237 Bioanalytical Analysis of Sunitinib Microparticles for Ocular Administration.

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Objectives: To understand the ocular distribution of sunitinib from extended release microparticles in non-GLP rabbit studies through the development of a fit-for-purpose assay utilizing plasma as a single surrogate matrix for all ocular tissues.

Methods: Sunitinib was extracted from rabbit plasma and homogenized ocular tissues in plasma by liquid-liquid extraction and analyzed by LC-MS/MS using reverse phase conditions. Peak ratios from calibration standard responses were quantitated using a quadratic fit ($1/x^2$ weighting).

Results: Graybug Vision has developed a sunitinib microparticle formulation as a potential treatment for wAMD. Development of this drug and dose selection requires evidence of controlled-release drug delivery to the desired ocular tissues with low systemic availability. To address this requirement, AIT Bioscience developed a fit-for-purpose dual range assay covering a quantitation range of 0.100 – 50,000 ng/mL. This method has been used successfully for over 2000 samples to support non-GLP in vivo rabbit studies. The assay demonstrates high levels of drug in ocular tissues and low to BLQ levels in aqueous humor and plasma.

Implications: The method described provides for the quantitation of high local and low systemic concentrations of sunitnib in plasma and ocular tissue. The use of plasma as a homogenization medium and surrogate matrix meets scientific rigor, provides ease of analysis in the lab and addresses ethical concerns of procuring scarce ocular matrices.

238 A quick and efficient approach to immunogenicity assay development and optimization using Design of Experiments

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Objective: Immunogenicity assessment of therapeutic proteins is a critical part of drug development. Using Design of Experiments (DOE), we developed an ECL based homogeneous bridging assay to support immunogenicity testing of an XmAb® (an antibody that is being developed as an oncology therapeutic) in Pre-Clinical and Clinical samples.

Method: We selected three critical assay conditions: concentration of capture reagent (biotin labeled XmAb®), concentration of detection reagent (ruthenium labeled XmAb®), and sample incubation time for optimization. For the responses, we evaluated background signal, sensitivity and drug tolerance. Each of these three conditions were tested at three levels (low, mid and high) in a total of sixteen combinations using a central composite design (CCD) determined by JMP software. The assay conditions predicted by DOE were confirmed with follow up experiments. These analyses included performing sensitivity curve, drug tolerance, matrix interference and cut point assays.

Results: The optimum assay conditions predicted by response surface model analysis of the data were 250 ng/mL of biotinylated XmAb®, 250 ng/mL of ruthenylated XmAb® and 75 minutes sample incubation. Using the predicted capture/detection reagent concentrations with 25-30min acid dissociation, 50-60min neutralization and 75-90min sample incubation, we were able to achieve drug tolerance of ≥ 25µg/mL at 100ng/mL and 250ng/mL of anti-drug antibodies (ADA). In conclusion, we developed two robust assays for immunogenicity testing of the XmAb®.

Implications: No matrix interference were observed while comparing cynomolgus monkey serum with human serum samples with comparable ADA recoveries, thereby enabling us to use this same assay to support both Pre-Clinical and Clinical studies.

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239 Development of High Sensitivity and High Throughput Immunoassays for PD-1 Cancer Immunotherapy

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Objectives: Programmed death-1 (PD-1) is an inhibitory receptor on T cells and overexpression of PD-1 leads to suppression of anti-tumor activity. Clinical trials inhibiting PD-1 have shown remarkable success and advances in understanding the upstream regulation of PD-1 may lead to new therapeutics. Thus measurement of this biomarker for diagnosis, disease progression, and therapeutic efficacy is crucial for immunotherapy. However, biopsy based immunohistochemistry is the current standard of care for assessment of PD-1. To create a non-invasive higher throughput assay, we developed an ELISA for soluble PD-1. We further aim to increase the sensitivity of the ELISA utilizing the NanoLuc® luciferase reporter, an ultra-bright luciferase with low background, and the Amplatto™ nanoparticle immunoPCR reporter.

Methods: The immunoassays are based on the sandwich ELISA developed by Somru BioScience**.** Briefly, a PD-1 monoclonal antibody was coated to a 96-well microplate to capture PD-1 and detected using a biotin conjugated PD-1 polyclonal antibody with HRP. In attempt to increase sensitivity of the ELISA, we are replacing HRP with a streptavidin conjugated NanoLuc® luciferase and an Amplatto™ streptavidin-DNA conjugated reporter.

Results: The ELISA meets FDA Bioanalytical Guidance and has a dynamic range of 200-4000 pg/mL with no cross reactivity against B7-H1, CD28, CTLA-4, and ICOS up to 10 ng/mL. No linearity assessment was performed as the C_{max} is expected to be below the ULOQ. Experiments and analysis using the NanoLuc® and Amplatto™ reporters are ongoing.

Implications: These assays may serve as valuable tools for ultrasensitive and rapid bioanalysis of PD-1 and future immunotherapies.

[1]Celerion Inc. [2]Somru BioScience Inc.

240 Title: High volume rapid turn-around clinical sample analysis: obstacles, solutions and lessons learned

Jocelyn Reid, Charles River Laboratories, Jonathan Reeves, Charles River Laboratories, Marjorie Ferguson-Gratton, Charles River Laboratories.

Objectives: Methodological and process optimization to streamline sample receipt, bioanalysis, quality control/assurance to meet data deliverables in a series of rapid turn-around clinical programs exceeding 24,000 study samples.

Methods: A pre-program stake-holder meeting was held between the Sponsor, the central clinical laboratory, and Charles River Laboratories for process optimization including: electronic sample manifest receipt, sample reconciliation, sample analysis, QC and QA of the data, reporting, and data transfers. Method optimization was performed to streamline the ELISA method previously used for lower throughput studies. Partial method validation of the optimized method included steps involved with: sample extraction, centrifugation, coating stability, extracted plate stability.

Results: The electronic sample manifests were revised to facilitate the 64 samples shipments received from the clinical site. Rolling dates for data submission were scheduled, so that QA'ed data was available every second week. Data was sent to the PK scientist in a rolling basis, to accelerate the PK analysis, while a final set of data was transferred to the central clinical laboratory using a revised data transfer process.

Implications: Due to the above process improvements, the assay failure rate was at approximately 6%, while the ISR sample pass rate was >96%. Using the new electronic manifest process, 20 of the 24000 samples received required reconciliation. The Sponsor requested deliverable dates were all met. These processes were implemented to other clinical studies in order to maximize throughput and to minimize manifest discrepancies.

241 HIV NRTI Program: Quantitation of Phosphate Anabolites in PBMCs Using LC-MS/MS.

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Objectives: We will discuss the quantitation of the monophosphate, diphosphate and triphosphate anabolites in Peripheral Blood Mononuclear Cells (PBMCs) for two compounds in the NRTI program. After the NRTI compound is introduced to the cell, the compound is phosphorylated into its active form, the triphosphate anabolite, inside the PBMCs. In Safety Assessment studies, the three phosphorylated anabolites are quantitated as there is conversion from the higher energy anabolite (triphosphate anabolite) to more stable energy anabolites.

Methods: Innovative quantitation methods for analysis of the three phosphate anabolites in PBMCs utilized Sciex 5500 Q-trap and Triple Quad mass spectrometers coupled with Acquity UPLCs. A combination of manual preparation on a wet ice bath and Hamilton Star Automated Liquid Handling System was used for the sample preparation using a dilution sample preparation procedure.

Results: Quantitation of the phosphorylated anabolite levels in PBMCs was challenging due to instability and specificity issues attributed in part to the fact that the anabolites of interest are closely related. We assessed impact of the specificity on the assay and made method improvements before validation. A further challenge was that samples have varying cell counts. We accounted for this in our validation procedures by assessing QCs in high cell count PBMCs. Exploratory validations were successfully completed, and Safety Assessment study samples were reliably quantitated. Implications: The challenges relating to specificity and varying cell counts associated with PBMC analysis were addressed in the validation so that sample analysis could be completed quickly and reliably.

242 Bioanalytical Challenges to Support HCV NS5B Nucleoside Inhibitor and its metabolite in Rat Milk.

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Objectives: To develop and validate a bioanalytical method for the determination of a NS5B nucleoside inhibitor, part of a combination therapy strategy for HCV, and its nucleoside metabolite in rat milk using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Methods: Preliminary experiments indicated a loss of the inhibitor in the milk matrix. Stabilization was achieved by acidification of the rat milk with formic acid. The inhibitor and its metabolite were extracted in acidified rat milk through protein precipitation, chromatographed with reversed phase column using gradient elution and detected on a Sciex API 5500 mass spectrometer equipped with a Turbo Ion Spray interface in positive ion mode with multiple-reaction monitoring. The standard curve range was 5.25-5250 ng/mL for both the inhibitor and its metabolite using 20 uL of acidified milk.

Results: Several challenges were encountered prior to sample analysis such as the inhibitor peaks observed in the blank milk samples and diluent injections which persisted in every run. A shutdown method with high organic wash at a slow flow rate was employed to resolve the inhibitor contamination issue. Another challenge was how to stabilize the inhibitor during the rat milk sample collection. Several collection procedures were investigated and rat milk collection method was optimized. This methodology ensures satisfactory quantitation for both the nucleoside inhibitor and its metabolite in acidified rat milk. All validation parameters met the acceptance criteria according to regulatory guidelines.

Implications: The validated method was successfully utilized to support a Good Laboratory Practices (GLP) rat lactational transfer study**.**

243 Hybrid immunocapture LC-MS/MS assay for analysis of insulin analogues in human plasma using MSIA.

Aaron R. Ledvina[1], Matt Ewles[1], Eric Niederkofler[2], Stephanie Cape[1]

Objective: To evaluate the feasibility of MSIA™ insulin D.A.R.T.'S in combination with conventional LC-MS/MS methodology for analysis of insulin analogues in human plasma over a clinically relevant range.

Methods: Insulin analogues lispro, aspart, glargine, were added to human plasma over the curve range of 70 pg/mL to 5,000 pg/mL using a 300 uL aliquot volume. Extraction was performed using MSIA insulin D.A.R.T.'S (Thermo) in combination with the Versette automated liquid handling platform. Human plasma was aliquoted, ISTD (bovine insulin) added, and dilution of samples performed using 10 mM *n*-octyl glucoside (NOG). Samples were vortexed and centrifuged to ensure that particulate matter was not exposed to the MSIA tips. Samples were subjected to MSIA extraction. LC Separation was performed using reversed phase separation, with eluent analyzed using an API5500 (Sciex) operating in MRM mode.

Results: The calibration range for lispro, aspart, and glargine was 70 pg/mL to 5,000 pg/mL. Adequate S/N was obtained for all three analytes at the LLOQ level. For Glargine, %CV at LLOQ level was found to be ~11%, for aspart 6%, and for lispro, 16%.

Implications: The work presented here demonstrates the feasibility of quantitation of insulin analogues from human plasma over a clinically relevant range using a hybrid assay comprising MSIA-based immunoextraction in combination with conventional LC-MS/MS analysis.

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244 Quantitation of Human Fc Fusion Proteins in the Presence of Human Matrices

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Objective: Acute Pancreatitis is a devastating disease with a mortality rate that remains at approximately 10% despite the advancement of medical care. The utilization of an Fc-fusion protein is an intriguing approach to the treatment of pancreatitis. Fc-fusion proteins have been shown increased half-life over their endogenous counterparts[1], and the longer duration provided by Fc-fusion proteins has the potential to help improve the treatment for acute pacreatitis. However, the analysis of an endogenous protein coupled to a human IgG can be challenging in the presence of human serum, where human IgG concentrations can range from 500 to 1500 mg/dL.

Method: Here we present a novel way of analyzing endogenous Fc-fusion proteins in human serum, where the analyte of interest is separated from potential interferences by the use of a simultaneous immuno-capture of the endogenous protein and Fc-fusion counterpart.

Results: After a wash, the captured analytes are then eluted off the plate using acid, and then are transferred to a new plate coated with an anti-human IgG antibody, which targets the human IgG of the Fc-fusion protein, leaving the endogenous protein unbound.

Implication: Using this solid phase extraction (SPE) technique, an Fc-fusion protein can be removed from potential matrix interferences, and can then be selectively captured over the endogenous counterpart, allowing for quantitation.

1) Strohl et al. Fusion Proteins for Half-Life Extension of Biologics as a Strategy to Make Biobetters BioDrugs. 2015;29(4):215-239.

245 A Workflow for Antibody Bioanalytical Analysis using Automated Sample Preparation and Sensitive Intact Protein based LC-QTOF Analysis.

Andy Gieschen , Shuai Wu, Alex Zhu, Steve Murphy, Agilent Technologies

Objective: Bioanalytical analysis of proteins is traditionally performed by ligand binding assays (LBA) or by monitoring signature peptides from digests based on targeted multiple reaction monitoring

techniques. LBAs are less specific and are affected by the presence of anti-drug antibodies in the samples. MRM based methods may not monitor unexpected drug. Here an intact protein workflow using AssayMAP sample preparation platform for affinity purification from biological matrices, followed by sensitive intact protein quantitation with high flow UHPLC and a newly developed QTOF is detailed.

Methods A commercially available ADC was spiked into rat serum at various concentrations. Biotinylated antigen to the ADC was immobilized on AssayMAP Streptavidin cartridges for affinity purification, using the Agilent AssayMAP Bravo automated sample preparation platform. **D**eglycosylation was carried out for half of the samples using the AssayMAP Bravo. Purified ADC samples were analyzed on a 1290 infinity II UHPLC coupled to a 6545XT AdvanceBio Q-TOF. Quantitation of both the intact and deglycosylated ADC were done using the peak areas of extracted ion chromatograms.

Results: The deconvoluted spectra showed a total of seven peak groups representing DAR values of 0-7. This workflow could detect down to 2 ng on-column for deglycosylated ADC with larger than 2 orders of dynamic range. The overall DAR value and the relative abundances of different DAR species can be accurately measured for each level including the LLOQ level.

Implications: A novel workflow for bioanalytical analysis of antibodies combining automated affinity purification and sensitive intact protein based LC-QTOF analysis.

246 Automated Bioanalysis Report Generation to Support Drug Discovery and Filing using Watson LIMS and StudyDoc

Stacy Ho[1], Larry Elvebak[2], Nick Levitt[2], Walter Korfmacher[1]

Objective: Develop automatic bioanalytical reporting tool to reduce the report writing time.

Method: At the drug discovery stage, various types of bioanalytical reports are needed. Some are just for internal use. Others may be used internally and for drug filing. Report content requirements for each type of report are specific and highly variable. In this project, we developed tools to automatically generate various types of bioanalytical reports in Microsoft® Word using Watson LIMS and StudyDoc. The tools are developed and tested using representative studies.

Results: We developed customized tools using commercial off-the-shelf software (Watson LIMS and StudyDoc) to allow us quickly, easily and comprehensively design and modify the content of the report according to report type. Using the customized tools, we can generate various type of bioanalysis report in just a few minutes. Types of reports that can be automatically generated include discovery bioanalysis reports with pharmacokinetic analysis results, filing ready method qualification and filing ready sample analysis reports.

Implications: It was found that automated report generation increased report writing efficiency by several folds. Because they are generated automatically, reports are more consistent and the time needed for verifying report contents is reduced.

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247 **Sensitive and Robust Quantitation of Intact Monoclonal Antibody using a Newly Developed Q-TOF Instrument.**

John Sausen, Alex Zhu Agilent Technologies

Objective: Bioanalytical LC/MS protein quantitation analysis is traditionally performed using signature peptides from digested samples using targeted multiple reaction monitoring techniques. Quantifying at the intact protein level avoids limitations of quantitation by peptide MRM, but faces separate challenges of sensitivity and reproducibility. This method uses novel chromatography and the 6545XT AdvanceBio LC/Q-TOF to give a highly sensitive and reproducible quantitative method with an impressive linear dynamic range for therapeutic proteins and its biotransformation products.

Methods: Formulated Trastuzumab was diluted in H2O containing 0.01% BSA (w/v) and 0.1% formic acid to cover a range from 0.01 ng/µl to 100 ng/µl. LC/MS analysis were performed on a 1290 infinity II UHPLC coupled to a 6545XT AdvanceBio LC/Q-TOF. Quantitation of the intact Trastuzumab was done using the extracted ion chromatograms peak areas.

Implications: This method was able to detect as low as 0.0316 ng of intact trastuzumab on column (corresponding to 31.6 ng/ml with 1 µl injection), with more than 3 orders of linear dynamic range (0.0316 - 50 ng on column). For all the levels including the LLOQ level, deconvoluted spectra clearly showed the major glycoforms with excellent mass accuracy confirming the identity of the mAb. The reproducibility for both the intensity and the mass accuracy is shown by a series of 100 replicate 10 ng injections. At this low level, the peak areas showed a precision level of only 1.5% RSD. For three major glycoforms, every data point out of the 100 runs was within 10 ppm mass error.

248 **A Novel bioluminescent based bridging immunoassay for anti-drug antibody (ADA) detection**

Marjeta Urh, Becky Godat, Rod Flemming, and Nidhi Nath , Promega Corporation

Objectives: A novel bridging immunoassay for sensitive detection of anti-drug antibodies (ADAs) in serum samples is described. Two aspects make the assay novel. First, is the use of bioluminescent protein (NanoLuc) as an antibody label resulting in a sensitive assay with wide dynamic range and a high tolerance for free drug in the serum. Second, is the method for labeling primary antibodies with NanoLuc, resulting in a shorter protocol with a single washing step.

Method: Detection of anti Trastuzumab antibody is used as model system for optimization of bridging immunoassay. Briefly, Trastuzumab labeled with Biotin (capture antibody) and NanoLuc (detection antibody) are mixed with serum sample containing anti Trastuzumab antibody and incubated for 1 h to allow formation of bridging complexes. Mixture is subsequently added to a white 96 well streptavidin plate and incubated for 1 h to capture bridge complexes. Plate is washed followed by addition of NanoLuc substrate and detection of bioluminescence.

Results: Two novel approaches for labeling Trastuzumab with NanoLuc were explored: first, covalent, and oriented attachment of NanoLuc and second, recombinant fusion of antibody and NanoLuc. Resulting bridging immunoassay had a LLOQ of 1.0ng/mL and a four-log order dynamic range, (± 20%

accuracy, and precision). Drug tolerance (defined as the ratio of free drug to ADA, detectable above cut point) of > 500 was obtained at 100 ng/mL which is better than the ratio of 250 recommended by FDA.

Implications: A one-step sensitive and robust ADA assay performed using widely acceptable ELISA format will be enabling for immunogenicity testing

249 Overcoming Challenges in Bioanalysis of Polyamines by LC-MS/MS: Trimethylpropyldiamine (TMPD)

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Objectives: The analyte, trimethylpropyldiamine (TMPD), was a degradant of a high molecular weight biological drug. Amines are volatile, ubiquitous compounds involved in a number of biological processes. The hydrophilic nature of these environmentally prevalent amines creates challenges for quantification in biological matrices. The objective was to develop a sensitive (pg/mL) and selective LC-MS/MS method for bioanalysis of TMPD in plasma.

Methods: Supernatants from protein-precipitated plasma samples were injected onto a 3.0 x 50 mm BioBasic SCX column operated under isocratic conditions using mobile phase consisting of 73.5:25:1.5 ACN:200 mM CH_3COONH_4:HCOOH. Electrospray-generated positive ions were detected with an API 4000 tandem mass spectrometer in MRM (multiple reaction monitoring) mode.

Results: While the final method was elegantly simple, identification and control of environmental sources of contamination, such as blood collection devices and disposable labware, were key to the successful validation and application of the method. The method was validated for plasma samples from several species (25 – 100 µL) with LLOQs from 25 to 75 pg/mL, and was successfully applied to study sample analyses.

Implications: The precautions and method approach developed for quantitative analysis of TMPD could be applicable to analysis of biological amines such as putrescine cadaverine, spermidine and spermine.

250 Novel, Simple Workflow for Bioanalysis of Biologics – Streamlined Approach to Evaluating Loading Capacity of a Revolutionary Sample Clean-up Device for Peptide and Protein Bioanalysis

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Objective: Traditionally, large molecules such as peptides and proteins were quantitated in complex biological matrices using ligand-binding assays (LBA) such as enzyme-linked immunosorbent assays (ELISA). LBA assays require targeting reagents that bind to specific structural regions of a molecule. Affinity based strategies can be very effective however, for some applications the utility of LBA approaches is significantly reduced since the capturing reagent may not can differentiate subtle difference in peptide structure (e.g. phosphorylation, glycosylation, etc.).

To address this and other scientific needs, the biopharma industry has moved to LC-MS, adding in specificity at the “detector-level” and enabling scientists to monitor multiple analogues and catabolites

simultaneously. Mass spectroscopy can address the specificity requirements for peptide/protein determination and quantification, however a protein/peptide specific sample clean up tool is required when analyzing complex samples (blood, urine, serum, etc.). A series of complex, time consuming, and expensive sample pretreatment procedures have been commonly adapted to the frontend of the LCMS workflow. Approaches such as solid phase extraction (SPE) and immune-affinity capture have been reported and their utility demonstrated. However, these approaches can require complex pretreatment, long method development time, use immune-affinity based reagent, and can be costly as well as labor intensive assays.

NanoMedical Systems (Austin, Texas) is launching its new product platform, the nHance™ Peptide Capture System (PCS) to revolutionize peptide quantification. Agnostic to any bioanalytical approach (LCMS, ELISA, etc.), this peptide enrichment solution provides access to analytes in the range of 500-10,000 Dalton molecular weight in complex biological samples through size exclusion and electrostatic interactions between the nHance™ PCS nanoporous surface and peptide(s) of interest. The experiments described herein focus on workflow, pretreatment and loading capacity for nHance™ PCS device. The nHance™ PCS technology affords users a workflow under an hour that requires minimal sample pretreatment and uses common every day reagents that are commonly available in an LC-MS laboratory. All devices which collect a broad biomolecule class within a serum-based sample in preparation for subsequent analytical isolation of a target analyte is potentially subject to loading effects, which can reduce binding and eventually recovery. The objective of this evaluation was to understand and characterize the loading of a representative peptide to provide optimized workflow starting points in assay method development for future molecules using this technology.

Methods The test biomarker, insulin beta chain, was used as representative peptide for evaluation of workflow, sample pretreatment and loading capacity of nHance™ PCS device. Known concentrations of IBC were spiked into surrogate matrix, 100% serum, and diluted serum at various dilution factors. And internal standard was used to compensate for instrument based variability (injection volume anomalies, suppression of signal matrix effects, etc.).

Additionally, MALDI was used to confirm decrease in complexity of matrix and increased ionization of low abundant proteins and peptides after simple sample pretreatment followed by nHance™ PCS device clean up.

Results: By using an experimental matrix incorporating a minimum required dilution (MRD) approach crossed with target analyte spiking at increasing concentrations, we were able to identify the maximum total loading (serum + peptide) that defines the operating point for 100% recovery for IBC using LC-MS/MS. For target analyte concentrations below 100ng/mL, a simple 10X dilution of the serum matrix assured recoveries of 50~100%. Looking at the recovery loss in the serum dilutions associated with various concentrations of the target analyte, including artificial high concentrations (1, 10, and 50µg/mL), it was possible to establish an approximate equivalent loading for serum as 50~100µg/mL. Because the 35 ± 5 Å pores of the nHance™ PCS remove proteins and other large serum components by size exclusion, this equivalent loading makes sense for the anticipated residual serum content that is made up of only small biologic molecules. The reduction in complexity of the matrix when implementing these various pretreatment workflows was further confirmed using MADLI. An advantage of this approach was that the MALDI scans were not reliant on a specific representative peptide. The data confirmed a decrease interferences from high abundant proteins and serum components and the

increased signaled due to increased ionization of low abundant peptides and proteins after simple pretreatment was implemented.

Conclusions: The nHance™ PCS device is shown to be a multi-dimensional sample clean-up and protein/peptide enrichment technique. In conjunction with the charge-based affinity models for the nHance™ PCS interactions, this study demonstrates the importance of surface area models that can be combined with these new loading response models to provide a reliable starting point for assay method development. In addition to being a highly flexible and tunable sample clean-up and enrichment tool for peptides and proteins, nHance™ PCS has greatly simplified workflow, is cost effective, requires no expensive reagents and further enables high-through put analysis.

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251 Development and Validation of a Sensitive and Rugged LC-MS/MS Method to Measure Phenylephrine and Chlorpheniramine in Human Plasma

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Objective: Phenylephrine is a sympathomimetic drug used as a nasal decongestant and Chlorpheniramine is used in the prevention of the symptoms of allergic conditions, both drugs are often formulated together to make allergy and congestion relief drug. Phenylephrine undergoes extensive metabolism to form conjugates to produce low parent concentration and very high conjugate metabolites in vivo. A very sensitive method is needed to measure the Phenylephrine parent concentration without any impact from conjugate metabolites; Chlorpheniramine occurs in high concentrations in the blood. This poster will summarize the method development and validation to overcome the challenges on the sensitivity, polarity difference and instability of the metabolite as well as combining measurement with Phenylephrine and Chlorpheniramine by using AB Sciex 4500.

Methods: Plasma samples were extracted using Strata X 96-well plate solid phase extraction (SPE) plates. Three hundred and thirty (330) µL of each sample was loaded on the sorbent. After washing the plate with organic solution, the analytes were then eluted out by using 500 µL of formic acid in acetonitrile, the eluents evaporated, and each sample reconstituted for Phenylephrine analysis. An aliquot of the reconstituted Phenylephrine samples were transferred to separate collection and further diluted with reconstitute solution for Chlorpheniramine analysis. Phenylephrine LC-MS/MS analysis was conducted on a Thermo Polar Advantage C18 column while Chlorpheniramine analysis was conducted on a Thermo Phenyl column, both using gradient conditions. The mobile phases for each analyte were identical and were 0.1% formic acid in 10mM ammonium formate in water and 0.1% formic acid in methanol for Mobile Phase A and B, respectively. The analytes were detected by MRM (168.0→ 135.0 for Phenylephrine, 171.0→ 135.0 for Phenylephrine-d3, 275.0→ 167.0 for Chlorpheniramine and 281.0→ 167.0 for Chlorpheniramine-d6) on AB Sciex 4500 by Turbo Ion-spray, positive ion mode.

Preliminary data: The LC separation was performed under gradient condition to better retain Phenylephrine and achieve good sensitivity. Based on the strong polarity of Phenylephrine, a very low

percent of organic solvent was included in mobile phase to separate the interference peak in plasma samples for Phenylephrine. However, the presence of the organic solvent caused Chlorpheniramine to elute late from the column and without adequate peak shape. Therefore, two separate injections were performed to get suitable run time and good peak shape for each of the analytes. Sample loading, wash and elution conditions for the Strata X 96-well plate (60 mg) were optimized to achieve the LLOQ of 10 pg/mL for Phenylephrine, with lower baseline and less matrix suppression. However due to expected high clinical concentrations of Chlorpheniramine, an aliquot of the reconstituted Phenylephrine sample was transferred to dilute further prior to injection to avoid saturation of the LC-MS/MS system. The recoveries for Phenylephrine and Chlorpheniramine were 81.0% and 101.4%, respectively. To investigate the stability of metabolites Phenylephrine-glucucronide, Phenylephrine-sulfate, and Chlorpheniramine-N-Oxide, benchtop stability was conducted at both room temperature and 4°C. The results showed that conversion from conjugates to parent drug was significant after 24 hours storage at room temperature, but was insignificant after 24 hours at 4°C. In addition, the conversion of metabolites was monitored during extraction and post-extraction storage and it was observed to have no impact on accurate determination of analytes in the presence of conjugates. The stability of F/T for 3 cycles at -70°C and whole blood stability at 4°C for 2 hours were assessed and the compounds were found to be stable with metabolites. The linear range of the method was 0.010–2.50 ng/mL for Phenylephrine and 0.20-50.0 ng/mL for Chlorpheniramine. The intra-day and inter-day coefficients of variation (%CV) for Phenylephrine were 0.4 – 8.7 and 2.8-7.3, respectively; for Chlorpheniramine were 2.8 – 7.7 and 3.5 – 5.3, respectively. The method was validated successfully and used for multiple bioequivalence studies. The ISR results showed all results met the acceptance criteria.

Novel Aspect: One time of extraction for a low concentration and strong polar analyte of Phenylephrine and combining with high concentration and less-polar than Chlorpheniramine.

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LC/MS/MS Bioanalytical Protocol for Determining the Degree of Non-Specific Binding in Multi-Well Plates

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Introduction: Sample preparation in quantitative bioanalysis often suffers from target compound(s) losses to exposed surfaces including commonly employed microplates. A number of factors have been described to reduce the degree of surface adsorption, including reducing the surface area-to-volume ratio, contact time, surface treatment, or addition of constituents to the sample to block active adsorption sites. Previous studies on adsorptive losses were often tested at high detection concentration based on either LC-UV or LC-MS. In the current work, we have employed LC tandem MS and LC-QTOF to evaluate the degree of non-specific binding of six known sticky compounds (COOH-THC, JWH-018, JWH-073, Cetrorelix, Chlorhexidine, and IGF-1).

Methods: The NSB experiments involved transferring aliquots of analytical standard QC samples (3 times their LLOQ in aqueous or aqueous methanol solvent) to microplate wells and comparing their LC/MS/MS analyte responses relative to the initial QC solution and the 2nd, 4th, and 6th transfer aliquots. After the transfers each plate was incubated at 4 °C for 24 hours while being covered with a sealing matt and wrapped in aluminum foil to allow time for further NSB adsorption. After the 24-hour incubation, aliquots of NSB samples were transferred to new wells and combined with the

corresponding compound's stable isotope internal standard (IS) followed by subsequent LC/MS/MS analyses.

Preliminary Data: In general, the NSB losses increased with additional transfers. For COOH-THC, the NSB losses of one commercial plate after 6 transfers were 16.8% while another commercial plate showed 22.6% analyte loss. For JWH-18 and JWH-73, the respective microplates showed lower NSB losses for these compounds (7.5% and 8.2%) compared to the other plate (26.2% and 25.0%). For cetrorelix, a similar trend was observed with NSB losses of 28% and 57.9%, respectively, while for chlorhexidine, the corresponding NSB losses were 27.4% and 35.2% after the six transfers.

Novel Aspect: NSB losses for two different microplates were evaluated at low analyte concentration using highly sensitive and selective LC/MS