Poster list
EBF Open Symposium
Barcelona

15th to 17th November 2023

Note: The full poster list including abstracts can be downloaded from the conference website.

Posters will be displayed in the exhibition hall.

Authors:

Poster boards are numbered, please find your poster number in the index and hang your poster in the appropriately numbered space in the exhibition hall.

To hang your poster please use double-sided tape available in the exhibition hall or from registration desk.

Please ensure that your poster is in place by the end of the first break on Wednesday and that it remains in place until the end of the last break on Friday

Floor plan for Exhibition Hall - Posters and Sponsors

B1: Tataa Biocenter	2-E: IDBS	B2: LabCorp	B3: Synexa Life Sciences	B4: Quality Assistance
B5: LabWare	B6: ACM Global Laboratories	B7: Worldwide Clinical Trials	3B: Bio-Rad	B9: Nuvisan
2-B: Celerion	B12: SGS	B13: Biotage	2-H: Gyros Protein Technologies	2-K: Evotec
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B28: Quanterix	B29: SVAR Life Science	B30: Eurofins ADME Bioanalyses	B31: Merck	B32: Frontage Laboratories, Inc
B33: Quotient Sciences	B34: up to data	B35: Ardena Bioanalysis BV	B36: Bioanalysis Zone	B37: WuXi AppTec
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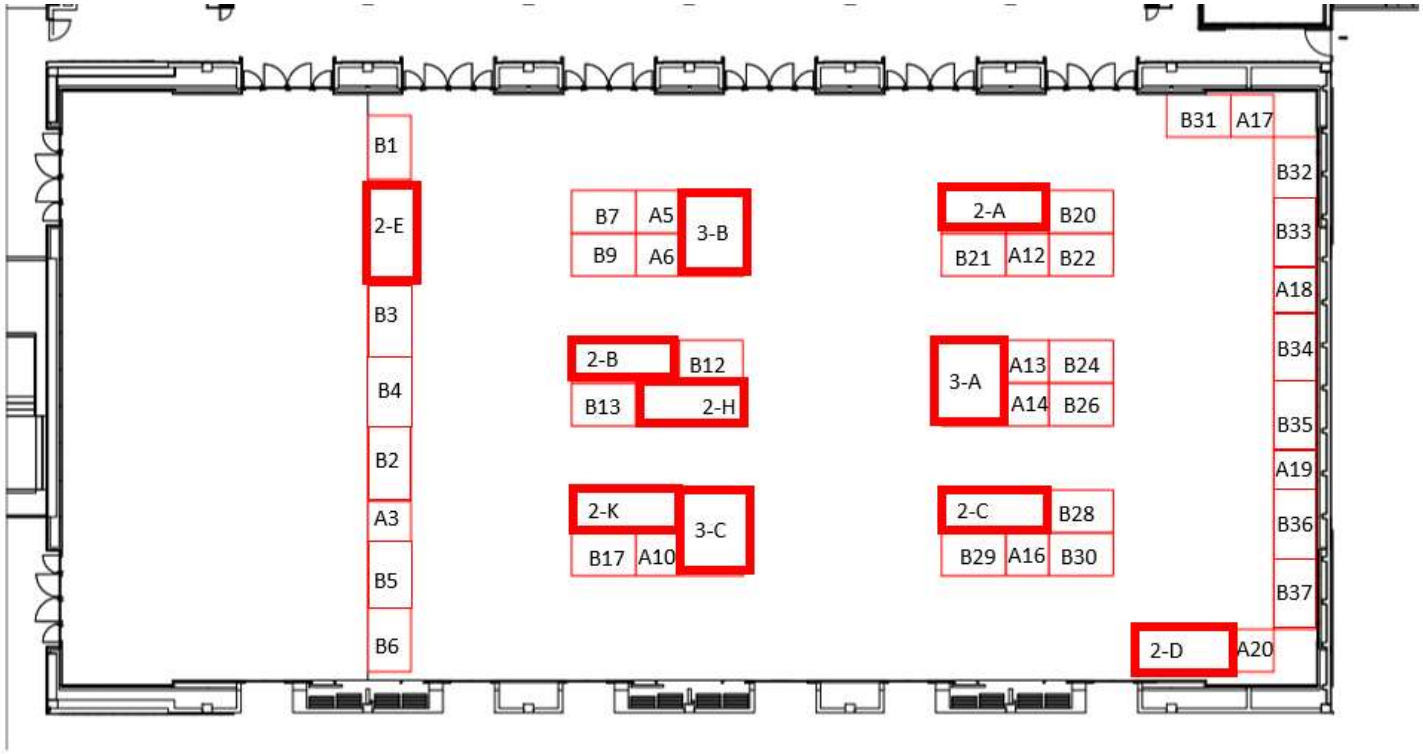

Hand-in-hand work between the Sponsor and the BA lab : a 5-year journey feedback toward the successful use of an IFN-γ ELISpot method supporting the clinical development of a new vaccine candidate

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Booth A12

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Developing reliable methods for monitoring cellular responses to a vaccine is akin to an obstacle course all along the process, from bedside - drawing blood from the patient - to bench - analyzing these samples for their immunogenicity. Understanding the context of use of the assay, including indication, expected magnitude of responses, injection regimen (prime +/- boost), sampling schedule, end use of data (dose-finding, correlates of protection, surrogate marker, statistical analysis…) determine the design and the level of validation of the assay.
We will illustrate how, during the clinical development of OVX836, an innovative universal flu vaccine candidate, Osivax, as the Sponsor, and Active Biomarkers, as the bioanalytical lab, have worked hand-in-hand to develop an IFN-γ ELISpot method and improve its robustness throughout the clinical development of the vaccine. We will showcase the steps allowing to move forward from an exploratory endpoint in phase 1 – which aims at exploring the kinetics of responses to the vaccine in relation with the route of administration and the dose of the vaccine – to a primary endpoint in more advanced phase 2 clinical studies. We will show how sample integrity, strict control of the critical reagents, and of the method along clinical sample analysis are the key elements that will determine the reliability and comparability of data across clinical studies.

Development and Qualification of a generic ligand binding assay for argenx lead molecules, on different platforms (ELISA-MSD-Gyrolab) to support Non Clinical Studies on several animal species.

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In this case study, the objective was to develop a generic ligand binding assay for argenx lead molecules, in order to support Non-clinical studies on different species (e.g Cynomolgus, Rat, Minipig, Rabbit).

New improved tools were generated in house to develop an robust generic assay. After tools selection, a ligand biding assay was developed on three different platforms, ELISA, Gyrolab and MSD), taking into account advantages and disadvantages of each technology.
Different factors were evaluated for the quantification of argenx therapeutics including matrix effect in preclinical matrices, assay sensitivity with lower limit of quantification and compatibility with micro sampling collection.

After resolution of several technical challenges (e.g dilution linearity), the selected assay presents an appropriate assay sensitivity with no or minimal matrix effect in biological samples and was successfully qualified at argenx for two lead molecules, illustrating the general applicability of the new tools.

Case study on the implementation of a regulatory compliant data platform for planning and execution, collaboration, review and reporting of bioanalytical studies.

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This poster will summarize the results of a project co-funded by the EU, which in close collaboration with large and small pharmaceutical companies and CROs exemplarily implemented a SaaS data platform to solve various issues related to bioanalytical studies.
For this purpose, the core workflows identified in discussions of the involved parties and implemented as exemplary individualized solutions: sponsor-clinic-laboratory collaboration and data exchange, laboratory workflow organization, results data review at the sponsor and in the laboratory, reporting incl. documents relevant for approval as well as long-term archiving of the accruing raw and regulatory data will be presented.
The used concept (SaaS) will be introduced in an easy-to-understand way, exemplary solution approaches demonstrated and the possibilities of deployment and extensibility discussed, as well as the principal differences to classical "central server" based approaches. Of course, in the framework of compliance with the GAMP and GxP regulations, that plays a superior role here.

Transfer of a GyroLab-based human IgG assay to a high-throughput LOCI-based assay format

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In the research bioanalysis department, we are responsible for the PK/PD analysis of several thousands of plasma samples from different in vivo animal studies using immunoassays. Within the department we have the following platforms to our disposal: ELISA, MSD, GyroLab and alphaLISA (LOCI). Some years ago, our department had established an Fc-targeted immunoassay for the detection of human (h) immunoglobin (Ig) G in pig plasma on our GyroLab platform. However, due to the large number of incoming samples, there was a wish for transfer to a more high-throughput method. As our department has established a fully automated LOCI-line, we decided to transfer the heterogenous GyroLab hIgG immunoassay to this homogenous LOCI platform. During assay development several antibodies were investigated and a shift in chosen antibody pairs was made. Increase in background seen in this homogeneous assay was tackled and several different hIgG constructs have since been analysed. All in all, we successfully established a new fully automated homogenous assay with maintained sensitivity providing us the ability to measure a large number of samples with limited hands-on time.

Short time line for LBA set-up with an anti-drug specific antibody: How to generate quickly high affinity monoclonal antibodies tools

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In bioanalysis, Ligang Binding Assay (LBA) have now become keys methods to detect or quantify biotherapeutic molecules (proteins, antibodies …).They give critical information to determine the safety and efficacity of the biotherapeutics. Typically, they are set-up with the presence of anti-drug antibodies to enable a specific binding activation.

Usually, LBA methods are widely used, for example, for PK assay, receptor assays etc, which required high sensitivity. Hence, one of the key success variable will be the high affinity and specificity of the developed anti-drug antibodies. These antibodies-tools can be generated by several ways: by polyclonal generation, with the rabbit as preferential host (the classical method in positive controls generation for immunogenicity assays). But also by monoclonal generation, here the interest is to produce very specific and sustainable antibodies.

Another essential variable for the project success is the required time for development and validation of the method. The anti-drug antibody generation can sometimes be the most time-consuming and complicated part. For this, the setting up of a high-throughput platform to generate high-affinity antibodies, can be decisive in the challenge of tight deadlines.

Here, we seek to develop specific mAbs against a therapeutic protein in order to have an antibodies panel, that can be used in several LBA method, as PK (sandwich format) and neutralizing assay. Thus, it requires to develop paired-antibodies and specific antibodies targeting the area involved in the interaction with the target.

Key aspects on re-optimization of a Ligand binding assay to get reliable data

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Ligand binding assays are widely used to quantify biopharmaceuticals, biomarkers, and to measure immunogenicity in preclinical and clinical studies. Key decisions are taken from the data, and it is therefore crucial that the results are reliable for its intended use.
A case study will be presented to highlight some key aspects to consider when improving a ligand-binding assay. An assay was transferred from a sponsor to a regulatory lab to perform PK analysis but showed issues during a first-in-human clinical study. There was a need to re-optimize the assay to get reliable data. The re-optimization was performed in an iterative way with step-wise identification of the most crucial parameters to ensure a cost efficient optimization. The iterative optimization included change in dilution buffer to reduce non-specific binding and replacing a commercial coated plate with edge effects with an in-house homogenous coated plate with low variation. As the clinical studies moved forward, the assay protocol was also optimized to increase the through-put. The re-optimized assay was fully validated and successfully used for sample analysis in phase 1 and phase 2 studies.

The Use of a Kinetic Immunoassay Format to Allow the Determination of Free Analyte
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The analysis of free analyte can be an important part of drug development since it can contribute valuable insight to the pharmacological effects of a therapeutic and the interactions between the biological drug and its target. The measurement of free analyte will depend on the affinity of the interaction the equilibrium between free and bound complex. Various parts of the analytical process can cause an equilibrium shift including incubation of reactants as part of the assay procedure as well as sample dilution to mitigate matrix interferences. While longer incubation times in plate-based assays may allow these shifts, the short (seconds) binding time of a microfluidic, CD-based platform minimizes or prevents shifts. In the first case study the free drug, bevacizumab (Avastin®) was measured with its target vascular endothelial growth factor (VEGF) as capture reagent using a microfluidic immunoassay platform to overcome these challenges, with ELISA for comparison. Up to 2-fold higher levels of free bevacizumab were observed using ELISA versus the microfluidic immunoassays, which can be explained by the very brief (seconds) exposure time of the sample to the affinity column for the microfluidic assay. In the second case study an assay was developed to quantify free IgE using omalizumab as capture. The assay was developed using 100% matrix to avoid sample dilution due to a low affinity interaction between drug and target. Results showed an over-estimation of Free IgE if the samples were diluted prior to analysis. These results point to the importance of selecting a kinetic analysis method for free analyte measurement dependent on the affinity characteristics of the interaction,

Rapid and Sensitive Simultaneous Determination of Fingolimod and Its Phosphate Metabolite in Human Blood Using Hybrid Extraction and LC-MS/MS
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This study presents a novel and efficient analytical method for the simultaneous determination of Fingolimod and its active phosphate metabolite in whole human blood. The methodology employed a unique hybrid extraction technique to enhance precision and reproducibility, resulting in sensitive detection at low concentrations. The analytical procedure involved protein precipitation followed by solid-phase extraction. The linear assay range extended from 10 to 5000 pg/mL for Fingolimod and its Phosphate metabolite. The chromatographic separation utilized gradient elution on a Kinetex biphenyl 5µ, (4.6mm × 100mm) column, followed by tandem mass spectrometric detection in electrospray positive ionization mode. The results of this study demonstrate the bioanalytical LC-MS/MS method's rapidity, sensitivity, specificity, and reliability in simultaneously quantifying Fingolimod and its Phosphate metabolite in human blood samples. This method has been developed and rigorously validated over the calibration range of 10 pg/mL to 5000 pg/mL. The bioanalytical methodology developed for the quantification of Fingolimod and Fingolimod Phosphate at a sensitive level, as described in this research, holds significant promise for the analysis of clinical trial samples. It offers precision, accuracy, and high throughput capabilities, making it a valuable tool in clinical research. Consequently, it has been successfully applied to analyze clinical trial samples. The utilization of Laboratory Information Management System (LIMS) software ensures the highest level of data integrity during method validation and the analysis of study samples. In summary, this method represents a robust and reliable approach for the quantitative analysis of Fingolimod and Fingolimod Phosphate, offering great potential in clinical research settings.

The quest for a highly drug tolerant neutralizing antibody assay: a combination of sample pre-treatment steps.

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In support of an argenx clinical program, a highly drug tolerant competitive ligand binding (CLB) neutralizing antibody (NAb) assay was developed. Based on modelling and simulation, it was anticipated that the Ctrough concentrations in serum under treatment would reach the mg/mL range in the highest dose group of the clinical study. This posed a major challenge as NAb assays generally do not achieve these high levels of drug tolerance (DT) using conventional methods. Therefore, a combination of two sample pre-treatment steps was evaluated during method development. Firstly, a polyethylene glycol (PEG) treatment was performed after spiking an excess of drug in the sample in order to form large(r) immune complexes. These complexes were precipitated by PEG during overnight incubation and subsequent centrifugation.
The following day, the PEG pellet was washed and acidified to dissolve the immune complexes. The subsequent neutralization step included an excess of biotinylated drug that was captured on a deepwell plate loaded with streptavidin coated resin which has a high binding capacity for biotin. Secondly, The ADA/NAb captured by the biotinylated compound were released by a second sample pre-treatment acidification step. Finally, the neutralized ADA/NAb are analysed in a CLB assay on the Mesoscale platform where biotinylated drug and sulfo-tagged target were used as capture and detection tool, respectively.
This innovative combination of two sample pre-treatment steps resulted in a drug tolerance of around 2 mg/mL during method development.

Computer/Modelling assisted evaluation of drugs on Non-alcoholic fatty liver disease (NAFLD)

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Introduction: The epidemic increase in non-alcoholic fatty liver disease (NAFLD) and the persistent resistance to pharmacological treatment require new approaches to study the molecular and functional changes underlying the progression of NASH and to develop new pharmacological interventions. NASH is a metabolic disease characterized by local and global metabolic abnormalities such as obesity, insulin resistance, and hepatic steatosis. There are currently several drugs on the market that target these metabolic derailments, including DPP-4 inhibitors, GLP-1RAs, and SGLT-2/1 inhibitors. Because in vivo studies of human liver metabolism are fraught with serious ethical and technical problems, mechanistic modelling of central liver metabolism, including the regulation of enzyme activities by their substrates, allosteric effectors, and hormone-dependent phosphorylation, has emerged as a promising tool for evaluating drug efficacy in silico.
Methods: By combining proteomic and transcriptomic signatures from biopsies with computational mechanistic modelling, we were able to quantify metabolic changes at a personalized level, identify potential targets for pharmacological intervention, and test the efficacy of approved drugs with known modes of action in silico in large clinically characterized cohorts.
Results: Using proteomic and transcriptomic signatures from 700 subjects (healthy/diseased) we conducted a comprehensive study of the efficacy of approved DPP-4 inhibitors, GLP-1RAs, and SGLT-2/1 inhibitors in patients with various stages of NASH, providing evidence for a novel approach to drug efficacy testing in early stages of the drug development process.

Targeted phosphor-proteomics elucidates drug's mechanism of action of Cetuximab.

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In drug development, understanding a drug's mechanism of action (MOA) is crucial for effective dosing and assessing potential toxicities by monitoring its impact on the target pathway in patients. A comprehensive understanding of MOA also opens up possibilities for identifying new drug targets and the development of combination therapies and biomarker strategies.
To achieve the precision medicine goal of improving response rates, we developed a robust, automated bead-based Western Blot platform for protein/phospho-protein profiling. This targeted platform has the capacity to simultaneously quantify up to 1,400 proteins, providing data on the activation status of key cellular signalling networks and proteins targeted by approved drugs or drug candidates, including those used in targeted cancer therapies (e.g., EGFR, HER2, PI3K, mTOR, ALK, and AKT).
For Cetuximab we compiled a list of relevant pathway nodes and their phosphorylation sites to assess activity in RAS/RAF/ERK, PI3K/AKT, and mTOR signalling pathways. In the here described project we utilized 242 total and phospho-antibodies to elucidate Cetuximab's mechanism of action. We obtained information on the activity of Cetuximab in signalling networks, spanning from receptor-level events to transcription factors, apoptosis, and proliferation. Appropriate media composition is shown to be crucial to evaluate Cetuximab sensitivity in vitro in patient-derived 3D models.

Development and validation of a qualitative qPCR assay as alternative approach for RCL monitoring

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Booth A10

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Chimeric antigen receptor (CAR)-T cell therapy is an emerging field for treatment of hematologic malignancies and other cancer types. To introduce new or corrected genes into the activated T-cells, lentiviral vectors are commonly used. Although lentiviral vectors are engineered to be replication defective, they potentially pose risks to human health, such as generation of a Replication Competent Lentivirus (RCL) capable of infecting non-target cells. qPCR assays for RCL monitoring are a rapid method to detect and usually quantify lentiviral genes, such as the envelope gene VSV-G (vesicular stomatitis virus G glycoprotein). Since FDA (Food and Drug Administration) guidance requires that all RCL positive results should be pursued by direct culture assay to obtain and characterize the infectious viral isolate, we concluded that a quantitative assay is not necessarily needed to detect RCL. Therefore, we developed an alternative approach for a highly sensitive, cost-efficient **qualitative** qPCR assay to assess the presence of the VSV-G sequence for the purpose of RCL monitoring.

Sensitive quantification of the protein targeting chimera (PROTAC) TL 13-112 in rat plasma using an LC-MS/MS workflow

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Proteolysis targeting chimeras (PROTACs) are endogenous protein degradation tools, capable of removing specific protein targets using a cell's own disposal machinery. PROTACs have evolved as a therapeutic modality, as several candidates have now moved into clinical trials. Sensitive and selective assays for high-confidence detection and quantification of PROTACs are needed to ensure safety and efficacy in the drug development pipeline and because PROTACs have expressed high potency in nanomolar drug concentrations

PROTACs were spiked into rat plasma at concentrations ranging from 10 pg/mL to 15000 pg/mL. Following protein precipitation, samples were vortexed and centrifuged at room temperature. The supernatant was transferred to a new Eppendorf tube and dried under nitrogen flow. Dried extracts were reconstituted prior to the analysis on a SCIEX 7500 system.

Calibration curves were constructed across concentrations ranging from 10 pg/mL to 15000 pg/mL. Individual concentrations were run in triplicate.

An LLOQ of 10 pg/mL was achieved for TL 13-112 and TL 13-110. No interferences were observed in the matrix blank (rat plasma) for either analyte. Strong linearity was achieved for both analytes with a linear dynamic range (LDR) of 3.2 orders of magnitude.

Accuracy was within ±11% and ±12% of the nominal concentration for TL 13-112 and TL 13-110, respectively. The %CV was <10% for both analytes.

Calculated values for accuracy and %CV were within the acceptance criteria at each concentration level.

Quantification of Oligonucleotide therapeutics in Plasma Using a New SPE Kit-Based Approach

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Booth A19

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Introduction/Purpose:

Oligonucleotide therapeutics, represent a growing class of drugs, with target specificity, low toxicity and higher potency. Bioanalytical assays are one of many assays required in support of pharmaceutical discovery and drug development efforts focus on molecules, such as oligonucleotides. LC-MS oligonucleotide sample preparation and quantification presents many challenges. This innovative work describes quantification of several modified oligonucleotide therapeutics including: an antisense oligodeoxynucleotide phosphorothioate, O-methylated antisense oligonucleotide, a GalNAc -SiRNA conjugate, and lipid modified oligonucleotide, from biological fluids using a kit-based approach and generic protocol.

Methods:

Using a prototype sample preparation and solid phase extraction kit with supplied protocol, oligonucleotides were extracted from plasma and urine (12.5-300 µL) using a proteinase K digestion sample pretreatment and anion-exchange SPE. LC-MS/MS quantification of signature peptides was performed using a triple quadrupole MS system. Separation was achieved using a UHPLC system with a sub-2 µm fully porous 130Å, 2.1 mm x 50 mm column (55°C), at a flow rate of 0.6 mL/min using a linear gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Final injection volume (5-15 µL) with a subsequent analysis time of 5 minutes.

Results:

The experiment resulted in reproducible quantification and recovery >75%. Sensitivity, linearity, accuracy and precision data met method validation requirements with mean accuracies ranging from 85-115% and mean % CVs ±15% Standard curves were linear over 3.5 orders of magnitude.

Conclusions:

This new approach allows for minimal to no sample prep method development achieving required high recovery and sensitivity with low sample volumes as 12.5 µL.

In vitro cellular approach for immunogenicity assessment of new developing NBEs

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Therapeutic monoclonal antibodies (mAbs) represent an increasing percentage of new drugs approved to treat a variety of chronic inflammatory diseases and cancers. However, these proteins can be recognized by the human immune system and can trigger the formation of anti-drug antibodies (ADA) with negative effects on drug pharmacokinetics/pharmacodynamics, safety, and efficacy. In view of this, developing preclinical assays to assess the immunogenicity risk is a key area of research for pharmaceutical companies. Our approach in Merck is to combine the in-vitro MHC-associated peptide proteomics (MAPPs) for the identification of NBE peptide sequences presented by major histocompatibility complex class II (MHC II) with the in vitro cell-based assay for the assessment of T helper cells activation and proliferation against NBEs. Here we present the assay development approach of two different cell-based assay formats, Peripheral Blood Mononuclear Cells (PBMCs) and Dendritic Cells/T cells (DC:T), to study T cell proliferation against entire NBE proteins and derived peptide sequences. Once the final assay is developed, it will be useful for estimating or predicting clinical immunogenicity with the final aim of contributing to the design of therapeutic antibodies with low immunogenicity during the drug discovery stage.

Enhancing chiral analysis using Supercritical Fluid Chromatography along with mass spectrometry in biological samples

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Supercritical Fluid Chromatography (SFC) represents a revolutionary approach in bioanalytical separation techniques, utilizing supercritical fluids as the mobile phase. A supercritical fluid, existing above its critical temperature and pressure, combines gas-like and liquid-like properties, offering unique advantages for separation of various compounds.
SFC employs supercritical fluids as the mobile phase. Supercritical fluids exhibit properties of both gases and liquids.
In this study, we harnessed the power of SFC for the analysis of chiral new chemical entity molecules, achieving remarkable results. Our investigation focused on chiral NCEs, encompassing one enantiomer and two diastereomers, totalling four distinct analytes.

The separation of diastereomers using traditional liquid chromatography (LC) was challenging task as it requiring extended runtimes of 40-45 minutes. In contrast, Supercritical Fluid Chromatography enabling diastereomers separation in a significantly shorter timeframe, typically within 20 minutes.
With SFC, we achieved exceptional enantiomeric separation and, notably, significantly reduced run times compared to conventional reversed-phase chromatography. Specifically, within a 20-minute runtime, we successfully separated the parent molecule, one enantiomer, and two diastereomers.
In other application, we achieved rapid separation of analytes, metabolites, and their enantiomers in as little as 10 minutes, while traditional LC systems required 25-30 minutes. Additionally, SFC exhibited superior baseline stability.

Conclusion: In summary, the utilization of supercritical fluid chromatography presents a transformative approach, particularly beneficial for chiral applications. It not only enhances analytical efficiency but also aligns with green technology principles, as it employs environmentally friendly carbon dioxide as the supercritical fluid, in contrast to traditional chromatography's use of organic solvents.

Evaluation of interferon signature as a biomarker in human biofluids by multiplexed immunoassay

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Interferons (IFNs) are widely expressed cytokines that are recognized as essential components of innate immunity and host defence. Different viral infections, chronic inflammatory and autoimmune diseases may produce unique IFN response signatures, which makes them intriguing biomarker candidates. The ability to simultaneously measure IFNs in biological samples by multiplexed immunoassay provides an ideal tool to evaluate the IFN signature across different diseases. We recently developed a customizable MILLIPLEX® Human Interferon Panel (Cat. No. HIFN-130K) that allows simultaneous detection of 9 biomarkers relevant to IFN biology including four type I interferons (IFNα2, IFNβ, IFNε, and IFNω), the type II interferon (IFNγ), three type III interferons (IFNλ1/IL-29, IFNλ2/IL-28A, and IFNλ3/IL-28B), and a soluble receptor IFNγR1 (CD119). Here, we report key assay characteristics of this novel multiplex assay including dynamic range, sensitivity, specificity, inter- and intra-assay precision, accuracy (spike recovery) and linearity of dilution/parallelism for each of the 9 biomarkers. We investigated serum and plasma samples from patients with autoimmune diseases (n = 20) and healthy donors (n = 16), observing detectability in at least 50% of the evaluated samples for all biomarkers. We also evaluated the IFN profile of plasma samples from individuals who had recovered from COVID-19 infection (PCR-negative at the time of collection, n = 5). Overall, our results highlight the performance characteristics of the Human Interferon Panel and demonstrate the utility of MILLIPLEX® immunoassays for interrogating the interferon response in human biofluids.

Mastering the Technical and Operational Hurdles to Obtain the PK Readout from a Complex Human Bronchoalveolar Lavage Study

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RG6006 is the first representative of a novel class of tethered macrocyclic peptide antibiotics active against Acinetobacter spp., including carbapenem-resistant Acinetobacter baumannii calcoaceticus complex (ABC) organisms.
It is essential to assess whether RG6006 can achieve adequate exposures in human lungs to achieve antibacterial effects for the treatment of pneumonia. Therefore, the generation of pharmacokinetic (PK) data in humans is important to support the progression of RG6006 from studies in healthy participants to studies in participants with lung infections.

In the presented study, we investigated the intrapulmonary penetration of RG6006 following administration of a single intravenous dose of RG6006 in healthy participants by performing a bronchoalveolar lavage. Challenging requirements were defined for PK evaluation, such as analysis of bronchoalveolar lavage fluid (BALF) and alveolar macrophage (AM) samples. Non-specific binding properties of the drug to plastic surfaces had to be considered for establishing a sample collection and sample handling process. Addition of additives like Tween© was not feasible due to the request to perform protein binding experiments in the BALF matrix.
By pulling bioanalytical and operational forces together, we established a study-specific sample handling and processing procedure overcoming challenges associated with 'sticky' compound characteristics. This enabled the analysis of several parameters from one biological matrix: PK (in BALF as well as in AM), urea content, and protein binding from healthy participant samples.

ADA screening and confirmation relationships in healthy and disease state drug-naïve subjects and the potential significance of low confirmatory cut points on patient stratification.

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A typical ADA validation was performed using 50 samples from both healthy and diseased drug naïve populations. A bridging assay using the MSD platform was used with an MRD of 10.

A small (<10 RLU), but significant ($p<0.05$) difference between the mean screening responses of healthy and diseased cohorts was observed. A broader range of screening values was seen in healthy samples compared with disease samples. Confirmation sample mean response difference was again low (<5 RLU) and also significantly different, ($p<0.05$).

An interesting difference, however, was observed in the relationship between screening and inhibition sample responses in the healthy cohort compared with the diseased cohort.

In the diseased group a relatively consistent difference between screening and confirmation responses was observed such that confirmatory responses increased in parallel with individual screening responses and hence the response difference between screen and confirmation samples was relatively constant. Conversely, in the healthy cohort the confirmation values are consistent, i.e. maximally 'inhibited' irrespective of screening value.

The latter is a more theoretically appropriate relationship in that the inhibited response forms the basis of the inhibition calculation in the current ADA assessment paradigm.

The differential relationship between disease state samples and healthy samples, together with inherently low cut-points and inhibition levels may lead to sub optimal use of the confirmation step in patient groups.

The implications for the confirmation step as currently configured will be discussed.

Developing novel Droplet Digital PCR methods for the quantification of gene and cell therapy targets

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Droplet Digital Polymerase Chain Reaction (ddPCR) technology allows PCR to be performed on a multitude of compartmentalised reactions simultaneously, giving positive or negative results for each droplet, and thus increasing assay sensitivity and accuracy in comparison with methods such as quantitative PCR (qPCR). A standard curve is not required for this direct quantification, improving set up efficiency and data accuracy. Furthermore, the sample partitioning also affords the assay an increased tolerance to the potential effects of PCR inhibitors existing within a range of biological sample types.
We have therefore established a ddPCR testing platform to allow for DNA or RNA targets to be accurately and efficiently quantified, even when the expected copy number is low. We have developed methods for assessing a range of DNA or RNA targets (with an additional reverse transcription step), which can then be validated to Good Laboratory Practice (GLP) standards to allow for sample analysis in regulatory studies. One example is an assay that we developed to detect and quantify a vector DNA target in mouse blood and tissues following administration of Chimeric Antigen Receptor (CAR)- T cell therapy.
Here we present data obtained during the development process for these assays and give an insight into the potential use of ddPCR in furthering our capacity to accurately quantify nucleic acid targets as part of the development of new gene and cell therapies.

Considerations for the conduct of cross-validations and statistical evaluation of data according to ICH M10 recommendations

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The ICH M10 recommendations for the conduct of cross-validations [1] has left open questions for the scientific community. Limited experience with statistical methods and lack of clear guidance for data evaluation and corresponding acceptance criteria may lead to confusion and uncertainties regarding cross validation conduct. Even though the EBF has provided some suggestions already [2], many laboratories are still following old procedures and have not yet implemented any changes since ICH M10 implementation.

Here we present case studies of previously conducted cross-validations to evaluate which statistical approach reflects best any method bias (e.g., Bland-Altman plot, Deming regression, Passing Bablok). LC-MS/MS and ligand binding assays with (also borderline) met and failed acceptance criteria (according to EMA guidance) are included. Strengths and limitations of each approach are discussed.

We address questions such as the need of separate approaches and acceptance criteria for QCs and study samples, the process if no study samples are available, stability considerations, follow-up actions for failed cross-validations, and when the involvement of PK scientists and modellers in the interpretation of cross-validation bias is required.

We propose a best practice, including experimental design, a framework for the subsequent data analysis, result interpretation and documentation of cross validation experiments.

[1] ICH guideline M10 on bioanalytical method validation and study sample analysis, EMA, 25 July 2022
[2] Tom Verhaeghe, on behalf of the EBF, ICH M10 cross validation & documentation: what now? 12th EBF Open Symposium 2022

Development and comparison of analytical methods by LC-MS for the analysis of antibody-drug conjugates

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The development of new analytical workflows is essential to characterize in-vivo behavior of next generation biotherapeutics, such as Antibody-Drug Conjugates (ADCs) that are complex molecules designed to target and kill cancer cells and composed of a biologically active agent covalently attached to a mAb via a chemical linker. These last years, the conjugation strategy has evolved from stochastic conjugation on lysine residues to more controlled approaches, allowing to get more homogeneous Drug-to-Antibody Ratio (DAR) profiles.
To support the development and the optimization of ADCs, it is essential to understand their pharmacokinetics behaviors (stability, antigen binding, clearance, biodistribution), by assessing the DAR in-vivo. Here, we have developed a full LC-MS workflow for the bioanalysis of Adcetris, an interchain cysteine-conjugated ADC (DAR 4) consisting of a mixture of covalent and non-covalent species. Quantification of the conjugated drug after a deconjugation step has been performed using LC-MS. In parallel, a middle-up MS approach combining immuno-enrichment and LC-MS analysis of LC and HC subunits after disulfide bond reduction has been set up to determine the DAR profile. Finally, bottom-up MS was used to quantify the total antibody species. All together, these approaches allow to obtain the PK profiles of all individual circulating DAR species.

A rapid and comprehensive characterization of the immune cell profile in human skin

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Human skin covers the surface of the body and is essential for the protection from external factors such as pathogens, chemicals and temperature. The immune cells of the skin are distributed as a complex network in the epidermal as well as the dermal layer and are crucial for tissue homeostasis and host defense. Dysregulation of this network can result in inflammation and skin disorders such as psoriasis or atypical dermatitis. Information about the immune cell profile in the skin can improve the understanding in the underlying mechanisms contributing to inflammation and can help to validate new therapeutic concepts. Understanding the skin reaction as injection site reaction or skin rush after treatment getting more in focus in clinical trials. Flow cytometric analysis provides an effective method to detect and quantify multiple immune cell subpopulations. However, due to limited access to fresh human skin biopsies, the establishment of flow cytometric analysis of tissue-derived immune cells remains challenging and is currently limited to T cell subpopulations in the skin. Here, we describe the methodical qualification of immune cell isolation from human skin by enzymatic and mechanical tissue breakdown and a following flow cytometric analysis of different T cell subpopulations, Neutrophils, Basophils, Eosinophils and innate immune cells. In summary, this study allows a rapid and comprehensive characterization of the immune cell profile of a small skin biopsy.

A case study on bioanalytical method development and validation for quantification of a prodrug in animal whole blood PK samples

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In this case study results will be presented for the bioanalysis and quantification of a prodrug, which is intended for the treatment of Malaria. Different aspects on the workflow for method development and validation experiments for whole blood PK samples of non-clinical species (rat and minipig) will be discussed.

Due to the instable nature of the prodrug, PK blood samples needed to be directly treated using ice-cold acetonitrile to ensure its reliable quantification after further sample processing for LC-MS/MS analysis.
Discussions on mass-spectral fragmentation behaviour, as well as the selection of a stable-isotopic-labelled internal standard on method performance will be made.
The results from the method validation experiments will be summarized, which enabled successful support of the GLP study programme in rat and minipig using this LC-MS/MS methodology.

Short RNA Quantification using Nucleic Acid Nanorobotics (NANs)

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For short RNA sequences, extraction from biological media, polyadenylation, or the presence of modified bases (2'-O-methoxy-ethyl, 2'-O-methyl or locked nucleic acids) can introduce high degrees of variability in quantitative analysis using PCR-based approaches.

This poster describes a novel and fit-for-purpose approach, Nucleic Acid Nanorobotics (NANs), for assaying short sequences such as microRNAs and modified short RNA-therapeutics including anti-sense oligonucleotides (ASOs). Our results demonstrate required assay parameters such as sensitivity, selectivity, and linear range for in a range of biological matrices including serum and cell media. A high-throughput method that incorporates multiplexing is also reported.

The validation of a Multiple Myeloma Minimal Residual Disease Flow Cytometry assay for use in multi-centric clinical trials

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Booth A3

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Flow Cytometry is a high-throughput and sensitive technique suitable for the detection of very rare cell populations amidst complex mixtures. Consequently, it has become a platform of choice for the detection of minimal residual disease (MRD) in various types of leukemia.
For Multiple Myeloma (MM) patients undergoing treatment, the detection of MRD helps determine if any cancerous cells escape treatment. This biomarker can constitute an important prognostic measure and help predict the likelihood of disease relapse and/or progression. Therefore, there is considerable interest around the use of MRD as a potential surrogate endpoint in clinical trials.
Labcorp Central Laboratory Services has validated and adapted a highly sensitive next-generation flow cytometry assay to detect MRD in MM patients based on EuroflowTM Consortium published recommendations (Kalina et.al., 2012) and the MM MRD assay developed by BD/Cytognos. Limit of Detection (LOD), Lower Limit of Quantification (LLOQ), precision and stability for key reported parameters of the assay, including abnormal plasma cells (PC) frequency, have been validated per published CLSI H62 guidelines and within CAP/CLIA compliance.
MM MRD assay validation experiments demonstrated high sensitivity (for abnormal PC, LLOQ was established at 23 events or 0.00039% (%Total Nucleated Cells, TNC)), acceptable assay performance (for abnormal PC intra-assay precision %CV was established at 7.67% (%TNC)) and confirmed published sample stability of 48 hours post-collection (Stetler-Stevenson et.al., 2016). Concluding that, MRD by flow cytometry offers a high-quality, sensitive, and robust approach to be used for multi-centric clinical trials and run in a CAP/CLIA clinical laboratory environment.

Spatial distribution of B cells and lymphocyte clusters for the treatment of non-small cell lung cancer

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The presence of tertiary lymphoid structure (TLS) in tumor tissues has been reported to be associated with good prognosis in several types of cancers including non-small cell lung cancer (NSCLC). However, the relationship between TLS spatial organization and the treatment response remains unknown in NSCLC. The purpose of this study was to evaluate the effect of the various stages of the spatial organization of the TLS from locally concentrated aggregates of immune cells to mature follicles in NSCLC and its relationship with the tumor microenvironment on anti-PD1 treatment response.
Frozen sections from retrospectively collected surgically resected NSCLC tumors treated with pembrolizumab therapy were used. The TLS in tumor tissues was detected by imaging mass cytometry staining and the difference in TLS spatial organization was compared to the features of the tumor microenvironment and the objective response rate of the patients.
TLS characterized according to their spatial organization within or adjacent to the tumor showed that the presence of tumor-associated TLS (TA-TLS) correlated with favorable response to anti-PD-1 therapy. The abundance and the spatial distribution of B cells allowed a better definition of the correlation between B cell subsets with clinical outcomes showing that the heterogeneity in these TA-TLS influences the predictivity significance to anti-PD-1 therapy.
Identifying the phenotypic heterogeneity of intratumor B cells and their functional connection to CD8 T cell helps optimally guide the anti-PD-1 treatment strategy and provides an opportunity for translation of B cell-based immunotherapies into clinics complementary to existing T cells strategies.

Developing a Luminex Method to Detect Nephrotoxicity Biomarkers in Rat Urine
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Conventional markers of acute kidney injury (AKI), BUN and serum creatinine, do not indicate early signs of kidney injury or stress. As such several markers have been proposed as alternative biomarkers for early monitoring of AKI. To that effect a Luminex panel of Clusterin, KIM-1 and Osteopontin was assessed, developed and validated for use on non-clinical safety assessment studies using rats as the test system. Measured in urine, these markers can be used to aid in the detection of AKI. To develop the method, the manufacturer's instructions were modified to produce reliable performance of QC samples at both the lower and upper end of the curves, whilst determining the endogenous concentrations of the analytes across a range of dilutions. This included assessing volume of sample, sample diluent, process timings and centrifugation speeds. To assess reproducibility and ultimately validate as fit for the context of use, parameters such as Parallelism, Dilutional Linearity, Selectivity and Stability were assessed. Parallelism was displayed between 1:4 and 1:16, indicating a minimum required dilution of 1:4. Dilutional linearity and selectivity were also assessed due to the low number of available individuals with endogenous concentrations of these analytes. Dilution linearity was displayed from 1:4 to 1:128 and selectivity showed no interference of analyte concentrations at low concentrations. Long term stability is on-going; however, initials stability assessments indicate multiple freeze thaws and 1 month stability are acceptable. The results indicate the Luminex immunoassay is a strong performing and reproducible assay which is fit for purpose.

Validation of a process specific ELISA for the monitoring of HCP
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The development of biological therapeutic products has been booming for years. A critical aspect for product manufacturing is the release of impurities such as host cell proteins (HCP), which affect drug efficacy and might compromise patient safety. HCP are proteins and polypeptides coming from the host organism, which may contaminate drug substance (DS) and that can be detected and quantified by sandwich ELISA using polyclonal sera raised against HCP. To quantify HCP, a generic HCP kit is usually used during each phase of the purification bioprocess, but from Phase III, it is strongly recommended to develop a process specific HCP ELISA kit. Another scenario may require to go directly with a process specific kit : when the general kit is no longer well adapted, because of a deficient coverage or because of the cross reactivity against the DS. As described in the guideline: "any cross-reactivity of the anti-HCP antibodies with the product may compromise the test method and yield biased results". Therefore, any contamination of the HCP antigen with product must be avoided to prevent the generation of anti-product antibodies." (Guideline FDA Residual Host Cell Protein Measurement in Biopharmaceuticals). In this context, the regulatory authorities require an adequate coverage from polyclonal antibodies (pAbs) on HCP population and to maintain HCP level in drug samples within 1-100 ng/mL. The aim of this poster was to present coverage and cross reactivity data, and to demonstrate validation of a process specific anti-HCP ELISA.

Development of specific assays for PK and PD monitoring of anti CD38 biotherapeutics

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The development of biotherapeutic drug products targeting CD38 has been shown to be successful for multiple myeloma patients (MM). The plasma cells from MM patients highly express CD38 on cell surface, making CD38 an attractive target for drugs. So far, 2 monoclonal antibodies (isatuximab and daratumumab) are currently available for patients.
While down regulation of CD38 on cell surface remains controversial since there is no commercial antibodies that bind to CD38 independently to isatuximab and daratumumab. In order to generate suitable antibody for determining the total number of CD38 molecules on plasma cells, we are developing several VHH targeting CD38 using extracellular vesicle (EV) as immunogen in llama. The antibody candidates are characterized by quantitative flow cytometry (qFC) and ELISA. Such an antibodies would be used in qFC for assessment of the total CD38 receptor density (CD38RD). In combination with a competitive antibody, the CD38 receptor occupancy (CD38RO) can be monitored as well for PD monitoring in clinical samples.
In parallel, BioCytex has developed several versions of assays for PK monitoring on plasma samples from patients receiving either isatuximab or daratumumab. The updated ELISA assay demonstrated higher sensitivity for anti-CD38 targeting antibodies.
Both fit-for-purpose PD monitoring qFC-based assays and PK monitoring ELISA-based assays are developed, validated and manufactured at BioCytex.
The preliminary characterization of the VHH or engineered minibodies on cell lines and whole blood samples by qFC is described and the main performances of the ELISA assay to quantify anti-CD38 targeting antibodies are discussed.

Multimodal stratification of predictive biomarkers in head and neck Cancers: A focus on cytokine-based immunotherapy

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Immunotherapy, particularly cytokine-based therapy, has been gaining traction in the treatment of head and neck cancers. However, not all patients respond to cytokine-based immunotherapies, and some may experience severe side effects. The ability to predict which patients are most likely to benefit from these therapies could greatly improve the efficacy and tolerability of treatment. In this context, the study of predictive biomarkers, such as the expression of immune checkpoint molecules like PD-L1 and TIGIT, and the profiling of cytokines within the tumor microenvironment, has become crucial.
A multimodal approach to stratify predictive biomarkers in head and neck cancers was used. The methodology was founded on combining metabolic, transcriptomic, and proteomic data to offer a comprehensive understanding of potential biomarkers. The data from different modalities were integrated using machine learning algorithms. This comprehensive dataset was analyzed to identify multimodal biomarker signatures that could predict patient responses to cytokine-based therapies.
Preliminary finding showed significant heterogeneity in the expression of PD-L1, TIGIT, and various cytokines across the tumor microenvironment. Certain multimodal biomarker signatures, which included specific patterns of immune checkpoint molecule expression, cytokine profiles, and immune cell infiltration, correlated robustly with patient responses to cytokine-based therapies.
Our study demonstrates the considerable potential of a multimodal stratification approach in deciphering the complexity of head and neck cancers and predicting responses to cytokine-based immunotherapies. By integrating metabolomic, transcriptomic, and proteomic data, we identified unique biomarker signatures that strongly correlated with therapy responses.

Highly sensitive analysis using EVOSEP-LC/MS assay for targeted PD-L1 and PD1 expression level for predicting response to immune checkpoint inhibitors

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PD-L1 and PD1 immunoassays have been traditionally used for predicting clinical responses to immune checkpoint inhibitors. However, there is no consensus about the antibodies used to determine PD-L1 and PD1 expression levels leading to uncertainty about the clinical value of these biomarkers across tumor types. In this work, we set to test a robust method to determine PD-L1 and PD-1 expression levels as compared to routinely used immunoassay at the analytical level and then use the Overall Response Rate to assess clinical utility.

Tumor tissues samples from resected non-small cell lung cancer patients further treated with pembrolizumab therapy were assessed for PD-L1 and PD1 expression by Evosep-LC/MS and ELISA immunoassay with different antibodies clones. A subset of serially diluted cases was evaluated for Evosep-LC/MS assay performance across a range of PD-L1 and PD1 expression levels shown to correlate with the Objective Response Rate of the patients: Complete Response and Progression Disease.

Assessment of PD-L1 and PD1 protein levels by Evosep-LC/MS demonstrated robust linearity across high and low expression range. Reproducible protein expression levels were obtained for Evosep-LC/MS and the ELISA kits tested, and both technologies delivered broadly comparable titration values. Still, targeting a given antibody clone biases the expression level evaluation, whereas such a bias is not observed from a label-free quantification assay such as the Evosep-LC/MS one. Evosep-LC/MS has the added advantages of being amenable to standardization and avoidance of interpretation bias for clinical use and provides a harmonized high throughput testing for biomarkers for predicting clinical response.

Mass Spectrometry Profiling of RNase T1 Cleaved ssRNA Oligonucleotides via Ion-Pair Reversed-Phase UHPLC-QTOF-MS

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Poster A19.

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mRNA has emerged as a promising biotherapeutic, particularly in the new generation of vaccines. Nevertheless, a long path has precluded the tremendous success of mRNA prophylactic Covid-19 vaccines, focused mainly on the in vitro transcription method of production set up and in the modification of the mRNA structure to enhance its stability and functionality.

Being able to detect and localize these modifications within the large molecule of mRNA has been one of the biggest challenges in the biopharmaceutical analysis field. Due to its large structure, small modifications in the nucleotides are difficult to detect when analysing the intact mRNA. This is why ribonuclease digestions offer a highly effective approach, mirroring the bottom-up methodology commonly used in protein analysis, known as peptide mapping. The recently defined oligomapping pathway aims to locate the modifications and verify the sequence of the entire mRNA molecule, with parallel ribonuclease digestions.

Therefore, a comprehensive study of RNase T1, one of the widely used specific ribonucleases, has been conducted. This study includes an examination of the cleavage products from custom designed oligonucleotides, and the assessment of the oligonucleotide-RNase T1 ratio and time of digestion.

The digested oligonucleotides were evaluated using ion-pair reversed phase UHPLC, coupled with an ESI-QTOF-MS. This allowed for the separation and individual characterization of the cleaved products. Their molecular weights were determined through deconvoluted MS spectra, and with a specific variant of data-independent acquisition mode, the MSMS spectra was provided, offering valuable insights into fragmentation and aiding in the elucidation of the oligonucleotide sequence.

Sensitive and Selective HPLC Determination of α-, β- and γ-Cyclodextrins and Hydroxy-Propyl- Cyclodextrins

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The HPLC-determination of α-, β- or γ-cyclodextrins and hydroxy-propyl-cyclodextrins is not easy because of different reasons:
1. Detection
Very weak UV-absorption
ELSD is possible but insensitive and unselective
MS/MS-detection is a good option

2. HPLC-separation
Challenging because of cyclodextrins not being single molecules
Very hydrophilic molecules, weak retention on C18
Good separation on NH2-columns because of many OH-groups
The combination of amino-columns (NH2) and MS/MS-detection is a very sensitive and selective combination for the determination of α-, β- and γ-cyclodextrins and hydroxy-propyl-cyclodextrins. The sensitivity for each substance is about 1 ng injected.

SENSITIVE AND SELECTIVE HPLC DETERMINATION OF POLYMERS IN BIOLOGICAL MATRICES AND PHARMACEUTICAL PRODUCT/SUBSTANCE

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The HPLC-determination of Polymers like Polyethylenglycol (PEG), Poloxamer (POL), Polylactic acid (PLA), Polyethylenimine (PEI), or Polyoxazolines (e.g. PEtOx or PMeOx) is not easy because of different reasons:

1. Detection
NMR would require large sample volume.
UV and FL do not fit (little or no native response).
ELSD is possible but insensitive and unselective.
ELISA cannot differentiate well between different molecular sizes of respective polymer.
MS/MS-detection is a very good option.

2. HPLC-separation
Challenging because polymers do not represent a single molecule but rather a molecule distribution around a certain molecular weight.
Possible on different large pore RP-columns, not only SEC.
LLOQs of below 100 ng/mL often possible, even in complex biological matrices such as plasma, whole blood or CSF.

Orthogonal Approach for AAV Immunogenicity Assessment: Evaluating Total and Neutralizing Antibodies
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Extensive studies of AAV vectors as carriers of gene therapy products has advanced the field from a promising new therapeutic concept into today's medical reality. However, several bottlenecks are still present during the development of gene therapy products hence meeting respective regulatory requirements has turned into a challenging process. Among them, the presence of antibodies against AAV has been shown to affect the outcome of in vivo AAV-mediated gene therapy. The most direct impact occurs during administration through capsid neutralization, or by triggering immune reactions such as activation of the complement system. Currently, it is recommended to analyze both the presence of total binding antibodies (TAbs) as well as neutralizing antibodies (NAbs). Here we present Svar's AAV immunogenicity solutions that allow for assessment of both TAbs and NAbs in two highly customizable assays. First, TAbs are assessed with a standardized immunoassay with a high sensitivity, allowing the evaluation of the presence of TAbs before or after administration of AAV vectors. In a second assay, NAbs are assessed using new two-component system for the detection and quantification of NAbs directed against recombinant AAV vectors, based on a reporter gene Platform These two platforms combine the advantages of both immuno- and cell-based assays. By offering reliable and customizable solutions for assessment and differentiation of humoral antibody responses against AAV vectors we respond to the expert demand for such assays. Svar's AAV immunogenicity solutions represent a unique toolbox designed to accelerate and support the clinical development of todays and tomorrow's AAV-mediated gene therapies.

Driving more sensitive and selective quantitation of highly potent inhaled corticosteroids in human plasma using accurate mass spectrometry
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For long-term asthma control, inhaled corticosteroids are highly effective anti-inflammatory medications. Fluticasone furoate, fluticasone propionate and mometasone furoate are inhaled corticosteroids commonly used to treat allergic conditions such as asthma and allergic rhinitis. Since the daily dose of inhaled corticosteroids is low, drug circulation in the blood occurs at low concentration levels. As a result, pharmacokinetic studies require highly sensitive and selective assays to quantify inhaled corticosteroids at pg/mL levels in biological matrices. Here, a method is presented for quantifying fluticasone furoate, fluticasone propionate and mometasone furoate using the ZenoTOF 7600 system. This accurate mass spectrometer provides exceptional selectivity for accurate and precise drug quantitation in complex biological matrices. All 3 corticosteroids were spiked into 300 µL of human plasma at concentrations ranging from 1 to 1000 pg/mL. A 700 µL aliquot of 30% (v/v) methanol in water was added to the sample and vortexed. Samples were centrifuged at 9400 rcf for 5 minutes. The dried samples were reconstituted in water with 100 µL of 50% (v/v) methanol in water. A 25 µL sample injection was used for analysis. Quantitation was performed using Zeno MRM^{HR} mode on the ZenoTOF 7600 system. LLOQs of 1 pg/mL, 1 pg/mL and 2 pg/mL were achieved in human plasma for fluticasone furoate, fluticasone propionate and mometasone furoate, respectively with linearity for the concentration range of 1 pg/mL to 1000 pg/mL with r^2 values of 0.992, 0.992 and 0.993 for fluticasone propionate, fluticasone furoate and mometasone furoate, respectively.

State-of-Art Instrument Integration Strategies

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Lab Automation of a (bio)analytical laboratory includes the integration of its analytical instruments. Instrument Integration will streamline the workflow of the lab, improve its throughput, and reduce errors. Correct set-up of instrument integration as well mitigates Data Integrity concerns during Regulatory Audits.

In this poster, the state-of-art strategies to integrate instruments to a LIMS or any applications are discussed, distinguishing the following instrument types:

- Direct Instrument Interfaces
    - Simple Instrument connections (balances, pH meters)
    - ASTM instruments (Clinical Analysers, Flow Cytometers)
- Software-to-Software Interfaces
    - Connecting to APIs (Application Programming Interfaces, like Empower, Chromeleon, Analyst)
    - Connecting to APIs using Web Services
    - File based interfaces (like AniML, Plate Readers, etc)

Strategies depend on the key characteristics of the instruments, like:

- Ease of integration
- Bi-directional interfacing
    - Information to be sent to the instrument
    - Information to be received from the instrument
- Regulatory Concerns – Data Integrity
- Raw Data Management
- Data Approval
- Implications when moving to a Cloud Solution

Developing an ultrasensitive PK assay using SIMOA: step by step to the target

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Single Molecule Array (SIMOA) is a powerful immunoassay technology that enables the detection and quantification of analytes at very low concentrations, supporting the development and discovery of novel therapeutics. Ultrasensitive immunoassays are typically utilized in biomarker research but there is an increasing need for highly sensitive PK assays in quantifying therapeutic drugs as well. These homebrew PK assays do not rely on ready-made kits, and, depending on the analyte of interest, can require extensive optimization during method development.

Here we describe several optimization steps that are typically needed when developing an ultrasensitive homebrew SIMOA PK assay. Such steps include, among others, screening and selecting the most suitable capture and detection reagents, optimizing the bead coupling procedure and testing different assay diluents and assay formats. In addition, we share our experience in fine-tuning the detector reagent biotinylation, titrating critical reagents and determining the most optimal MRD of the assay. Finally, we tackle some of the most typical issues encountered during SIMOA PK assay development.

The successful establishment of a robust and sensitive SIMOA PK assay is a sum of various parameters: high quality capture and detection reagents being among the most critical ones. In addition, careful planning and execution of the other optimization steps can help in reaching the targeted sensitivity and getting the most out of an in-house developed SIMOA PK assay.

A validated Method For the Quantification of Protacs – 3 – Gefitibib in Rat Plasma to Support Rodent PK Studies

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Booth A10

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Proteolysis Targeting Chimeras (Protacs) are a new class of drug molecules which work by mobilizing the ubiquitin–proteasome system to achieve proteasome-mediated degradation of the target protein via the cell machinery. Protacs consist of three main components i) target binding moiety, ii) linker and iii) ubiquitin E3 ligase binding moiety. They can be considered “large small molecule” and as such share many of the attributes such as synthesis scale up, cost of manufacture, shelf life, stability and route of administration. Protacs eliminate all functions of the protein, hence provide differentiated pharmacology and do not require target binding moieties that inhibit protein function. They also significantly increase the number of “drugable” proteins opening up the possibility for new safer medicines. Gefitinib is a tyrosine kinase inhibitor for the treatment of non-small cell lung cancer, it is a potent EGFR inhibitor interrupting cell signalling, it was withdrawn in 2012. To compare the pharmacokinetics of Protacs-3- gefitinib with that of gefitinib we developed a rapid reversed – phase LC-MS/MS based assay for the quantification both compounds in a single 3-min methodology. The samples were prepared by protein precipitation of 10 µL of plasma with 40 µL acetonitrile using gefitinib (d6) as an internal standard (50ng/mL). The limit of detection for Protacs-3-gefitinib was determined to be 20pg/mL with a dynamic range of 20 – 10,000pg/mL. Gefitinib showed a limit of detection of 100pg/mL with a dynamic range of 0.1 – 1,000ng/mL. The assay was subjected to the 3- day validation with a CV ranging from 5 – 10%. Freezethaw studies (n = 4) show no reduction in measure concentration for the batch QCs between sampling occasions. This methodology was applied to the analysis of rat and mouse plasma samples following the IV administration of gefitinib and Protacs-3-gefitinib at 10mg/kg to determine the pharmacokinetics and detect major metabolites.

AUTOMATED WORKFLOW TO STUDY MICROSOMAL CLEARANCE AND ANALYSIS OF METABOLITES USING COLLISION-INDUCED DISSOCIATION AND ELECTRON-ACTIVATED DISSOCIATION MS/MS DATA

Ferran Sánchez

Poster A20

SCIEX

Ferran Sánchez

Studies of *in vitro* metabolism of drugs in human and animal tissues help to predict the metabolic clearance rate of compounds and identify major metabolism pathways.
A software-aided methodology was developed to quantitatively study microsomal clearance and qualitatively identify the soft-spots for metabolism.
Datasets from CID and EAD were applied to predict the sites of metabolism. Various drugs were incubated at 37°C in human hepatocytes and rat liver microsomes at concentrations of 1-5µM. Samples were quenched with acetonitrile at 0-, 30-, 60-, 90-, 120- and 240-minute. Analysis was performed using a data-dependent acquisition method with CID & EAD fragmentation on the ZenoTOF 7600 system. Molecule Profiler software was used for the analysis of microsomal clearance and prediction of biotransformation sites. The DDA data provided excellent MS/MS coverage of TOF MS peaks of interest for both CID and EAD acquisitions. Automatic prediction of metabolites based on MS1 data and structural elucidation using the precursor and metabolite-specific fragment ions were performed. Several phase 1 and phase 2 metabolites were identified and studied. The Molecule Profiler software enabled processing and analysis of both CID and EAD data in a single file. The interpretation of the site of metabolism was enabled by the automated assignment of the structures by the software based on the relative weighting of EAD and CID MS/MS spectra on the scale of 1-100%. A correlation analysis was performed for drugs and metabolites by processing result table files from various time points. Metabolite and fragment identification were performed with less than 5 ppm error.

HPLC-MS/MS based quantification of human monoclonal antibodies targeting SARS-CoV-2 in the presence of endogenous SARS-CoV-2 antibodies in human serum.

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Despite widespread vaccination campaigns against SARS-CoV-2, there is a need for antibodies as a passive immunity option for treatment and prophylaxis, particularly for immunocompromised individuals. To address this need, Aerium Therapeutics is developing fully human, long-acting antibodies targeting the SARS-CoV-2 spike protein. A bioanalytical method to quantify fully human monoclonal antibodies in a population with widely varying anti-spike antibody titers is required to investigate the safety and efficacy of these antibodies in clinical trials. To this end, we have developed an HPLC-MS/MS assay to quantify the monoclonal antibodies (AER001 and AER002) without interference from endogenous anti-spike protein antibodies by targeting signature peptides spanning the monoclonal antibodies' CDR regions. By developing and comparing affinity capture and ammonium sulphate precipitation, we show that both procedures allow accurate and precise quantification of AER001 and AER002 in human serum with comparable sensitivity. We selected ammonium sulphate precipitation due to its simplicity and lower cost and performed full bioanalytical method validation. The assay was also validated for human nasal lining fluid extract with a 50-fold lower limit of quantification compared to serum and was shown to deliver similar sensitivity to previously published antibody affinity capture HPLC-MS/MS assays. Finally, we show that the CDR-derived unique signature peptides are also generated by tryptic digestion of blank serum in some individuals, an important caveat for HPLC-MS/MS strategies targeting human monoclonal antibodies. In summary, the presented results are crucial for the clinical testing of two investigational COVID-19 monoclonals and have broader implications for HPLC-MS/MS assays for human monoclonal antibodies in situations where an excess of endogenous antibodies with overlapping specificities are present.

Structural elucidation of conjugation drug metabolites by utilizing novel electron-activated dissociation (EAD)

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Collision-induced dissociation (CID) is the fragmentation approach widely used in LC-MS/MS qualification and quantitation of drugs and their metabolites. In many drug metabolite identification studies, conjugation metabolites are found to have multiple binding potentials to parent drugs. It is a great challenge to locate the binding sites by CID due to the information lost by highly selective cleavage at these sites. Novel electron-activated dissociation (EAD) developed in a QTOF system produces varied fragmentation patterns and helps to generate additional or different fragments than CID. These fragments can be crucial to locating the metabolic modification sites, especially for conjugations.

A series of drug compounds was incubated with rat liver microsome in the presence of NADPH, UDPGA and GSH at 37°C for 60 minutes. After incubation, the reaction was terminated by adding acetonitrile. The supernatants were obtained by centrifugation, evaporated under vacuum and injected into the LC-MS.

After LC-HRMS full scans on the tested drugs and their metabolites, EAD and CID fragmentations were conducted for MS/MS spectra. The fragmentation patterns were compared, and the efficiency in structure elucidation was evaluated.

Metabolite profiling identified several conjugation metabolites, including glucuronides and GSH adducts by characteristic mass EAD showed the potential to fragment the molecular ions more extensively than CID. Most importantly, the EAD-specific fragments included the ones created by breaking the relatively stable bonds on the parent drug motifs but keeping the relatively weak conjugation bond intact. By this means, EAD significantly assisted identification of the conjugation binding sites.

Novel method for quantifying Anti-AAV Neutralizing Antibodies using AVV-producing Reporter Gene based assay
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The extensive study of AAV vectors as carriers of gene therapy products has advanced the field from a promising new therapeutic concept into today's medical reality. However, several bottlenecks are still present during the development of gene therapy products hence meeting respective regulatory requirements has turned into a challenging process. The use of bioassays facilitates the progression through the different developmental phases as they can be used to address key parameters for regulatory acceptance including vector potency or determination of neutralizing antibodies (NAbs). Here we present a new two-component system for the detection and quantification of NAbs directed against recombinant AAV vectors, based on a reporter gene Platform. This platform can be used for detecting and optimally quantitating anti-AAV NAbs directed against the capsid in a test sample. The availability of reporter cells in a frozen, thaw & use format, obliviates the need for cell culture or specialized equipment and provides a means for obtaining highly reproducible results superior to those obtained using the same cells maintained in culture. Furthermore, the time necessary to run the assay is half of the time required in the current alternatives. These features combined allow the advantages of a cell-based assay to quantify neutralizing anti-AAV antibodies, as recommended by regulatory agencies, to be combined with the versatility and ease of an immune-detection assay for use in AAV-based gene therapy trials.

Automated Extraction and Quantitation of Per and Polyfluoroalkyl Substances (PFAS) in Bioanalytical Matrices Determined using UHPLC-MS/MS
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The aim of the poster is to provide an automatable, robust, high-sensitivity method for the clean-up of 31 PFAS compounds from biological fluids for analysis using UHPLC-MS/MS. Optimised sample preparation techniques using reverse-phase polymeric SPE and Weak Anion Exchange SPE were compared to a novel multifunctional sorbent extraction utilising a solvent crash/filtration-based procedure. Methods were compared for recovery, ion suppression, phospholipid content, calibration curve performance and overall sensitivity. An optimised extraction procedure for the multifunctional sorbent was developed. Parameters such as evaporation efficiency, crash solvents, crash ratios, sample pre-treatment and sample/solvent first protocols were investigated. A final method of a 1:7 sample: solvent crash ratio using unmodified acetonitrile in a solvent first protocol produced the best results. Dilution and injection with an ammonium acetate solution provided consistent recoveries above 80% for the range of PFAS compounds with RSDs typically below 5%. Removal of >99.9% phospholipids was demonstrated. This optimised extraction procedure was transferred to an automated sample preparation platform. The use of an automation system demonstrated comparable calibration curves and LOQs compared to a manual, vacuum method. Calibration coefficients were >0.99 and LOQ's were between 0.1-0.4 ng/mL. The extraction demonstrated a low PFAS residue contribution from the multifunctional sorbent and the automated sample preparation platform, vital for a reproducible extraction. Using a novel multifunctional sorbent extraction combined with automated sample extraction gave clean samples and consistently high recoveries are demonstrated across the range of PFAS compounds when compared to SPE providing a reproducible, robust method ensuring sensitivity at low concentrations.

Increasing capacity and accuracy through automation using liquid handler MicroLab Hamilton Star/Starlet

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The need to deliver drugs into the market quickly for the benefit of the patients is very compelling. To expedite the drug development process, automation can play a great role in providing large analysis in a fast and precise manner. Frontage Laboratories (USA) has utilized liquid handler Hamilton MicroLab Star/Starlet to develop pharmacokinetic (PK) method which was used to obtain data with high accuracy and precision.

Accuracy and precision runs were performed manually or using automation. For automation, MicroLab Star 8+4-channel instrument was used to prepare Standard Curve from drug stock. The curve ranged from 100 to 50,000 ng/mL. The Quality Controls (QCs) were prepared by the same drug stock at the following concentrations: ULOQ 50,000 ng/mL, HQC 37,500 ng/mL, MQC 9,000 ng/mL, LQC 300 ng/mL and LLOQ 100 ng/mL. The Standard Curves, QCs and Samples were transferred into Deepwell plates and diluted to MRD 1:40. Addition of Blocking buffer, Capture buffer, Detection buffer, and Read buffer was done using Hamilton Starlet 96-head instrument. Results: The %CV between different runs and operators are below 7%, and the %RE are below 12%. %CV and %RE are lower than manual run.

Each Hamilton method could be programmed and modified easily for different drug concentrations, curve points, tip sizes, volumes, and tubes. We are one of the first and few CROs that have validated such methods to be used for large scale sample analysis. Frontage is committed in investing more time and effort to get more methods developed for its diverse client base.

Development of innovative bioanalytical methods using LC-MS for the quantification of a fusion protein in tissues to support pharmacokinetic studies

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A fusion protein is currently developed for the treatment of rare disease related to an enzyme deficiency syndrome in pharmaceutical industry. This recombinant protein results from the combination of an antibody-derived structure and the deficient enzyme. To support pharmacology and toxicology studies and to estimate the therapeutic index, assessment of the in vivo stability of this fusion protein and characterization of biodistribution, both in plasma and in several targeted tissues, is essential. However, the quantification of such biotherapeutics in tissue is still challenging because, for instance, of the low in vivo concentrations, the (off)-binding to target, or the inconstant extraction recovery of the multiple forms of the biologics, among others. In this poster, we describe the development and optimization of a sensitive bioanalytical workflow, using a combination of immunocapture (IC) and liquid chromatography-mass spectrometry (LC-MS), for the quantification of a fusion protein in several mouse tissue samples (brain, liver, quadriceps, and heart). Using these methods, we were able to successfully monitor both the total fusion protein and the total Antibody-derived structure forms, with very low limits of quantification (e.g., 0.15 nmol/kg in liver).

Assessment of alternative DNA extraction methods from micro samples of common matrices collected for vector shedding purposes in clinical trials.

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Advancements in patient management and the experience for patients on clinical trials is always being improved. In addition, during the COVID pandemic changes to the way that clinical trial patients have been sampled, were also required with many home testing kits being more readily available with micro samplers being employed. Pediatric clinical trials also need to use smaller sample volumes too.
This means we need to consider the ways in which we may receive clinical patient samples may change in the future and we should prepare in some part to expect to see sample volumes and methods of collection change or vary.
Here we would like to share preliminary data from our investigation of new methods for extracting DNA from micro samples of several different matrices commonly collected in vector shedding assessments.

Optimisation of a fully automated oligonucleotide assay to minimise metabolite interference

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A dual hybridisation assay was developed on a fully automated microfluidic immunoassay platform. The hybridisation reaction was performed in the mixing chamber of the Mixing CD. It was shown that similar performance was achieve to offline incubations which allowed the assay to be fully automated.

One limitation of dual hybridisation assays is the potential to detect metabolites. Although the nuclease cutting assay format was tested, the dual hybridisation assay format gave improved sensitivity.

Metabolite interference was assessed using N-1, N-2 and N-3 metabolites at both the 5' and 3' end of the parent oligonucleotide therapeutic. Using the automated assay format, the only significant interference was demonstrated for the N-1 metabolite at the 5' end. It was however possible to reduce the interference by the addition of a nuclease wash across the affinity column.

This Nuclease Enzyme Wash Dual Oligonucleotide Hybridisation Assay (NEW DHA) assay format was shown to improve the metabolite interference. The flexibility of the microfluidic approach would allow for method modification to improve metabolite dependent on the expected levels and the chemistry of the analyte.

Characterisation of high mass product ions for LCMS insulin assays and some comparative hybrid vs SPE assay data

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Mass spectrometry-based bioassays for large peptides commonly use product ions with a higher mass to charge ratio than the precursor ion to improve method specificity. The high mass product ions used in the insulin assays developed at Quotient for the analytes Glargine, Glargine M1, Glargine M2 and Bovine Insulin were partially characterised. Mass differences between precursor and product ions were calculated using data acquired on a SCIEX 6600+ TripleToF instrument and, where possible, putative structural assignments made. In the case of Glargine and Bovine Insulin this was possible, however, this was not possible for Glargine M1 and Glargine M2. We tentatively suggest that the Glargine product ion is the Glargine precursor ion that has undergone the neutral loss of 9 water molecules. In the case of Bovine Insulin, we tentatively suggest that the precursor ion loses Asparagine from the C-terminal position of the B-chain peptide.

We also present some data from a comparative study designed to compare a hybrid immunoaffinity based extraction method for human insulin levels in volunteers with results derived from a solid phase extraction method. The two methods show good agreement and a positive correlation.

Development of a 2-in-1 Hybrid LC-MS/MS method for quantification of total antibody and total ADC levels in mouse plasma

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Antibody drug conjugates (ADCs) are one of the fastest growing anti-cancer drug types currently in development, combining the specificity of monoclonal antibodies with the cytotoxicity of small molecule payloads. Due to their complex nature, ADCs present unique bioanalytical challenges, such as the need to quantify total antibody, total ADC and free payload levels in every sample.
Here, we present data on the development and use of a method for simultaneous quantification of total antibody and total ADC levels in mouse plasma. The ADC consists of a human monoclonal antibody with MMAE-derived payload, conjugated at a specific site. Quantification of total antibody levels is achieved with a generic human IgG tryptic peptide while quantification of total ADC levels relies on the tryptic peptide conjugated to the linker-payload. Capture was achieved with commercially available biotinylated anti human antibodies and strep magnetic beads. SiluMab was employed as internal standard. An LLOQ of 10.0 ng/mL was achieved for both total antibody and total ADC levels, using only 20µL of mouse plasma. The developed method was subsequently used to support mouse PK studies.

Bioanalysis of Endonuclease Cas9 in Plasma Conducing to Clarify CRISPR-Cas9-Mediated Genome Engineering Using Immunocapture Followed with LC-MS/MS Screener Application

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A new and improved genome-editing method using CRISPR-Cas9 technology can greatly reduce mutations and enable the development of more efficient therapies with reduced incidence of undesired genetic mutations. To clarify the CRISPR-Cas9-mediated genome engineering, the bioanalysis of streptococcus pyogene Cas9 protein in rat and monkey plasma was developed. The detailed methodology and results will be presented in this poster.

Instead of the traditional LBA method, a magnetic bead-based immunocapture followed by liquid-chromatography coupled with mass spectrometry (LC–MS/MS) was used to greatly reduce interferences and achieve a higher sensitivity. Applying a bottom-up approach, the method used immunocapture against the N-terminal while choosing signature peptide one near the C-terminal and peptide two located at the middle of the Cas9 sequence for qualitative analysis and quantitative analysis of Cas9, respectively, to ensure the entire protein was covered.

As a result, a 2,000 times curve range and high sensitivity (LLOQ=5.0 ng/mL) were achieved with optimal reproducibility. We have applied this method to quantify Cas9 in rat and monkey plasma. The toxicokinetics data provided more insights to better understand the mechanisms and metabolism of gene editing in vivo.

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Quantification of short peptides using a stable-isotope-labeled labelling agent

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Stable-isotope labelled (SIL) internal standards are usually prepared for quantitative bioanalysis. However, the application of SIL internal standard is often restricted by the cost and/or time of obtaining this type of internal standards.
6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (13C3) is a stable-isotope-labeled labeling agent and can be used to prepare internal standards from non-labeled analyte peptides. We have developed a reliable method to quantify short peptide and amino acids. This method is very useful when multiple analytes need to be analyzed in a biological matrix. Different cases are presented in this poster.

Endogenous Large Peptide/ Protein Biomarker Quantitation in Human Plasma Using Orbitrap LC-HRMS for Clinical Research

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In the practice of medicine, peptide and protein biomarkers are becoming integral analytical targets during drug development, clinical diagnosis, and disease treatment. LC MS based approaches are increasingly used for the quantification of peptide and protein biomarkers. In addition to well-known issues associated with small molecule endogenous biomarker assay development, the bioanalysis of large peptides and proteins present further bioanalytical challenges that stem from their endogenous nature, and it is challenging to confirm that recombinant derived reference standards truly depict their endogenous counterparts.
Triple quadrupole mass spectrometry (MS) has been successfully used for small peptide quantitation. However, large peptides and proteins are too big for direct MS. As such, proteolytic digestion is used to yield smaller peptides, which serve as surrogates for the biomarker(s) of interest (bottom-up). Recently, LC-HRMS has made important inroads into the quantitation of intact protein. The Orbitrap Exploris 240 LC-HRMS has high-resolution capability (up to 240k) that can fully resolve isotopic clusters of different intact protein charge states (~20 to 30 kDa). By leveraging this high resolution, and by using SIM mode, we can extend the molecular weight range for intact quantitation, without the need for deconvolution, up to ~30 kDa.
In this presentation, the utility of exploring LC-HRMS for the purposes of quantitation of intact protein biomarkers and a comparison to the bottom-up results are provided. We describe practical approaches for implementation of these procedures with regulated endogenous biomarker LC-MS assays. As an example, we present the measurement of endogenous human IgG light chain in human plasm.

A Quick and Robust LC-MS/MS Method for Quantitation of R-/S-Beta-hydroxylbutyrate in Rat Plasma

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Beta-hydroxybutyrate (BHB) is the primary circulating ketone body that serves as an alternative fuel to glucose. It is also a useful biomarker for diabetic acidosis in clinical diagnosis. A sensitive bioanalytical method with capable chiral separation of both R-/S-BHB is required for the routine clinical sample analysis. The application of chemical derivatization would, therefore, effectively enhance the detectability of carboxylic acids in electrospray ionization, while the attachment of a special function group on the target compound could significantly improve its chromatographic behaviour. In this study, a sensitive and robust analytical method capable of quantifying both R-/S-BHB from plasma is reported.

Detection was achieved using a Waters Aquity I Class UPLC coupled with Waters Zevo TQ-s tandem mass spectrometer employing an electrospray ionization (ESI) in the positive ion mode with multiple reaction monitoring. Linear regression calibration curve was established from 0.5 to 100 µg/mL in plasma.

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In our study, a completely new method was developed, in which a simple protein crash instead of LLE/SPE was applied. The unfriendly solvent was replaced with acetonitrile/water, and the amount of reaction reagents was reduced by 5X. The chemical derivatization reaction could occur at room temperature within as short as 30 min. In this way, a high reaction efficiency has been achieved with low interference in LC-MS analysis. A unique chiral HPLC condition with 6.5 min run time has been established, by which the baseline separation of derivative R-/S-BHB was achieved with minimum MS interference. Excellent linearity was obtained with correlation coefficient higher than 0.995