1. **A Versatile Multi-Matrix LC-MS/MS Method for the Quantitation of Short Chain Fatty Acids (SCFAs). Allan Jaochico, Dewakar Sangaraju, Sheerin K Shahidi-Latham, Genentech, Inc.**

Objectives: Short chain fatty acids (SCFAs) are a group of organic carboxylic acids with a linear or branched aliphatic tail of 2 to 6 carbon atoms in length that have an important role in human health and biological homeostasis. A multi-matrix reversed-phase liquid chromatography tandem mass spectrometric method (RP-LC-MS/MS) was developed for the accurate quantitation of SCFAs using stable isotope labeled internal standards in various biological matrices, such as human plasma and urine.
Methods: A high throughput 96-well plate format optimized derivatization and liquid-liquid extraction procedure was developed and used for sample preparation. Mass spectrometric analysis of SCFAs was performed using selected reaction monitoring transitions in positive electrospray ionization mode. As SCFAs are endogenously present, a surrogate matrix approach was used for quantitation, and the method was qualified over a linear calibration range of 25.20-2,500.00 ng/mL.
Results: Intra- and inter-assay precision indicated by percent relative standard deviation (%RSD) was less than 9.21% for low, medium, and high quality control samples (QCs). The accuracy of the method ranged from 86.85 – 115.59% of nominal concentration for within-run and between-run QCs. Stability of SCFAs were established in plasma and urine under bench top (Room Temperature, 5h and freeze thaw (-20±10°C, 3 cycles)) conditions.
Implications: The aforementioned reversed-phase LC-MS/MS method was used to quantify and compare the SCFA levels in human plasma and urine of inflammatory bowel disease (IBD) patients versus control human subjects.

2. **Method Development and Qualification of a Method to Measure Free and Total Colistin from Colistimethate by LC-MS/MS. Shelby R. Anderson1, Bradley King1, Steve Gorman2, Jennifer Vance1, Ray Bakhtiar2, 1 AIT Bioscience, 7840 Innovation Blvd, Indianapolis, IN 47268, USA, 2 Teva Branded Pharmaceutical Products R&D, Inc., Teva Pharmaceuticals, 145 Brandywine Parkway, West Chester, PA 19380, USA**

Objective: A novel bioanalytical LC-MS/MS method was required to measure free colistin A and colistin B in rat plasma as well as the concentrations of colistimethate A and colistimethate B (or their partially hydrolyzed derivatives).
Method: The method was developed over the range 50-50,000 ng/mL of colistin (18.9-18,900 ng/mL colistin A; 31.1-31,100 ng/mL colistin B) in K2EDTA rat plasma using Polymyxin B as internal standard. Total colistin is determined by the complete hydrolysis of colistimethate in a rat plasma sample aliquot with 0.5N sulfuric acid followed by isolation of the resultant colisitin via weak cation exchange (WCX) SPE. WCX SPE retains the polycationic colistin but not the polyanionic colistimethate. Free colistin is determined by subjecting a second sample aliquot directly to WCX SPE to separate colisitimethate from colistin. The resultant SPE eluates are quantitated for colistins A &B by LC-MS/MS.

Result: Extraction recoveries for colistin A, colistin B, and polymyxin B from the WCX SPE plate are approximately 80%. The pH 9 buffer used during extraction effectively eliminates most colistimethate from the samples and free colistin levels do not increase significantly over time in extracted samples.
Implication: A single method has been developed for directly measuring both free and total colistin A and B, and also indirectly measuring colistimethate A and B or their partially hydrolyzed derivatives. The critical SPE extraction uses pH control to fractionate colistimethate from colisitin for LC-MS/MS analysis.

3. Development of PK and Anti-Drug Antibody Assay with High Drug Tolerance for Bevacizumab (Avastin®) and its biosimilars. Neelam Chavan1, Sumit Kar, Curtis Sheldon Rafiqul Islam, Celerion Inc.

Objective: Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody approved globally for treatment of multiple types of cancer. It is a popular target for biosimilar developers as the patents on Avastin® will expire in July 2019 in US and in January 2022 in Europe. In case of bevacizumab, it is particularly challenging due to the fact that a high level of drug is expected in clinical samples. The mean trough concentration range is 127 ± 29 ug/ml (range 77–155), and the mean peak concentration is 149 ± 13 ug/ml (range 113–157).
In addition, endogenous VEGF can interfere with the detection of Bevacizumab and ADA. This high level of drug concentration combined with VEGF interference necessitates the development of highly reliable assays for the biosimilar. Here, we present a PK assay with ELISA platform and an immunogenicity assay that utilizes solid phase extraction with double acid dissociation (SPEAD).
Method: PK assay for total bevacizumab is sandwiched ELISA, wherein VEFG165 was the capture and anti- bevacizumab (HRP) as detection antibody. The Immunogenicity assays method utilized a modified solid phase extraction with acid dissociation (SPEAD) assay with Sulfo-tag detection. Samples containing bevacizumab and ADA were pre-dissociated and neutralized using proprietary buffers before incubation with biotinylated bevacizumab on a streptavidin coated plate. The ADA was then dissociated, neutralized, and coated onto a MSD plate and detected using sulfo-tag labeled bevacizumab.
Results: The assays were developed for use in human serum with inter-assay precision and accuracy to be < 10%. The sensitivity was 50 ng/mL and 20 ng/mL in PK and ADA assay respectively. The linearity of the bevacizumab assay was established up to 250 ug/mL, also the stability and interference parameters met the validation criteria as per regulatory guidelines. The typical single dissociation SPEAD assay format only achieved a drug tolerance of 10 ug/mL. In modified SPEAD assay, 100 ng/mL of ADA was detectable in the presence of 100 ug/mL of bevacizumab.
Implications: Thus, highly sensitive and tolerant assays to the presence of drug and VEGF, and reproducible method to detect bevacizumab and ADA were developed to support the development of biosimilars.

4. **Development and Qualification of a Sensitive and High Throughput Cell-Based Neutralizing Antibody Assay for Bevacizumab. James Hnilo, Curtis Sheldon, Rafiqul Islam, Celerion Inc.**

Objectives: Bevacizumab (trade name Avastin) is a humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A. Protein-based therapeutics like Bevacizumab have the potential to induce immune responses in the body. One adverse immune response is the generation of neutralizing antibodies (NAbs) that can bind to the drug and reduce efficacy and potentially safety of the therapeutic. Cell-based assays have emerged as the industry standard in detection of NAbs because they mimic the method by which these antibodies exert their effect in a living biological system. The US FDA and the European Medicines Agency (EMA) have recognized the importance of these cell-based NAb assays and have recommended them whenever possible. We have successfully qualified a cell-based Bevacizimab NAb detection assay based on Promega's KDR-NFAT assay technology. The assay is fast, high-throughput, sensitive, and tolerates high levels of human serum for neutralizing antibody detection in human serum samples.
Methods: The reporter gene assay is based on a HEK293 cell line stably expressing vascular endothelial growth factor receptor 2 (VEGFR-2 or KDR) and the luciferase reporter gene under regulation of the nuclear factor of activated T cells (NFAT) response element. Bevacizumab is pre-incubated with serum containing either a control NAb or unknown serum sample for 1 hour, followed by addition of VEGF165 and another pre-incubation of 30 minutes. This pre-incubation is added to KDR-NFAT cells in microtiter plates, incubated for 6 hours at 37oC/5%CO2, and luciferase activity is then measured.
Results: The KDR-NFAT assay is robust with a dynamic range for VEGF165 response of 1 – 100 ng/mL in the presence of 33.3% pooled human serum (30 lots) and an EC50 of the response of 7.8ng/mL. At a VEGF165 concentration of 30ng/mL (EC90), Bevacizumab dose response assays yield an IC50 of 200ng/mL with a dynamic range of 30ng/mL – 2ug/mL. An anti-Bevacizumab neutralizing antibody was tested in a human serum background of 33.3% and demonstrated an EC50 of 276ng/mL. Additionally, individual lots of serum were tested to determine NAb assay cut point, matrix interference of the positive control and drug tolerance limit for Bevacizumab in the NAb assay.
Implications: This qualified high-throughput cell-based assay is an important tool for evaluating human serum for the presence of neutralizing antibodies to Bevacizumab.

5. **Approaches to Improve Sensitivity and Drug Tolerance by Reducing Target Interference during Development of an Anti-drug Antibody Assay. Amanda J. Daugherty, Sarah K. Peters, Curtis E. Sheldon, Celerion Inc. Christina U. Lorentz, Aronora Inc.**

Objectives: An anti-drug antibody (ADA) assay was developed for a novel drug whose target is prevalent in normal human plasma. The soluble drug target interferes with the ADA assay by

blocking binding sites on a drug-coated microplate. Method sensitivity and drug tolerance are primary issues for developing ADA methods for this type of molecule.
Methods: Many approaches were evaluated to avoid the inference and eventually the assay was developed to include sample processing that uses anti-target antibodies to deplete the soluble target prior to sample analysis. Many efforts were made to improve drug tolerance and increase sensitivity of the assay including; using target depletion to remove excess drug from the sample, transferring the method to the MSD platform, and adding excess target to complex and remove drug prior to target depletion.
Results: For this assay, soluble target removal allowed for evaluation of ADAs present in the samples and sensitivity was verified to be as low as possible using samples in target-free plasma.
Implications: A variety of methods can be tested to reduce target interference and improve drug tolerance in other assays with soluble drug targets. The samples in target-free plasma show better sensitivity than normal human plasma verifying that the ADA assay was sensitive with the method selected.

6. Overcoming Challenges in the Development & Validation of an LC-MS/MS Assay for the Quantitation of PF-06755347 (GL-2045) in Human Plasma.

James P. Saunders, Matthew Blatnik, Eugene P. Kadar, Jeffrey Kurz, Denise M. O'Hara, Rick C. Steenwyk, and Christopher L. Holliman, Pfizer, Inc.

GL-2045 is a recombinant biologic of covalently linked, ordered multimers of human IgG1 Fc, that has been granted orphan drug designation to treat chronic inflammatory demyelinating polyneuropathy (CIDP), a rare neurological disorder.

Objective: Due to the nature of GL-2045 and that of the plasma, a specific and sensitive IgG purification reductive-alkylation proteolytic digestion LC-MS/MS quantitation method was developed for determining GL-2045 pharmacokinetics in human plasma. Enzyme linked immunosorbent assays (ELISA) were not appropriate because GL-2045 is an IgG and endogenous IgG's interfere with GL-2045 ELISA selectivity.
Methods: A novel IgG purification utilizing Melon™ Gel was successfully implemented to purify GL-2045 from plasma. The resulting eluant was reduced with dithiothreitol, alkylated with iodoacetamide and digested with chymotrypsin. The cysteine-alkylated S225-peptide (SLSPGKERKCCVECPPC) from the IgG2 hinge region was identified as a selective and specific amino acid sequence to GL-2045 for LC-MS/MS analysis.
Results: Melon Gel binds non-antibody serum proteins such as albumin, and allows IgG's to elute from the gel preparation. Incorporating the use of Melon Gel, instead of IgG based immunocapture, allowed for GL-2045 recovery without the competition of endogenous IgG's. The LC-MS/MS plasma assay was successfully validated with a lower limit of quantitation (LLOQ) of 25.0 ng/mL (+/- 30% CV) and an upper limit of quantitation (ULOQ) of 10,000 ng/mL (+/- 25% CV).
Implications: This LC-MS/MS assay provides a sensitive and selective method for the quantitation of GL-2045 in human plasma, which can enable understanding of the pharmacokinetics of GL-2045 following administration in future clinical studies.

7. **Development of a Robust Neutralizing Antibody (NAb) Assay with High Drug and Target Tolerance to Support Clinical Development of an anti-TFPI Therapeutic Monoclonal Antibody (mAb). Yuhong Xiang, Chuenlei Parng, Jean Donley, Elena Seletskaia, Darshana Jani, Teresa Caiazzo and Boris Gorovits, Pfizer Inc.**

Objective: Our drug is a fully human monoclonal antibody against Tissue Factor Pathway Inhibitor (TFPI), a natural inhibitor of extrinsic coagulation pathway. Blocking of TFPI function can increase coagulation activity in hemophilia patients. Endogenous TFPI exhibits different isoforms and can bind to lipoproteins and proteoglycans. Circulating target and residual drug generate significant concerns when developing robust NAb assay. MSD competitive ligand binding NAb assay with high target and drug tolerance was requested to support ongoing clinical studies.
Methods: Double acid affinity capture elution approach was used to mitigate drug interference and a robust target removal strategy was employed to enhance target tolerance. Assay sensitivity, drug tolerance, target interference, selectivity and precision were evaluated.
Results: The validated NAb assay sensitivity was 313 ng/mL based on PC performance (mouse anti-drug monoclonal antibody). The drug tolerance and target tolerance were established at 50 µg/mL and at least 1200 ng/mL of TFPI, respectively, with a positive control at the 1µg/mL level. The screening cutpoint factor is 0.78. Other assay performance characteristics, including precision and selectivity, were evaluated with acceptable results.
Implications: This validated method demonstrated a superior drug and target tolerance to warrant specific and precise characterization of the ADA responses in support of ongoing clinical studies.

8. **Qualification of an Ultrasensitive Non-Clinical Pharmacokinetic Assay for a Biologic in Presence of Interfering Target Using the Quanterix Simoa™ HD-1 Platform. Patrick Breslin, Rajitha Doddareddy, Tong-yuan Yang, Biologics Development Sciences, Janssen BioTherapeutics, Janssen Research & Development, LLC.**

Objective: To develop and validate an ultrasensitive PK assay capable of measuring analyte in the femtogram range of a peptide therapeutic.
Method: The Quanterix Simoa™ HD-1 technology was implemented in developing a PK assay in dog serum for a biologic peptide. LC/MS and Western Blotting were used to identify an interfering agent in the assay. Thermal heating was used to denature target so that the therapeutic could be measured.
Results: A PK assay was developed to measure analyte in non-clinical dog serum study samples. Preliminary development experiments demonstrated a matrix dependent interference causing low recovery of analyte. LC/MS was implemented to confirm the interference was caused by varying levels of therapeutic target receptor in individuals. Western Blot analysis confirmed this result. Thermal stability of the therapeutic was leveraged by heating diluted serum samples at a

specific temperature to denature target receptor while not destroying integrity of the therapeutic. The range of quantification of the assay was 0.06 to 15.36pg with an MRD of 1:4 (maximum dilution 1:4000). Selectivity passed at 240fg levels in 9/10 individuals confirming this result.
Implications: Descriptions of methods for using Simoa™ technology for PK analysis in a non-clinical regulated setting are rare. This method also describes using LC/MS and Western Blotting technology to isolate matrix interference issues common to PK method development and an innovative solution to measure PK in the presence of that interference.

9. **A Rapid Response to Regulatory Questions Following a Biosimilar Submission including Assay Development, Validation and Sample Analysis Strategies to Reproduce Originator Submission Data. Gwen V Eak, Pharmaceutical Product Development, Bruce C Stouffer, Pharmaceutical Product Development**

Objectives: The intent of the project was to develop an assay and reanalyze selected samples from a biosimilar protocol to demonstrate similarity to results generated during analysis to support the originator submissions.
Methods: The difference in the results from the biosimilar study was thought to be a result of the assay platform used for testing. The initial GyroLab XP assay utilized a dual capture process so the second assay platform validated was an ECL assay with a master mix incubation. After sample results were generated with the ECL assay and reviewed it was determined that the results were still not similar to the originator submission data. With a very small amount of time remaining before the response to the regulatory questions were due a classic sandwich ELISA assay was developed. A subset of the study samples were tested to determine if the results resembled the originator data. The results were promising and after optimization the same subset of study samples were reanalyzed using the ELISA platform.
Results: The optimized ELISA assay resulted in sample data from the biosimilar study that closely resembled the data generated in the originator submissions. The data was generated and reported in time to meet the agency's deadline.
Implications: Since regulatory authorities are comparing biosimilar study data to originator submissions there will be a demand for rapid responses to any differences in data observed by the agencies. The demand for the additional work to support biosimilar submissions will increase as t Solution Based Homogeneous Immunoassays for Characterization and Screening of Antibody-FcRn Interactions
Nidhi Nath, Becky Godat, Rod Flemming, and Marjeta Urh
Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711
E-mail: nidhi.nath@promega.com

10. **Solution Based Homogeneous Immunoassays for Characterization and Screening of Antibody-FcRn Interactions, Nidhi Nath, Becky Godat, Rod Flemming, and Marjeta Urh, Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711**

Objective: Here we report a novel solution-based homogeneous immunoassay to measure interaction between human FcRn and antibodies. Assay is based on NanoBiT, which is a small and extremely bright bioluminescent protein complementation reporter. NanoBiT subunits (i.e., 1.3 kDa peptide (SmBiT) and 18 kDa polypeptide (LgBiT)) can be fused or chemically conjugated to a variety of interacting proteins and antibodies to interrogate their binding characteristics in solution

Method: A competitive immunoassay format is used to derive binding affinities of antibodies to FcRn using NanoBiT. Human IgG labeled with LgBiT (Tracer) is incubated with human FcRn labeled with SmBiT to enable complementation between SmBiT and LgBiT resulting in bright luminescent signal. The addition of unlabeled antibodies (samples) result in competition with tracer for binding to FcRn and subsequent decrease in signal. Apparent affinity constant of antibody for FcRn is determined from IC50 values using Cheng-Prusoff equation.

Results: A variety of human antibodies of various isotypes was tested and IC50s were obtained. Assay is specific since no binding is seen with human F(ab)2 and mouse antibodies. Methionine oxidation of antibodies is known to decrease the binding affinity between antibody and FcRn and in fact, we saw a 10-fold decrease in affinity with the FcRn assay.

Implications: A quick (30min), simple, add and read assay for measuring FcRn antibody binding will allow screening of large libraries of antibodies to tune the pharmacokinetic properties of therapeutic antibodies. Moreover, this assay may minimize the artifacts resulting from immobilization as has been widely reported.