Tuesday April 9 Posters

Poster T01: "Validation of a Dried Blood Spot Boanalytical Method for Perampanel Analysis in Pediatric Studies**"**
Poster Presenter: Dr. Luca Matassa *(Eisai, Woodcliff Lake, NJ, USA)*

Introduction: Perampanel is a first-in-class, orally administered, highly selective non-competitive AMPA-type glutamate receptor antagonist, developed by EISAI for epilepsy. A DBS-LC-MS/MS method has been developed and validated in order to analyse perampanel in heparinised blood samples from paediatric studies.
Dried blood spots (DBS) have been shown to be a useful means of collecting, storing and shipping blood samples for quantitative drug analysis which provides advantages over conventional plasma collection. Moreover, due to low sample volume that DBS uses, it is a method of choice when it is comes to paediatric studies..
Methods: A 20 uL dried blood spot on FTA DMPK A card is punched (6mm punch) and the subsample is solubilized by shaking in 150 uL of 90/10 methanol water containing internal standard (IS) stable label peramapanel. An aliquot of the solution is diluted with an equal volume of 50/50 methanol/water, centrifuged at 4C for 5 min prior to injecting 10uL on a reverse phase column (Chromolith RP18e 100x3mm) at 40C under gradient conditions. The detector was a Sciex API5500 Qtrap operated in positive ion ionspray mode. Quantitation was achieved monitoring precursor/product ions for analyte and IS (350/219 m/z perampanel; 356/219 IS) at retention time 2.5 minutes using 1/x2 linear weighted regression.
Result: A full validation according to FDA and EMA guidance was conducted in human blood. Assay linearity was demonstrated over 7 validation runs with R-squared greater than 0.995. The intra-run accuracy and precision was between 95.2 -107.6% and 3.1-12.6%, respectively, at four concentration levels (LLQ, low QC, mid QC, high QC) demonstrating the repeatability of the analytical method from 1 to 500 ng/mL. The matrix factor in 6 lots of control blood was 1.0 for analyte and IS. Control blank matrix showed no interference at the LLQ. Punch tool carryover and autoinjector carryover were not found to impact assay performance. Analyte and IS recovery was 80% across all 3 QC levels with imprecision less than 5%. A 2-fold dilution factor was validated. The specificity of the method for perampanel at the LLOQ in presence of 10 other commonly used AEDs, individually or pooled all together, (valproic acid, phenobarbital, lamotrigine, topiramate, oxcarbazepine, carbamazepine, levetiracetam, zonisamide, phenytoin, primidone) was demonstrated. A blood/plasma ratio of 0.88 was determined, allowing the correlation between blood and previous study plasma results. Short term autosample perampanel extract stability and perampanel stability in blood was demonstrated. Perampanel long term stability on DBS was demonstrated for 363 days at room temperature.
Novel Aspect / Conclusion: The fully validated DBS-LC-MS/MS method was successfully applied to analysis of paediatric study clinical samples.

Poster T02: "Unexpected Results for Sample Collection and Handling Stability Assessment for Sumatriptan in Human Plasma**"**
Poster Presenter: Ginny James *(Celerion, Lincoln, Nebraska, USA)*

Introduction: Determination of sample collection and handling stability (SCHS) is a requirement for validation of bioanalytical methods. SCHS of sumatriptan for 120 minutes did not meet pre-defined acceptance criteria. As sumatriptan was stable in plasma for 23 hours at ambient temperature, it was hypothesized that partitioning of sumatriptan between plasma and red blood cells was not immediate and was impacting the results of the early time points.
Methods: Whole blood was fortified with sumatriptan at 0.150 and 100 ng/mL for target plasma concentrations of approximately 0.300 and 200 ng/mL. The samples were incubated in an ice-water bath, at ambient temperature, and at 37°C for multiple time points between 0 and 120 minutes. At each time point, samples were centrifuged, and the plasma layer was immediately frozen at -20°C. For samples in an ice-water bath for 30 minutes, the red cell fraction was also stored at -20°C for testing. The collected plasma samples were analyzed using a validated method for the quantitation of sumatriptan in human plasma. Red blood cells were analyzed with the same chromatographic and instrument conditions after a protein crash of the cellular material.

Result: The initial comparison for 0 to 120 minute samples held at ambient temperature failed acceptance criteria (approximately 65% of control). Additional comparisons of multiple time points between 0 and 60 minutes at ambient temperature showed a steady concentration decline to approximately 60% of control occurred between 0 and 30 minutes. There was negligible difference (less than 8%) between concentrations of the 30 and 60 minute samples. Comparisons of multiple time points between 0 and 60 minutes in an ice-water bath demonstrated the same pattern, but at a slower rate. Summation of the concentrations discovered in the plasma and red blood cell fractions at the 30 minute time point in an ice-water bath were equivalent to the amount of sumatriptan spiked into the whole blood. Samples at multiple time points between 0 and 60 minutes at 37°C showed a steep drop in concentration between 0 and 10 minutes to approximately 70% of control with no further significant reduction between 10 and 60 minutes.
Novel Aspect: The temperature dependence, concentration plateaus, and accuracy of the total amount of sumatriptan in the plasma and red blood cell fractions all supported the original hypothesis. Disequilibrium in the partitioning between the plasma and red blood cell fractions, an artifact caused by the preparation of fortified samples for testing, should be considered as a potential cause of apparent analyte instability in whole blood.

Poster T03: "Investigating Matrix Interference in Bioanalysis of Antiarrhythmic Cardiac Drugs**"**
Poster Presenter: Dr. David Bell *(Supelco/Sigma-Aldrich, Bellefonte, PA, USA)*

Introduction: For efficient therapeutic drug monitoring, it is important for clinicians to have access to fast and robust methods for accurate assessment of drug efficacy. Industrial trends toward highly specific LC/MS applications over traditional ELISA type immunoassay has resulted in the need for high-speed chromatographic assays along with simplified sample preparation. The goal for this study was to develop a robust bioanalytical method utilizing a combination of fast chromatographic separation along with selective sample preparation.
Methods: The first portion of the study focused on chromatographic conditions for resolution for several cardiac drugs. The basic characteristics of the antiarrhythmic agents make them candidates for HILIC chromatographic separation. The second portion of this study was to evaluate the effectiveness of sample preparation and the impact of sample matrix on the assay. Using protein precipitation technique overlap of endogenous phospholipids with the target analytes resulted in significant ion suppression. Plasma samples were then processed using a HybridSPE-Phospholipid 96 well plate which showed depletion of the phospholipid matrix with no additional sample processing.
Result: HILIC separation allowed for direct analysis of precipitated plasma samples without the need for additional sample solvent exchange and the high acetonitrile content mobile phases also offered an additional benefit in increased analyte response using ESI+ MS detection. The targeted phospholipid selectivity of the HybridSPE-Phospholipid technique enabled simplified sample processing with no detectable phospholipid matrix interference, while exhibiting excellent analyte recovery from plasma samples. This application demonstrates how combining selectivity in both sample preparation and chromatographic separation allows for a simplified and efficient bioanalytical method resulting in a high precision and accurate assay.
Novel Aspect: Combined application of HILIC chromatography and phospholipid depletion to bioanalytical methodology.

Poster T04: "Chromatographic selectivity of some therapeutic peptides and lipids synonymous with LC-MS matrix effect on a silica hydride-based phase**"**
Poster Presenter: Marc Moussallie *(Huntingdon Life Sciences, Somerset, NJ, USA)*

Introduction: An examination of the chromatographic selectivity offered by a Type C™ silica (silica hydride surface) stationary phase, known to exhibit aqueous normal phase (ANP) chromatography as well as reversed-phase (RP), with particular regard to human plasma-abundant lipophilic interferences which are linked to matrix effect. Three representative peptide therapeutics were also included in the chromatography, appropriate to the increasing abundance and importance of such methodologies. An RP type B silica-based column was used for critical comparative purposes.
Methods: Test solutions were injected with isocratic LC conditions across a range of % acetonitrile to calculate retention factors, giving an indication of resolving power, and to monitor chromatographic

peak integrity, paying particular regard to over-retention (or otherwise) of lipids as this has direct implications for signal drift. These tests were performed at both high pH (9.0) and low pH (3.0). The silica hydride-based column was the Cogent Diamond Hydride phase, 50 x 2 mm and 4 µm, from Microsolv Technology Corporation, and the type B silica-based column was GL Sciences' Inertsil ODS-3 of equivalent dimensions. Front end instrumentation consisted of a Waters Acquity UPLC system, and an AB Sciex API 4000 with Turbo Ionspray gas phase ion production provided the tandem mass spectrometric detection.
Result: The propensity for aqueous normal phase (ANP) retention on the silica hydride-based phase was strong and extensive in comparison with the silica-based comparator, and the lipophilic interferences in question were readily eluted using the ANP mode, a contrast to over-retention issues with accompanying implications for method ruggedness typically found with silica-based phases.
Novel Aspect / Conclusion: The first direct investigation of the selectivity offered by Type C™ silica for lipophilic interferences endogenous to human plasma that have important implications for matrix effect in LC-MS/MS. These interferent compounds were observed to be readily eluted early and resolved from suitably-chromatographed peptidic analytes using bimodal retention (ANP & RP) on the silica hydride based Diamond Hydride phase, in stark contrast to pronounced interferent over-retention and inevitable build-up on the comparator type B reversed-phase column. The important region of ANP retention was far more extensive on the silica hydride-based column compared to the NP/HILIC region of the silica-based column.

Poster T05: "Streamline the process and improve the data quality**"**
Poster Presenter: Dr. Hong Gao *(Vertex Pharmaceuticals, Cambridge, MA, USA)*

Introduction: Discovery in vivo study sample bioanalysis desires to both fast turnaround and high data quality to allow quick decision making on compound selection. Neither of these two aspects should be sacrificed for the other. To achieve both of increasing Lab efficiency and improving data quality at the same time, we have streamlined the bioanalysis process using custom designed tools and methods. In consequence, the Lab consumable cost has also been reduced significantly and the FTE time were optimized.
Methods: 1. Custom- designed shorter cluster polypropylene tubes were used to omit the step of extracts transferring during sample preparation of plasma, dry blood spot (DBS) and tissue homogenate samples.
2. Custom-designed DBS card holder and automated DBS sample preparation system were developed to accommodate the DBS card sample analysis.
3. Custom-designed superior high speed centrifugation device is applied on animal tissue sample preparation and analysis.
Result: 1. The bioanalysis was greatly benefited from the sample preparation without extracts transferring, especially for DBS card extraction.
2. A fast turnaround DBS screen PK study platform has been developed to accommodate early compounds selection. Non-specific binding and compound instable issue has been minimized through dry blood spot card sampling and analysis.
3. Tissue sample bioanalysis was accelerated and cleaner extracts were achieved by applying ultra high speed centrifugation.
Novel Aspect / Conclusion: Custom-designed devices speed up the bioanalysis, increased Lab efficiency and improved data quality.

Poster T06: "Detection of Fentanyl Analogs and Metabolites in Human Plasma Via Automated Solid Phase Extraction and Liquid Chromatography Tandem Mass Spectrometry**"**
Poster Presenter: Dr. Courtney Callahan *(Centers for Disease Control and Prevention, Atlanta, GA, USA)*

Introduction: Fentanyl is an opioid analgesic, similar to morphine in action, often used by physicians for surgical analgesia in patients. Unfortunately, there are also several analogs of fentanyl that are illegal, e.g. China white, and are used non-medically. Due to the potential to be abused or misused, a method for rapid and sensitive screening of human blood for legal and illegal fentanyl analogs and metabolites simultaneously is necessary to indicate exposure.

Methods: Human plasma samples were pretreated with acetic acid to denature proteins prior to automated sample preparation using a 96-well SPE plate. This high-throughput approach minimized human error and increased efficiency through the simultaneous extraction of numerous samples. Nine fentanyl analogs and three metabolites were measured in human plasma using reverse phase liquid chromatography tandem mass spectrometry coupled with isotopically labeled internal standards.
Result: The limit of detection was 0.1 ng/ml, and 0.5 ng/ml could be quantified with acceptable precision. Furthermore, fentanyl analogs and metabolites could be detected in only 250 ÂµL of human plasma.
Novel Aspect / Conclusion: Therefore, this method is a valuable analytical tool for investigating medically prescribed fentanyl at low doses as well as differentiating from illegal fentanyl analogs.

Poster T07: "The Analysis of Human Parathyroid hormone 1-34 (Teriparatide) by LC-MS/MS: Challenges and Lessons Learned**"**
Poster Presenter: Erin Chambers *(Waters, Milford, MA, USA)*

Introduction: Teriparatide, a treatment for osteoporosis, is a recombinant form of a human parathyroid hormone fragment. Traditional assays for Teriparatide involve time-consuming, laborious immunoassays and/or multidimensional or nano-flow LC. Immunoassays can suffer from significant cross-reactivity and lack of standardization. Teriparatide itself suffers from non-specific binding and poor solubility. Teriparatide is rapidly absorbed and eliminated, with a half-life of 1hr. At the clinical dose (20 Âµg) plasma levels are ~50pg/mL, making detection by LC-MS/MS even more difficult. This work represents a single, simple method for quantifying Teriparatide in human plasma which uses analytical scale sub-2Âµm LC and fast, selective sample prep in 96-well format to achieve an LOD of 15 pg/mL and a dynamic range of 15-500 pg/mL in human plasma.
Methods: Teriparatide and the IS (PTH 1-38) were spiked into human plasma. 200 ÂµL was precipitated with basified ACN and the supernatant extracted using polymeric reversed-phase SPE sorbent in 96-well plate ÂµElution format. Teriparatide was eluted in 50 ÂµL 60% ACN/5% TFE/1% TFA. Absolute recovery after PPT and SPE was 90% (+/- 2.5%), from human plasma. Extracted samples were directly injected onto a 2.1 x 50 mm sub-2 Âµm charged surface hybrid C18 column on a low dispersion LC system and eluted using a linear gradient from 15-50% B over 3.6 minutes at 0.4 mL/min. Mobile phase A was 0.1% HCOOH in water and mobile phase B was 0.1% HCOOH in acetonitrile.
Detection was performed with triple quadrupole MS in ESI+ mode.
Result: Detection limits for the SPE-LC/MS method for human parathyroid hormone 1-34 (Teriparatide) in human plasma are similar to traditional immunoassays. The pH and ratio of organic used during the PPT pretreatment eliminated protein binding and high abundance proteins, improving specificity whilst yielding Teriparatide recovery of 100%. During SPE extraction, composition of the wash and elution steps were carefully modified to eliminate non-specific binding, adsorption to the SPE plate, and to maximize solubility and thus recovery for Teriparatide. The SPE format used concentrates the sample without evaporation, eliminating adsorptive losses typical of large sticky peptides during evaporation.
For large peptides such as Teriparatide, it is often difficult to produce selective MS fragments without yielding very small, non-specific fragments. The triple quadrupole used in these experiments produced fragments ranging from m/z 700-1000. These fragments provided a distinct selectivity advantage (significantly reducing endogenous background) relative to use of lower m/z, and often initially more intense, immonium ion fragments.
A novel C18 chromatographic column with a charged surface stationary phase produced peak widths for Teriparatide that were 3X narrower than traditional C18 columns using formic acid mobile phases. The charged surface of this column reduces secondary interactions and mimics the peak shape benefit for peptides historically achieved through the use of TFA in reversed-phase systems. Standard curves were prepared in human plasma from 15 pg/mL to 500 pg/mL. The CV of all points on all standard curves was 4% and average accuracy was 101%.
The detection limit for extracted human plasma samples was <15 pg/mL. In addition, QC samples, prepared at 5 concentrations (from 20- 250 pg/mL) in 6 independent sources of human plasma, yielded mean accuracies for all levels from 90-106%, easily meeting FDA criteria. The CV of the matrix factors across 6 plasma lots was 5.65%.

Novel Aspect / Conclusion: Higher m/z MS fragments, multi-step 96-well extraction and charged-surface LC columns for ultra-sensitive quantification of Teriparatide (rhPTH) in human plasma.

Poster T08: "Phase-I 'Proof of Principle': Immunization With Virosome-Gp41-Derived Antigen Induces Mucosal Antibodies With Antiviral Properties To Reduce Risk Of HIV-1 Infection**"**
Poster Presenter: Dr. Jan Detmers *(Chimera Biotec GmbH, Dortmund, Germany)*

Introduction: Mymetics has developed a proprietary virosome technology and judicious antigen design, aiming to provide a first line of defense through mucosal protection, as well as a second line of defense against infection by generation of blood antibodies.
We previously demonstrated that mucosal IgA/IgG induced by vaccination with influenza virosomes encompassing HXB2 rgp41 and modified P1, protect non-human primates (NHP) against vaginal heterologous SHIV challenges, in the absence of seric neutralizing antibodies. Correlation was observed between induction of HIV-1 transcytosis-blocking and ADCC activities from cervicovaginal antibodies and protection. We have now investigated if mucosal antibodies with similar antiviral properties can be induced in women during a Phase-I trial.
Methods: This is a double-blind, placebo-controlled Phase-I study, involving 24 healthy women randomized in 2 groups to monitor safety, tolerability and immunogenicity of the vaccine MYM-V101 (virosomes-lipopeptides P1): group 1: 10 Î¼g/dose and group 2: 50 Î¼g/dose. In each group, 8 subjects received the vaccine and 4 subjects received the placebo (virosomes without HIV-1 antigens) through intra-muscular (weeks 0/8) and intra-nasal (weeks 16/24) administrations. Ultra-sensitive ImperacerÂ® methods have been developed and validated prior to supporting the pre-clinical and phase-I clinical trial.
Result: The vaccine MYM-V101 was considered safe and well-tolerated by both groups, when administered intramuscularly and intranasally. Within two months, all vaccinees developed lipopeptide-specific serum antibodies in the high dose group. Using the ImperacerÂ® technology, lipopeptide P1-specific mucosal IgG and IgA antibodies were detected in the majority of vaginal and rectal secretion samples tested. Vaginal antibodies against the lipopeptide P1 exhibited strong inhibition of HIV-1 transcytosis. Vaginal antibodies of the placebo recipientsâ€™ showed no or weak inhibition.
Novel Aspect / Conclusion: This study shows that virosome-gp41 is a safe vaccine and induces promising mucosal anti-HIV-1 responses.

Poster T09: "Determination of Imatinib in Cynomolgus monkey plasma and CSF Using an AB Sciex 6500 QTrap: Extending Linear Dynamic Range**"**
Poster Presenter: Dr. Anahita Keyhani *(Charles River Laboratories, Senneville, QC, Canada)*

Introduction: Imatinib is an oral anticancer-targeted drug functioning as a tyrosine kinase inhibitor (TKI). Since most TKI's are characterized by inter-individual variability pharmacokinetics, there are renewed efforts being made for treatment optimization in targeting adequate drug exposure. In the current pre-clinical investigation involving tolerability and toxicity of three new formulations of imatinib in Cynomolgus monkey, it was necessary to develop an LC-MS/MS method capable of supporting a concentration range spanning four orders of magnitude (1.0 - 10 000 ng/mL). Previously, such a dynamic range would challenge the saturation limit of a pulse-counting detector, resulting in problematic curve fitting. Therefore, concentration ranges are often truncated, with repeats a necessity for samples whose response exceeds the upper limit of quantitation. However, with the recent introduction of the Ion Drive High Energy Detector in the Sciex 6500 QTrap, ultra-fast pulse counting (108 cps) with a higher saturation point is feasible, without loss in low-end sensitivity. We therefore evaluated the applicability of this new detector for establishing a quantifiable linear dynamic range for imatinib.
Methods: Samples of Cynomolgus monkey plasma and CSF (50 uL) were fortified with imatinib-d3 and diluted with 4% phosphoric acid. Extraction involved a strong cation exchange mechanism using a Waters OASIS MCX sorbent with elution in ammoniated methanol. Extracts were diluted in mobile phase and chromatographed by UHPLC on a Shimadzu Nexera LC-30AD using a Waters Acquity CSH C18 stationary phase (1.7 um; 2.1 x 100 mm) operated under gradient conditions. Parent ions were formed by positive electrospray with MRM transitions m/z 494 > 294 (imatinib) and m/z 497 >

394 (imatinib-d3) monitored at 35 eV using an AB Sciex 6500 QTrap. A Shimadzu Sil-30AC autosampler equipped with Pt-coated needle and peristaltic pump was configured for external, internal, and injection port rinses using a combination of optimal rinse solvents.
Result: Given the high nitrogenous content of imatinib, chromatography on a number of C18 reverse phase columns was challenged by significant peak tailing in the absence of ion pairing agents. While the use of TFA or TEA/AcOH reduced tailing, significant ionization suppression was noted. Therefore, a Waters charged surface hybrid (CSH) stationary phase designed for separating basic compounds without the requirement for ion pairing agents was used with optimal mobile phase components 0.1% HCO2H (A) and MeOH/0.1% HCO2H (B) in a ballistic gradient 30 â€“ 95% B / 30 sec at 0.7 mL/min (45°C). Imatinib and imatinib-d3 co-eluted at tr 1.2 min. Under these conditions, tailing was minimized to a baseline width of 3 sec.
Using a strong cation exchange mechanism for extraction with 2% HCO2H (aq) and MeOH wash stages furnished an average recovery of 85% from both CSF and plasma, without matrix effect. Sample pre-treatment with 4% phosphoric acid was required to disrupt protein binding while at the same time protonate the methylated piperazinyl nitrogen required for SPE trapping. The final extract dilution factor was determined by the accuracy and precision of the QC-LLOQ response from six different control donors, in addition to the saturation level of the ULOQ. A 40-fold dilution factor proved optimal, furnishing an average LLOQ signal-to-noise ratio of 50:1 corresponding to 125 fg on-column; accuracy was 97.8% with 3.3 % CV (CSF), and 100 % with 5.98% (plasma). The ULOQ of 10 000 ng/mL furnished a height response of 7.5e7 cps without detector saturation. Calibration curves were linear and weighted 1/x2 with correlation coefficients > 0.9990. In a pre-validation feasibility assessment, all acceptance criteria were met for selectivity, accuracy, and precision from six different control donors.
Autosampler carry-over is a potential challenge for assays encompassing four-to-five orders of quantitation. In addition to careful titration of the extract dilution factor in order to minimize the on-column injection amount of imatinib, it was necessary to devise a multi-stage autosampler rinse procedure involving basic and acidic washes, since an external needle rinse alone resulted in a response after the injection of ULOQ equal to the LLOQ. Carry-over could be reduced < 20% by performing an internal needle rinse with IPA/H2O/NH4OH, IPA/H2O/HCO2H, and MeOH/H2O/HCO2H. An external active peristaltic pump rinse and injection port rinse with IPA/H2O/HCO2H were also required.
Novel Aspect / Conclusion: In pre-clinical toxicology studies, drug bioavailability is oftentimes unknown. Consequently, to minimize the occurrence of sample repeats it is advantageous to have as large a dynamic range as possible. The practical quantitation range is dictated by the saturation point of the detector and the required lower limits of sensitivity, each of which must be carefully titrated for low-end accuracy and precision and the minimization of carry-over. In the current assay we report for the first time the successful quantitation of imatinib over four orders of magnitude by leveraging the Ion Drive High Energy Detector in the Sciex 6500 QTrap.

Poster T10: "The role of internal standards in high-sensitivity protein quantifications with LC-MS/MS**"**
Poster Presenter: Mark Warren *(PRA, Lenexa, KS, USA)*

Introduction: Ligand binding assays (LBA) are currently the most popular approach to large molecule bioanalysis. However, the recent developments in LC-MS have turned these instruments into a viable technique for the accurate quantification of proteins in biological fluids.
Because proteolytic peptides are significantly better candidates for LC-MS/MS quantification than proteins, in the case of high sensitivity quantifications, a proteolytic digestion is used to cleave the protein into peptides, one of which is subsequently used for quantification. Is the use of a Stable Isotope Labeled (SIL) protein internal standard required to correct for the variability resulting from such a digestion step? Can other internal standardization approaches also be applied?
Methods: A review of the literature identified several internal standardization approaches and work-flows in use in the field. The model compound salmon calcitonin was spiked in 6 separate human plasma lots at 50.0 and 100 pg/ml. These pools were prepared using three different work-flows and their appropriate internal standards.

The work-flow consisted of reversed phase SPE (Waters OASIS HLB) followed by a trypsin digestion. The digest was separated by reversed phase chromatography on a 100*2.1mm 1.7 µm CSH-C18 (Waters) column and detection was done with a Xevo-TQS MS-detector (Waters).
Result: The Accuracy and Precision results for six different internal standardization approaches are shown, as well as the results obtained without using an internal standard.
Novel Aspect / Conclusion: When a digestion step has been well optimized, it does not introduce significant variability into the work-flow. The use of SIL-protein internal standards is not an absolute necessity, comparable results can be obtained with peptide internal standards, which are added into the work-flow after the digestion step.

Poster T11: "Development of four ultra-high sensitivity LC-MS/MS methods for the determination of four new compounds in human plasma in support of microdosing studies**"**
Poster Presenter: Dr. Yu-Luan Chen *(Sunovion, Marlborough, MA, USA)*

Introduction: To support a microdosing study, one of the biggest challenges is the availability of an ultra-high sensitivity bioanalytical assay for the concentration measurement of plasma PK samples. In this study, four highly sensitive LC-MS/MS methods with LLOQ of 0.5 pg/mL were developed for the accurate quantification of four compounds in human plasma, respectively. Deuterated compounds (d8- for first 2 and d4- for the rest) were used as internal standards for the corresponding analytes.
Methods: In each method, target analyte and IS were extracted from 0.500-mL human plasma by protein precipitation coupled with solid-phase extraction on HLB cartridge (for Compound-1) or by liquid-liquid extraction with 6-mL 1:1 MTBE/Ethyl Acetate (for other three compounds). The extracts were dried down at 45 °C under a nitrogen stream. The residue was reconstituted in 100 uL of 2:8 MeOH-H2O. A 25-40 uL of resulting sample was injected into LC-MS/MS (API 5000 or Waters Xevo TQS) for analysis. Reversed-phase HPLC separation was achieved on an XBridge C18 column (100 x 2 mm, 5u). The mobile phases A and B were 0.1% NH4OH in H2O and acetonitrile, respectively. A gradient at a flow rate of 0.3 mL/min was operated at 22-35% B for first 3.5-4.5 min followed by climbing up to 60% B for another 0.5-1.5 min (gradient varies by compounds). MS/MS detections were at transitions of m/z 341â†'244 for Compound-1 (CE 25 EV), m/z 355-112 for Compound-2 (CE 28 eV), m/z 358-275 for Compound-3 (CE 32 eV), and m/z 387-254 m/z for Compound-4 (CE 15 eV) in ESI+ mode.
Result: The retention times of these four compounds were 4.2, 4.8, 4.3, and 4.5 min, respectively. The validated linear range was 0.5-500 pg/mL for all four analytes based on the plasma volume of 0.5-mL. The coefficients of determination for the standard curves were 0.998, 0.996, 0.997, and 0.997, respectively. Intra- and inter-assay precision and accuracy were within 15% CV and within 7.0% bias for all four compounds. The extraction recoveries were 55% for Compound-1, 67% for Compound-2, 40% for Compound-3, and 85% for Compound-4. All four compounds were stable for 3 cycles of freeze/thaw, 24 h bench-top, and at least 2 months of plasma sample frozen storage at -70°C. The matrix effect tests showed a 12%, 1%, 28%, and 50% suppression for above compounds, respectively. The above methods have been qualified and successfully applied to the analysis of human plasma PK samples collected from the microdosing studies with these compounds.
Novel Aspect / Conclusion: Each of these 4 methods allows for the quantification of corresponding compounds as low as 0.5 pg/mL in human plasma, which is satisfactory to support microdosing PK measurement.

Poster T12: "A Free PK Assay With Perceived Limitations Elucidated with an Appropriate Theoretical Model**"**
Poster Presenter: Dr. LaKenya Williams *(Bristol-Myers Squibb, Princeton, NJ, USA)*

Introduction: The challenges associated with developing accurate free PK assays are documented in literature and widely known in industry. While models can predict the concentrations of total and free drug, the actual concentrations of free drug may differ from predicted values. All models take into account the dissociation constant, drug concentration, and target concentration. However, the minimum required dilution (MRD) causes a shift in the binding equilibrium that should be accounted for when measuring free drug levels. A model which factors in the effect of MRD was used to evaluate the suitability of the free PK assay at different MRD factors.

Methods: A free PK assay for a biotherapeutic was developed using electrochemiluminesence (ECL). The assay utilized a biotinylated mouse anti-drug mAb to capture drug on a streptavidin coated plate and a ruthenylated rabbit anti-drug pAb was used for detection. The anti-drug mAb for the free PK assay was shown to bind to the same epitope as the target when screened using bio-layer interferometry. The final qualified assay utilized a 10 fold MRD.
Result: To evaluate the effect of MRD on the free levels of drug, a titration experiment was run at varied levels of drug and target and MRDs of 5, 10, 20, and 50 fold. Without considering the MRD, models of the equilibrium at equimolar concentrations of drug and target (~50 nM) suggested that only 4 nM would be detectable in the free PK assay. The actual recoveries were 13, 15, 19, and 21 nM at 5, 10, 20, and 50 fold MRD, respectively. The 225 - 425% bias from the theoretical value was believed to be a limitation of the assay and an indication of poor reagent selection. However, a model that factors in the MRD was later used to further evaluate the suitability of the assay. Using the new model, the theoretical values at the same level of drug and target are 10, 13, 18, and 25 nM at the respective MRDs. This confirmed that the assay was detecting free drug within 30, 15, 6, and -16% of the theoretical value at 5, 10, 20, and 50 fold MRD.
Novel Aspect / Conclusion: To evaluate the effect of MRD on the free levels of drug, a titration experiment was run at varied levels of drug and target and MRDs of 5, 10, 20, and 50 fold. Without considering the MRD, models of the equilibrium at equimolar concentrations of drug and target (~50 nM) suggested that only 4 nM would be detectable in the free PK assay. The actual recoveries were 13, 15, 19, and 21 nM at 5, 10, 20, and 50 fold MRD, respectively. The 225 - 425% bias from the theoretical value was believed to be a limitation of the assay and an indication of poor reagent selection. However, a model that factors in the MRD was later used to further evaluate the suitability of the assay. Using the new model, the theoretical values at the same level of drug and target are 10, 13, 18, and 25 nM at the respective MRDs. This confirmed that the assay was detecting free drug within 30, 15, 6, and -16% of the theoretical value at 5, 10, 20, and 50 fold MRD.

Poster T13: "Quantitative Determination of a 20-mer Antisense Oligonucleotide in Monkey Tissues using LC/MS-MS**"**
Poster Presenter: Shane Karnik *(Pyxant Labs, Colorado Springs, CO, USA)*

Introduction: In order to demonstrate dose proportionality of an antisense oligonucleotide AOT in Monkey tissue (13-week study), a bioanalytical method was developed and validated for the respective quantitation of a therapeutic 20-mer antisense oligonucleotide in monkey tissues using LC/MS-MS.
Methods: Tissue samples (liver, spleen, nasal scrape, brain and kidney) were homogenized under liquid nitrogen. 60 mg portions were then separately weighed and processed using a Precellys tissue homogenizer and a homogenate buffer. Extracts were cleaned up by a liquid-liquid extraction. The aqueous portion was then transferred to HLB blocks for SPE cleanup. The final eluate was evaporated to dryness and reconstituted. Samples were then analyzed by negative Ion-Spray LC/MS-MS in the MRM. Analytes were chromatographed by reverse phase HPLC employing a Waters XBridge C18 column under isocratic conditions. A Sciex API 4000 mass spectrometer was used for detection. The validated method monitored three different MRM transitions corresponding to three different charge states at m/z 720 (n = 10), 800 (n = 9), and 900 (n = 8) m/z.
Result: The LC/MS/MS assay was validated over a linear range of 0.05 to 10.0 ug/g. Full validations were performed for monkey lung and liver tissues. Control liver tissue was used for the respective partial validations for rare matrices (fetal liver, nasal scrape, trachea-bronchial lymph nodes, brain, kidney, and spleen). Validation experiments included intra- and inter-day precision and accuracy, selectivity/specificity, extraction recovery, matrix effect, lower and upper limit of quantification and stability tests. The accuracy was 97% to 104% and the precision was 7% to 11%. Acceptable selectivity/specificity was demonstrated by examining multiple lots of liver and lung tissues for ion suppression and interferences. The LLOQ percent theoretical was 92% and the precision was 5%. Stability tests demonstrated that the compound proved acceptably stable.
Novel Aspect / Conclusion: Bioanalytical performance and full validation of the 20-mer antisense oligonucleotide quantitation method, coupled with in-life conduct produced "textbook" dose proportionality in multiple tissue types, including meeting CDER Guidance performance requirements set for plasma assays for tissues.

Poster T14: "Fit-for-Purpose Mass Spectrometry Bioanalytical Method Validation Considerations for Oligonucleotide Therapeutics**"**
Poster Presenter: Dr. Christopher Tudan *(Pyxant Labs, Colorado Springs, CO, USA)*

Introduction: The 2012 EMA Guidance and AAPS White Papers do not describe regulatory or industry expectations pertaining to the validation of RNA therapeutics via LC-MS/MS. Small molecule LC-MS/MS and large molecule LBA validation approaches and expectations are described. Due to relatively reduced sensitivity and lower analyte recovery of oligonucleotides from biological matrices with LCMS/MS, therapeutic RNA quantitation remains hybridization-based assays. Despite greater sensitivity, hybridization assays lack the necessary selectivity and dynamic range required to quantitate a specific therapeutic oligo for pre-clinical and clinical studies. Mass spectrometry methods, but not hybridization, can quantitate drug-related materials including: 3' and/or 5' chain-shorted metabolites; N-1 nuclease truncations, phosphorylation, oxidation, depyrimidization and depurination resulting from metabolic processes; shortmers/longmers; incomplete removal of protecting groups during synthes is; oligo degradation products; chemical substitutions at the 2-hydroxyl group such as 2-OMe, 2-F or 2-MOE, and/or phosphorothioate modifications. Respective metabolic truncations and alterations, drug modifications, or target mismatches would result in over-quantitation when analyzed via hybridization techniques. Moreover, when analysis of RNAi duplexes is conducted, synthetic impurities in single strands can be carried over into the duplex. Since mass analysis is capable of differentiating the mass differences and thus discriminating the analyte of interest versus any of the abovementioned impurities, mass spectrometry remains ideal for RNA quantitation. Furthermore, tandem mass spectrometry and the utilization of HPLC with online or pre-HPLC SPE are ideal for the quantitation of therapeutic RNA and associated delivery modifications or peptides.
Methods: We present a process for the full validation of bioanalytical methods for the quantitation of RNA therapeutics. A comprehensive table describing method validation tests, test formats and acceptance criteria is demonstrated, as well as examples and respective pre-validation strategies is described. Further, a strategy for pre-validation/method development is illustrated; a necessary prerequisite for robust and validation.
Result: Mass spectrometry is not without its own set of problems, including poor extraction efficiencies from plasma or tissue, strong cation adduction, wide charge state envelope and large molecular weights, all of which contribute to reduced sensitivity. Although bioanalytical strategies continue to be developed to address such challenges, the validation of these methods becomes substantially more comprehensive in order to obtain methods of sufficient robustness and reproducibility to ensure data integrity.
Novel Aspect / Conclusion: An effective approach to RNA bioanalytical method validation is effectively described.

Wednesday April 10 Posters

Poster W01: "The Demonstration of Selectivity in a LC-MS/MS Assay Where an Observed Suppression Occurs During the Bioanalysis of Samples from a Combinational Drug Study in Patients**"**
Poster Presenter: Dr. Kevin Carleton *(inVentiv Health Clinical Lab, Princeton, NJ, USA)*

Introduction: Multiple Reaction Monitoring (MRM) using a triple quadrupole mass spectrometer is widely accepted as a highly selective technique that limits the potential for matrix or co-administered drug interferences. As the number of combinational treatments evaluated in clinical trials has increased the past decade, this technique has proved even more valuable than ever. A bioanalytical LC-MS/MS method was validated for a highly potent protease inhibitor used against the Hepatitis C virus. This validation consisted of the standard experiments as outlined in the FDA Guidance for Industry (May 2001). In addition, numerous other experiments were carried out in the validation or shortly thereafter. One of these additional experiments was an Internal Standard normalized Matrix Factor (MF) experiment. This experiment aimed to determine whether the internal standard successfully reduced the variability of the assay due to effects such as the presence of the biological

matrix, known metabolites, degradation products, or co-administered drugs. The result of that experiment was a MF of 1.02 suggesting that the IS would compensate for any of the potential interferences.
With a validated method in hand, numerous clinical studies were supported with this assay for the quantitation of the protease inhibitor compound. However, it was noted that in some of these studies, namely one combinational study in which up to 5 medications were being evaluated, the internal standard response was quite variable from sample to sample. So variable in fact that a percentage of samples required reanalysis for the compound when the sample's IS response did not meet the SOP minimum requirement; 40% of the mean IS response in the analytical run. Additionally, upon reanalysis, it was found that the same result (low IS response) was observed. A reportable value was unable to be obtained for many of these samples due to the SOP requirement. As these samples may contain valuable information for the study and for the combinational drug product, an experiment was designed to support the removal of the SOP requirement for minimum IS response.
Methods: Five samples with suppressed IS response were selected along with five samples of typical IS response from an analytical batch. Individual samples with BLQ measured concentrations of the protease inhibitor compound were selected to minimize the impact of the experiment. These samples were reassayed in duplicate at a dilution with the high QC sample at a 1:5 ratio. A 50 microliter aliquot of the unknown sample was added to 200 microliters of the high QC sample. These ten samples were analyzed in a batch with front and back calibration curves and typical run qualifying QCs (low, mid, high and dilution).
Result: All ten samples back-calculated accurately within ± 15% of the nominal high QC concentration when a 5x dilution factor was applied. The five samples with suppressed IS response again demonstrated IS suppression in this experiment; however, the analyte response was also suppressed at the same percentage yielding an accurately back-calculated result.
Novel Aspect / Conclusion: The results of this experiment using incurred samples support the validation results from the IS-normalized matrix factor experiment. The internal standard used in the bioanalytical assay will compensate for any matrix effects, such as the presence of the biological matrix, known metabolites, degradation products, or co-administered drugs. These results allowed for the acceptance of previously non-reportable data, according to the SOP. Moving forward, the SOP requirement of a minimum IS criteria for this compound was removed from the method SOP based on the outcome of this investigational experiment.

Poster W02: "Quantitation of Amifampridine and its Major Metabolite in Human Urine: the Importance of Extensive Investigation of Selectivity during Method Development**"**
Poster Presenter: Xiao Liu *(Biomarin Pharmaceutical, Novato, CA, USA)*

Introduction: Amifampridine phosphate, also known as 3, 4-diaminopyridine phosphate, is a small molecule drug for Lambert-Eaton myasthenic syndrome (LEMS). It is currently marketed in Europe as FIRDAPSE® and in Phase 3 clinical development in the United States. Quantitation of amifampridine, the active moiety of FIRDAPSE, by LC/MS/MS has proven to be challenging due to the low molecular weight (MW 109.13) of the analyte. AGEPS-EPHP (France) used HPLC coupled with electrochemical detection to quantitate amifampridine in human serum. More recently an LC/MS/MS method was developed by SGS Cephac Europe using derivatization with danzyl chloride. Here we report, for the first time, an LC/MS/MS method for direct measurement of amifampridine and its major metabolite, N-(4-amino-pyridin-3-yl)-acetamide, in human urine with easier sample preparation and increased sensitivity.
Methods: The analytes and the internal standards (IS) were extracted from human urine by solid phase strong cation exchange. Amifampridine and N-(4-amino-pyridin-3-yl)-acetamide were chromatographed on the Luna HILIC column (50 x 3 mm, 3 □m particle size, Phenomenex) and the XBridge HILIC column (50 x 3 mm, 3.5 um particle size, Waters), respectively. The eluant was introduced directly to a triple quadrupole mass spectrometer (4000 QTRAP) operating in positive electrospray ionization mode. MS/MS quantitation was achieved by monitoring the fragmentation of 110.0-93.0 for amifampridine, 152.1-110.0 for N-(4-amino-pyridin-3-yl)-acetamide, 113.1-96.0 for IS1 (3,4-diaminopyridine-d3), and 155.1-111.0 for IS2 (N-(4-amino-pyridin-3-yl)-d3 acetamide).
Result: A number of HILIC columns were screened during the method development. The Atlantis HILIC Silica column and the Luna Silica(2) column first appeared to perform well for both

amifampridine and N-(4-amino-pyridin-3-yl)-acetamide. But further investigation of selectivity revealed an interfering peak for N-(4-amino-pyridin-3-yl)-acetamide in 2 out of 26 individual lots of human urine. An XBridge HILIC column was optimized to separate the interfering peak from the peak of interest and was selected to analyze N-(4-amino-pyridin-3-yl)-acetamide.
The final method had a standard curve range of 0.5-500 ng/mL for amifampridine and 1-1000 ng/mL for N-(4-amino-pyridin-3-yl)-acetamide. The intra-batch (n=6) mean accuracy was 99.0-108% for amifampridine and 97.5-110% for N-(4-amino-pyridin-3-yl)-acetamide. The inter-batch (n=18) mean accuracy was 101-105% for amifampridine and 101-108% for N-(4-amino-pyridin-3-yl)-acetamide. The intra-batch (n=6) relative standard deviation (RSD) was 0.6-3.6% for amifampridine and 0.7-5.2% for N-(4-amino-pyridin-3-yl)-acetamide. The inter-batch (n=18) RSD was 2.8-3.8% for amifampridine and 1.9-4.1% for N-(4-amino-pyridin-3-yl)-acetamide. The sample extracts demonstrated viability after storage at 6 □C for 72 hours. The samples were shown to be stable after five freeze/thaw cycles, after storage at room temperature for 24 hours, and after being stored at -70C for 101 days.
Novel Aspect / Conclusion: The first LC/MS/MS method is reported for direct measurement of amifampridine. The work highlights the importance of extensive investigation of selectivity in human urine method development.

Poster W03: "Development of a Stable-Isotope-Labeled Universal Monoclonal Antibody (SILUMab) Standard**"**
Poster Presenter: **Melissa Radabaugh** *(Sigma-Aldrich, St. Louis, Missouri, USA)*

Introduction: Monoclonal antibodies are increasingly becoming primary therapeutic agents for a host of human diseases. The ability to accurately assess the pharmacokinetic (PK) properties of biotherapeutics has become an essential part of monoclonal antibody development and characterization. While ELISA assays are typically utilized for this purpose, they require lengthy development of custom antibodies and can be prone to matrix effects. Quantitative liquid chromatography mass spectrometric assays are being increasingly implemented for this application due to their superior specificity, sensitivity, and reduced matrix effects. These methods typically rely on analyte-specific stable-isotope-labeled peptides as internal standards. While good reproducibility and relative quantitation can be achieved with such peptide-based approaches, the accuracy of absolute quantitation is subject to error associated with protein fractionation, enrichment, and proteolysis steps that generally occur prior to introduction of the stable-labeled peptide. A more ideal alternative strategy involves the use of a stable labeled full length antibody internal standard that can be introduced early in the analytical workflow, overcoming processing variability. To this end, we have developed a stable-isotope-labeled universal monoclonal antibody (SILUMab) standard, which can be used as an internal standard for quantification of monoclonal antibodies as well as Fc-fusion therapeutics. Due to the overlap with the common sequences in the Fc region with candidate antibodies, there is universal utility, thus eliminating the need for the production of candidate-specific stable-isotope-labeled internal standards.
Methods: A stable labeled IgG1 monoclonal antibody was expressed in CHO cells grown in serum-free 13C6 15N4 ARG/13C6 15N2 LYS enriched media. The antibody was then purified and label incorporation was determined to be >99% by mass spectrometry. Sequence coverage was confirmed by peptide mapping. The full length antibody was then used as an internal standard to assess quantification of a commercially available biotherapeutic in canine plasma by MRM-based LC-MS/MS. The analagous tryptic peptides were used for quantification and were chromatographically resolved.
Result: The resulting curves generated were linear and reproducible. The SILUMab afforded the ability to quantify a currently prescribed biotherapeutic in a complex biological matrix from a pre-clinically relevant species.
Novel Aspect / Conclusion: The use of a full length stable labeled monoclonal antibody standard provides the potential for more accurate quantification of biotherapeutics than do stable labeled peptides. The fact that this antibody acts as a universal internal standard allows for more rapid and cost effective quantification of preclinical biotherapeutic candidates.

Poster W04: "The Challenges of Developing and Characterizing Clinical PK ELISAs for Antibody Drug Conjugate Therapies**"**
Poster Presenter: **Dr. Helen Davis** *(Genentech, South San Francisco, CA, USA)*

Introduction: Antibody drug conjugates (ADCs) are a novel class of therapeutics for cancer treatment. An ADC is composed of a monoclonal antibody (mAb) covalently bound to a cytotoxic drug. ADCs combine the target specificity of a mAb with the cell killing activity of a potent cytotoxic agent avoiding the systemic toxicity observed with traditional chemotherapies. The ADC is a complex mixture; each mAb can have different amounts of cytotoxic drug conjugated to various conjugation sites. Biotransformation (e.g., deconjugation of cytotoxic drug) in vivo further increases the complexity of ADC so appropriate bioanalytical strategies and assays are needed to assess the pharmacokinetics (PK) and safety of ADCs. For example, several ADC analytes may be measured for PK evaluation.
This presentation will focus on a ligand-binding assay, an ELISA, designed to quantitate the total ADC in circulation, which could be fully conjugated antibody, partially unconjugated antibody and fully unconjugated antibody. Because of the ADC complexity in vivo differs from that of the standard used for quantitation, this type of assay should be developed to ensure that the assay reagents bind equally to the ADC with different drug to antibody ratio (DAR). Examples of assay challenges to develop and characterize clinical PK ELISAs for ADCs will be reviewed.
Methods: Special reagents, such as ADC fractions with different DAR and plasma stability experiments, were utilized to assess the suitability of ELISA formats to accurately detect the changing DARs of the ADC in vivo using a standard with a fixed average DAR. Assay formats were evaluated using the ADC target antigen or anti-complementarity determining region (CDR) mAb as capture reagents and generic anti-human IgG antibodies, anti-Fab framework, the ADC target antigen or anti-CDR mAb for detection reagents.
Result: Specific assay formats for accurately measuring ADC with different DAR required a specific capture reagent (ADC target antigen or anti-CDR mAb) and specific detection reagent (anti-CDR mAb or anti-Fab framework). Generic anti-human antibodies were sensitive to drug load and could not be used for detection.
Novel Aspect / Conclusion: Developing PK ELISAs for ADCs requires understanding biotransformation of the ADC in vivo and extensive characterization using special reagents that represent the components of the ADC mixture in vivo. For clinical assays, only specific detection reagents provided suitable assays. Generic anti-human IgG detection reagents did not equally recognize all the DARs as the presence of the cytotoxic drug may sterically hinder the binding to the antibody portion of the ADC.

Poster W05: "Micro dosing studies: a consideration on analytical technology choice**"**
Poster Presenter: David Higton *(Xceleron, Germanton, Maryland USA)*

Introduction: Micro dosing has been used as an investigative pharmacokinetic tool for approximately 10 years. Initial skepticism of the value of these studies was followed by investigative clinical trials to understand the circumstance when they provide useful data and this has led to routine use. When first introduced, accelerator mass spectrometry coupled with LC fractionation (LC+AMS) was the only technology that could provide the sensitivity required for these studies. Over the years, LC-MS/MS sensitivity has improved so that it is now viable to use this technique for micro dosing studies, and a decision needs to be made on what technique to use.
Methods: We have considered the relative merits of the technologies and the implications of using these techniques in terms of clinical study design along with the potential impact on the overall progress of the study. The authors' expertise in analyzing samples from microdosing studies by LC+AMS and LC MS/MS from over 50 microdosing studies was captured to share this knowledge more widely.
Result: A decision tree is presented that allows the correct decision to be made on whether to use AMS or LC-MS/MS for these analytical challenging studies. The decision tree is further expanded with detailed information on the background to the questions and the facets that should be considered when determining the optimum pathway. It considers the impact of the clinical study design and the aims of the study and the challenges in that will be faced when using either of the instruments. The vagaries of both LC+AMS and LC-MS/MS will be compared to allow decisions to be made on the likelihood of success depending on the chosen pathway.
Novel Aspect / Conclusion: Previously, there has been a lack of an objective comparison between LC+AMS and LC-MS/MS. Sufficient experience has now been gained in these techniques. This has

allowed this decision tree to be produced that will provide a valuable starting point when faced with the bioanalytical challenge presented by microdosing studies.

Poster W06: "Achieving Regulatory Approval for Accelerator Mass Absolute Bioavailability Studies**"**
Poster Presenter: Dr. Stephen Dueker *(Eckert & Ziegler Vitalea Science, Davis, CA, USA)*

Introduction: Bioanalytical method validation includes all procedures required to demonstrate that the quantitative determination of the concentration of an analyte in a biological matrix is reliable for the intended application. Many of the principles, procedures and requirements for bioanalytical method validation are common to most all forms of analytical methodologies with core parameters being accuracy, precision, selectivity, sensitivity, reproducibility, linearity, dilutional integrity, limit of quantification and stability. There are no universally accepted criteria for Accelerator mass spectrometry though a recent white paper published by European Bioanalysis formum outlines some basic expectations specific to the AMS. AMS is a method of directly quantifying the concentration of a rare isotope (<parts per billion) versus a common isotope in a uniform matrix derived from a defined biological sample. AMS shares many characteristics with quantitation by isotope decay counting (e.g., LSC), which often does not use internal standards or compound-specific calibration. AMS is now firmly a part of regulated bioanalysis, particularly for absolute bioavailability studies. The frequency of these studies has prompted a need to define a general approach to AMS method validation. We present recently published data showing our approach to validation and subsequent bioanalysis that successfully supported a regulatory request for a marketed product, Onglyza.
Methods An intravenous microtracer study was performed were a 5 mg unlabeled oral dose was given concomitantly with a 14C labeled microdose (<100 ug, 250 nCi). Plasma study samples were analyzed in three batches, with supporting standards and QCs from 0.025 to 15 DPM/mL (1.19 to 1144 pg/mL)using a validated method. Accuracy and Precision were set at +/- 15% except at the LLOQ which was 20%. ISR acceptance criteria was set at +/- 10% of the original value.
Result: All study sample batches and the ISRs passed according to set acceptance criteria similar to those used for regulated LC/MS analysis. The mean absolute bioavailability of saxagliptin was ~50% in the 6 subjects. ISRs fell well within acceptable limits while the pharmacokinetic results indicated good agreement in the terminal phase half-life values between the intravenous and oral routes.
Novel Aspect / Conclusion: Absolute bioavailability data from this study was as part of the IND submission package for approval of Onglyza in Australia and received subsequent approval. A traditional method approach was enlisted to prove that AMS methodology can meet or exceed more standard performance criteria for regulated bioanalysis. More limited approaches are being engaged so long as the method can be traced with QCs and method specific acceptance criteria that meet the objectives for the clinical study. This was the first time, to our knowledge, that an absolute BA study with the IV [14C]microdose was used in support of a regulatory approval.

Poster W07: "A GC-MS(MS) Method for Quantification of 4β-hydroxycholesterol (4βHC) from Bological Matrices using Electron Capture Negative Ion Chemical Ionization**"**
Poster Presenter: Dr. Jim Settlage *(inVentiv Health Clinical, Princeton, NJ, USA)*

Introduction: Oxidative metabolites of cholesterol are generated enzymatically and therefore have the potential to serve as biomarkers for disruptions in metabolic pathways and to monitor drug-drug interactions. Additional hydroxylated metabolites such as 4α-hydroxycholesterol (4αHC) are generated non-enzymatically. Since 4βHC is a product of CYP3A4, changes in its concentration in a biological matrix can be used to infer induction or inhibition of that enzyme. The use of 4βHC as a biomarker requires not only its isolation from the biological matrix but its isolation from the other hydroxyl isomers.This method was developed to focus on that application in human plasma.
Methods: The method utilizes 50 µL of matrix and a d7-SIL analog is added as internal standard. The sample is saponified by heating with ethanolic KOH and then extracted with hexane. The organic phase is evaporated to dryness and then derivatized first with 2,5,bis trifluoromethylbenzolychloride in anhydrous pyridine and then with MSTFA+1% TMCS. Samples are then evaporated and reconstituted in 35 µL of undecane and transferred to autosampler vials. From each vial 1 µL is injected. Chromatography is carried out on a DB-5 column with hydrogen carrier and samples are ionized using methane as the reagent gas. The analytes may be monitored in etiher MS or MS/MS

mode but because of the weak and non-specific fragmentation observed for these derivatives, we chose to use a single quad method.
Results: The method covered a biologically relevant dynamic range of from 2.0 to 200 ng/mL and demonstrated good linearity (R ≥m0.999 over 4 analytical runs). Quality control samples at 18, 80 and 180 ng/mL yielded interday %CVs of 4.0, 6.6 and 3.5 % respectively (n=8 with an error of <2%). All biologically relevant hydroxylated cholesterol isomers including 4αHC were chromatographically resolved from 4βHC
Novel Aspect / Conclusion: Recent methods have been published which utilize derivatization and LC/MS/MS methods to measure 4βHC .
We chose an alternative derivatization scheme and used GC since the LC methods offered no savings of time in sample preparation. Instead, the GC method utilizes the high separation power of capillary gas chromatography and a unique derivatization scheme that,by itself, isolates the analyte from other isomers which derivatize differently. The precision is sufficient to detect even small changes in the endogenous levels of 4-β hydroxycholesterol and has been used to successfully monitor drug-drug interactions in a clinical study (the results of which will be published elsewhere).

Poster W08: "Estimation of the effect of Yumberry on the bioavailability of Diclofenac in rabbits by the complementary utilization of UPLC-QTOF AND UPLC-triple quadrupole optimization process**"**
Poster Presenter: Dr. Mohamed AlAjmi *(King Saud University, Riyadh, SaudiArabia)*

Introduction: Yumberry is aqueous juice of Myrica rubra. Yumberry has been reported to possess antiinflammatory effects. It is used routinely as refreshing juice. The concomitant use of Yumberry and diclofenac occurs frequently and unfortunately no reports were found in literature to illustrate any sort of interaction between this functional food and diclofenac.
Moreover, the literature lacks any information about the effect of Yumberry in the plasma on the transitions of diclofenac.
Therefore this study is designed to study the effect of Yumberry on bioavailability of diclofenac and the method development and validation of diclofenac in biological matrix.
Methods: Two groups of rabbits (n-6) were treated either with Diclofenac (50 mg/animal) alone or diclofenac (50mg/animal) and yamberry (10ml/animal). Method development were performed using UPLC-QTOF. The internal standard chosen was codien. The blood was centrifuged and the plasma was spiked with codeine as internal standard. The plasma was then diluted 10 times and loaded in the system where the accurate mass of parent compound was determined as well as required trasitions. The method is validated during the development of analytical method.
The quantitiation was done using UPLC-triple quad. Utilizing agilant optimizer software. Mass parameters and acquisition methodology are fully customized to the current experiment.
Result: Administration of Yumberry juice before diclofenac increase the absorption of diclofenac 30 min by 32% (table 1) but the rate is decreased progressively thereafter. The effect was not significant p>0.05 (unpaired t-test, n=6).
Table 1. Effect of Yumberry drink on diclofenac bioavailability in rats

Time	Diclofenac Concentration (ng ml-1)					
Treatment	0.0 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h
diclofenac alone	17.13	20.71	24.74	31.64	20.25	18.56
diclofenac+yumberry	15.51	26.59	19.58	15.76	8.66	13.8.

Novel Aspect / Conclusion: Bioavailability of Dicolfenac after Yumberry juice consumption has been quantified in biological matrix (plasma) without need for preciptation after 10 folds dilution using guard column. The transitions are determined using UPLC-QTOF and the drug was quantified using UPLC-Triple quad.

Poster W09: "Intra-lab Comparison between Exploratory LC-MS/MS and Ligand Binding assays for Adalimumab from rat serum following intravenous administration to male Sprague-Dawley Rats**"**
Poster Presenter: Michael Koleto *(InVentiv Health Clinical Labs, Princeton, NJ, USA)*

Introduction: While ligand binding assays (LBA) are considered the gold standard for quantifying large molecules such as antibodies, the time and cost for reagent and assay development can be a limiting factor at certain stages of a development program. Liquid chromatography tandem mass

spectrometry assay (LC-MS/MS) is being considered more frequently as an alternative for rapid support for new large molecule therapeutics. Here we examine the comparability of LBA and the LC-MS/MS methods for the determination of Humira (Adlimumab) in rat serum.
Methods: IN-LIFE A ten-day single dose intravenous rat study was conducted through a partnership with Product Safety Labs. Serum samples were collected and stored frozen (-20°C) for subsequent analysis by both an electrochemiluminescence assay (ECLA) and LC-MS/MS assay on the same day.
LC-MS/MS method: Adalimumab calibrants calibrants (0.5 to 500 µg/mL) and QCs (1.5, 30, and 300 µg/mL), blanks and study samples (25 ÂµL) were transferred to a low-binding 1-mL 96-well plate followed by 75 µL of methanol to precipitate serum proteins. The protein pellet was isolated by centrifugation, the supernatant was removed and pellet reconstituted in ammonium bicarbonate solution containing 10% methanol followed by vigorous mixing. A stable-label proteotypic peptide internal standard (Furlong et al 2012) and aqueous trypsin was added to each resuspended pellet and the plate was incubated at 60°C for one hour while the plate was agitated. The plate was then centrifuged and each extract was directly injected onto the LC-MS/MS system. Analyses were performed using a reverse phase HPLC method followed by selected reaction monitoring of both the stable-label and unlabeled proteotypic peptide in positive electrospray ionization mode.
ECL-ELISA method: A 100 µL mixture of biotinylated TNF-alpha and Sulfo-tagged goat anti-human antibody (1:1; 200 µg/mL) was incubated with study samples (50 µL) for 4 hours at room temperature with gentle shaking. An aliquot (25 µL) was subsequently transferred to a blocked (C-block) streptavidin-coated MSD plate and incubated for 1 hour at room temperature. The plate was washed and read buffer (1X, 50 µL) was added before reading the plate.
Result: Each method met objectives following a one-day qualification that included precision and accuracy assessment (<±20%). Run qualifying samples (QCs) included with each sample batch met acceptance criteria. The same QCs were analyzed within both the ELISA and the LC-MS/MS methods. Measured concentrations for the QCs were within ±20% of nominal. Sample concentrations as measured by LC-MS/MS ranged from 2.60 ug/mL to 28.6 ug/mL, while those measured by ELISA ranged from 4.09 ug/mL to 27.1 ug/mL, indicating a good degree of concordance between the different technologies. Mean concentrations derived from each method were regressed against each other and produced a slope of 1.063 with a coefficient of determination (R2) of >0.90.
Novel Aspect / Conclusion: Good concordance was observed between the two technologies. The LC-MS/MS procedure is non-complex, rapid, and does not require immunocapture separation. This approach would be suitable for rapid quantitation of large molecule therapeutics in preclinical species.

Poster W10: "Quantification and Qualification of Intact Insulin by a Sensitive and High Throughput LC-HR/AM MS**"**
Poster Presenter: Hongxia Wang *(Thermo Fisher Scientific, San Jose, CA, USA)*

Introduction: Human insulin secreted by pancreas regulates blood sugar level. Therefore, insulin has been widely used to treat diabetes. The quantitation of intact insulin is preferable than peptide digests by eliminating variable modifications during sample processing, especially for the pharmacokinetics study. In recent years, there is a trend towards the use of high resolution mass spectrometry (HRMS) for fast screening and subsequent quantitation as an alternative to overcome the limitations of nominal resolution provided by triple quadrupole MS. A high throughput generic UHPLC-HRMS assay is demonstrated for intact insulin analysis using Q-Exactive Orbitrap MS. The qualitative information provided during quantitation of analytes within one injection is a crucial advantage to accelerate drug development process by reducing instrument analysis time and sample consumption.
Methods: Calibration solutions of human insulin (MW 5,808 Da) and bovine insulin (MW 5,777 Da) were prepared by serial dilution of stock solution (10mg/mL) in rat plasma. Samples were analyzed on Thermo XRS UHPLC pump and Open Accela autosampler interfaced with Q-Exactive Orbitrap with HESI source. Insulin was separated by 3-min gradient from 20 % to 90% acetonitrile in water with 0.1% formic acid at flow rate of 500µl/min on a PLRP-S, 2.1x 50mm column. The MS method was set at full scan (70,000 resolution) in positive mode. Selected ion monitoring(SIM) method was also employed for sensitivity comparison.
Result: A simple, sensitive and high throughput LC-HR/AM MS method was developed for the qualification and quantitation of insulin in rat plasma matrix. In the presence of large amount of high background noise, selectivity was greatly increased by using high resolving power at 70,000 (FWHM).

Insulin samples were analyzed by full scan mode for the charge state distribution envelope. Three most abundant ions and its isotopes at [M+4H]4+ [M+5H]5+ and [M+6H]6+ were selected for the quantitation. SIM at 70,000 resolution effectively isolated the analyte of interest from interferences in rat plasma spiked with insulin. To minimize the adsorption on LC-MS system, three different carrier solutions, BSA protein, BSA tryptic digests and rat plasma, were evaluated. Good linearity was observed over the range of 1ng/mL-100Âµg/mL with correlation coefficients (r2) greater than 0.99. Lower Limit of quantitation (LLOQ) was detected at 1ng/mL with precision <15 (% RSD). In summary, the intact insulin is used to demonstrate the extraction recovery. Accurate quantitation results were achieved without the need of trypsin digestion. While high confident identification of insulin could be completed with fragmentation accurate mass data.
Novel Aspect / Conclusion: UHPLC-MS by HR Orbitrap provides simple, sensitive and fast identification and quantification of intact Insulin.

Poster W11: "Challenges in the Development of a Dual universal LC-MS/MS assay for quantifying human and humanized monoclonal antibodies in animal matrices**"**
Poster Presenter: Dr. Song Zhao *(PPD, Richmond, Virginia, USA)*

Introduction: Traditionally, ligand-binding assays (LBA) have been used for the quantitation of mAbs. Recently, LC-MS/MS has attracted more attention due to shorter development times, wider linear range, and better specificity. Herein, we present improvements to the previously described "universal" LC-MS/MS assay capable of quantifying human or humanized IgG1 or IgG4 monoclonal antibodies in animal matrices. The applicability of the improved "dual" universal assay was demonstrated through the successful evaluation of three different human therapeutic mAbs and will be discussed herein.
Methods: The quantitative analysis of intact mAbs using LC-MS/MS is not practical. Instead mAbs are digested and proteotypic peptide(s) are used as surrogate analytes for quantitation. For the work presented , eight proteotypic peptides , four from the heavy chain and four from the light chain, were evaluated as potential surrogate analytes for the quantitation of the intact mAb. An intact, isotope-labeled human monoclonal antibody was used as the internal standard.
Analytes are extracted from a 25 µL aliquot of animal matrix using magnetic beads coated with Protein A. The bound analytes and internal standard are subjected to "on-bead" proteolysis with trypsin, following standard digestion procedures (denature, reduce, alkylate, digest). Chromatography is performed using a binary gradient, consisting of 0.1% formic acid and 0.1% formic acid in acetonitrile and a Waters BEH C18, 2.1 mm x 50 mm, 1.7 µm column, operated at 65°C. Due to the intrinsic differences in sensitivity for the eight tryptic peptides and the amino acid sequence difference between IgG1 and IgG4, two tryptic peptides, one from the heavy chain VVSVLTVLHQDWLNGK, (the peptide from the original universal peptide assay) and one from the light chain, TVAAPSVFIFPPSDEQLK are used quantitation.
Result: Summarized results for three different human therapeutic mAbs using LC/MS/MS

VVSV peptide

	mAb1(IgG1)	mAb2(IgG4)	mAb3(IgG1)
LLOQ(µg/mL)	0.050	0.0125	0.0125
ULOQ(µg/mL)	36	36	36
LLOQ(Bias)	1.59	4.59	1.55
LLOQ(CV)	6.72	0.54	16.7
ULOQ(Bias)	-5.58	-12	-4.5
ULOQ(CV)	2.77	2.68	4.11

TVAA peptide

	mAb1(IgG1)	mAb2(IgG4)	mAb3(IgG1)
LLOQ(µg/mL)	0.050	0.050	0.050
ULOQ(µg/mL)	100	100	100
LLOQ(Bias)	1.44	-3.5	11.1
LLOQ(CV)	13.6	1.32	N/A
ULOQ(Bias)	-3.24	-5.79	1.06
ULOQ(CV)	1.42	0.3	9.88

Novel Aspect / Conclusion: Several enhancements to the previously described universal LC-MS/MS assay for the quantitation of human and humanized monoclonal antibodies, in animal matrix, has been successfully demonstrated. The tryptic peptides above are found in many human and humanized mAb therapeutics and are proteotypic in many animal species. These important features are critical for establishing a "dual universal assay", which would allow the same methodology to be used for numerous humanized mAb therapeutics in many different animal species. Improvements to the 1st generation universal peptide LC-MS/MS assay, including incorporation of a second, light chain-based

peptide, thus introducing the concept of a “dual” universal peptide assay; use of an isotope-labeled mAb; Protein A based extraction..

Poster W12: “Development and Validation for a Combined LC-MS/MS Method for the Quantitation of Buprenorphine, Naloxone, 6ÃŸ-Naloxol and Oxycodone in Human Plasma**”**
Poster Presenter: Kyla O'Brien *(inVentiv Health Clinical Lab, Princeton, NJ, USA)*

Introduction: The purpose of this study was to develop and validate an LC-MS/MS method for the quantitation of buprenorphine, naloxone, 6ÃŸ-naloxol and oxycodone in human plasma. For the quantitative analysis of above analytes three separate validated methods were used. The current methods require more than 0.5 mL of plasma samples, time consuming, not being cost-effective and may anticipate variable results. The objective of this study was to develop a combined LC-MS/MS method to minimize the sample volume, increase the throughput and minimize the cost for sample analysis.
Methods: In this method, buprenorphine, naloxone, 6-naloxol and oxycodone, with their corresponding deuterated internal standards, were extracted by solid phase extraction (Empore MPC-SD Extraction Disc Plate). The extracts were chromatographed on a Luna Silica, 3µ, 2 x 50 mm column with gradient elution using water/acetonitrile based mobile phase solutions containing formic acid and ammonium formate. The analytes and their internal standards were detected under positive MRM mode on a MDS Sciex API 5000 with a turbo ionspray interface.
Result: The method showed a linear range with a weighting of 1/x2 for buprenorphine and naloxone over the range of 0.025-12.5 ng/mL and 0.0100-5.00 ng/mL respectively. The method showed a quadratic range with weighting of 1/x2 for 6-naloxol and oxycodone over the range of 0.0100-5.00 ng/mL and 0.100-50.0 ng/mL respectively. Accuracy and precision for buprenorphine, naloxone, 6-naloxol and oxycodone were all within the ±15% acceptance criteria. These LOQ ranges are similar to the previously validated methods. Buprenorphine, naloxone, 6-naloxol and oxycodone showed stability for up to 20 hours when stored at room temperature, through 6 freeze/thaw cycles and for up to 42 days when stored at 20°C. Processed samples showed stability for up to 83 hours stored at 5°C.
Novel Aspect / Conclusion: A combined, robust and rugged LC-MS/MS method was developed with similar LOQs for the quantitative analysis of buprenorphine, naloxone, 6-naloxol and oxycodone in human plasma. The current validated combined method offers many advantages to use this method for the analysis of clinical sample with minimal sample volume, minimal analysis turnaround times in cost-effective manner.

Poster W13: “Development of an Immuno-PCR Method with extreme robustness against the presence of endogenous counterpart for clinical PK study bioanalytical support of replacement therapy Fc-Fusion protein**”**
Poster Presenter: Dr. Beena Punnamoottil *(Chimera Biotec GmbH, Dortmund, Germany)*

Introduction: Quantification of an analytical target in the presence of an interfering substance is a prime challenge in development of specific ligand-binding assays (LBA). Biogen Idec is developing a novel recombinant Fc-fusion protein as a long lasting replacement therapy. Bioanalytical support of the Phase-II/III study for this product required an LBA method without interference by the endogenous counterpart present in patient plasma at varying levels or by the Fc portion of immunoglobulin.
Methods: The Imperacer LBA technology enabled sufficient analytical sensitivity despite a high minimal required dilution (MRD) of the clinical sample. A sandwich ImperacerÂ® assay setup was realized by surface immobilized capture antibody for binding of the therapeutic moiety and synthesis of a tailored Imperacer antibody-DNA conjugate for detection of the Fc fusion protein via a human Fc domain-specific antibody. The 1:100 MRD needed to avoid competitive binding by the endogenous protein was compensated by exponential amplification and detection of marker DNA of the Imperacer conjugate by real-time PCR.
Result: The GLP validated Imperacer method provided a standard curve range of 20 ng/mL to 9000 ng/mL therapeutic Fc-fusion protein. Assay parameters met validation criteria in pooled human plasma depleted of the therapeutic as the standard curve matrix. No significant interference was seen from endogenous protein that may be present in up to 250-fold excess or from IgG present at >300,000-fold

excess. Dilution integrity and linearity were confirmed, allowing expansion of the quantification range to 50,000 ng/mL by additional sample dilution.
Novel Aspect / Conclusion: An Imperacer method for quantification of a therapeutic Fc fusion protein free of interference from an excess of endogenous counterpart was developed and validated according to industry standard guidelines and whitepapers. A standard curve range from 20 to 9000 ng/mL was achieved with an MRD of 1:100. The assay was successfully used in the analysis of disease state clinical samples to support a global Phase II/III clinical trial.

Poster W14: "MS-based absolute quantification of proteins**"**
Poster Presenter: Dr. William van Dongen *(TNO Triskelion, Zeist, The Netherlands)*

Introduction: Biotechnology increasingly delivers highly promising protein-based biopharmaceutical candidates to the drug development funnel. The importance and rapid growth of protein therapeutics necessitates equal improvements in bioanalytical techniques to support toxicokinetic (TK), pharmacokinetic (PK), and bioavailability data collection.
Methods: Due to its flexible applicability and high selectivity, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most likely analytical technique to cope with the high demands on development time, high-throughput capacity, accuracy and precision. This is in contrast to the limited dynamic range of traditionally applied ligand binding assays (LBA) and the associated issues of non-specific binding, cross-reactivity and immunogenicity that seriously complicate accurate quantification.
To become more than competitive with LBA's, LC-MS/MS has to overcome several main challenges associated with the complexity of the matrix, particularly the wide dynamic range of endogenous proteins. Furthermore, more stringent demands on LC-MS sensitivity are expected, considering the increasing specificity and potency of novel biopharmaceutical compounds.
Result: An overview is provided of current considerations and achievements on the traditional "seven critical factors" in LC-MS/MS based protein quantification, including: (1) internal standardization, (2) protein purification, (3) enzymatic digestion, (4) proteotypic peptide selection, (5) peptide purification, (6) liquid chromatographic separation, and (7) mass spectrometric detection with respect to optimal sensitivity, accuracy and precision.
Valuable suggestions for optimization are borrowed from adjacent fields, such as targeted proteomics, protein biomarker and peptide bioanalysis. In addition, novel concepts, focusing on intact protein quantification, are discussed. Such an advanced bioanalytical approach holds enormous promise for simplification and improved throughput, precision and reproducibility of the bioanalytical workflow.
Novel Aspect / Conclusion: This overview provides various suggestions for optimization, with respect to optimal sensitivity, accuracy and precision, of the traditional protein cleavage- isotope dilution mass spectrometry workflow as well as some novel concepts focusing on intact or larger molecule quantification. The results based on an extensive survey of current literature have been considered for generalization of the bioanalysis of therapeutic proteins.