, Randox was the preferred platform, and of the multiplex panel investigated, IL-6, MCP-1, IL-8 precision was good and all sample ranges were covered. For VEGF and TNF α precision was good, but for IL-1 β LLOQ the method was not sensitive enough.

The meeting was concluded with a panel discussion led by the co-authors of this conference report and CT Viswanathan. In this panel discussion, the audience was reconnected with the aim of this conference: how can we stimulate emerging technologies to become accepted and fully integrated in regulated bioanalysis? Two discussion papers have been written from this discussion [3,4].

The EBF is a nonprofit organization comprised of European pharmaceutical companies and CROs and has created a broad European network for discussions on scientific, technological and regulatory topics of bioanalytical interest [101]. The slide decks of this meeting have been made available as PDF renditions on the conference website of the 3rd Focus Meeting [102].

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