Tuesday, July 29, 2014

Poster Session: 9:30 AM - 5:00 PM

Mass Spectrometry Applications

A-375

Tandem mass method for disaccharide units of urinary glycosaminoglycans from MPS patients

C. Chuang, H. Lin, C. Tsai, S. Lin. *Mackay Memorial Hospital, Taipei, Taiwan*

Background: Identification of acid mucopolysaccharide by liquid chromatography/ tandem mass spectrometry method (LC-MS/MS) of predominant disaccharide units of glycosaminoglycans (GAGs) (chondroitin sulfate, CS; dermatan sulfate, DS; heparan sulfate, HS) after methanolysis is validated and applicable for mucopolysaccharidosis (MPS) phenotype determinations.

Methods: A total of 76 urine samples were collected and analyzed, including 9 MPS I patients, 13 MPS II, 7 MPS III, 8 MPS VI, and 39 normal controls. Urinary GAG was first precipitated by Alcian blue method followed by a treatment of 3N HCl methanol. The protonated species of the methylated disaccharide products were detected by using a multiple reaction monitoring experiment. Internal standards, [$^2H_6$] CS, [$^2H_6$] DS and [$^2H_6$] HS, were in-house prepared by deuteriomethanolysis of CS, DS and HS.

Results: The within-run and between-run precisions were good, and the recoveries were 94.3% for DS and 95.1% for HS. Linearity of DS and HS was calculated individually and the correlation coefficients (r) were 0.9914 for DS and 0.9935 for HS, respectively. One particular disaccharide for each GAG was selected, in which the parent ion and its daughter ion after collision were *m/z* 426.1→236.2 for DS (*m/z* 432→239 for dimmers derived from [$^2H_6$] DS) and *m/z* 384.2→161.9 for HS (*m/z* 390.4→162.5 for the [$^2H_6$] HS dimmer). The results were correspondent well when comparing with the two-dimensional electrophoresis method. The quantities of DS and HS were determined, which were varied from one MPS phenotype to the others, and the results can be used to evaluate the severity of MPS subgroups, as well as the amelioration of follow-up after enzyme replacement therapy (ERT).

Conclusion: The modified LC-MS/MS method for MPS phenotype determination is specific, sensitive, validated, accurate and applicable for simultaneous quantifications of urinary DS and HS. This method can help to make correct diagnosis of MPS patients and evaluate the effectiveness of ERT.

A-376

Improving detection limits of prohibited substances and therapeutics by Solid Phase Microextraction (SPME) coupled to LC-MS/MS

F. Ruparelia[1], J. Pawliszyn[2], N. Reyes-Garcés[2], G. Augusto Gómez-Ríos[2], B. Bojko[2]. [1]*IONICS Mass Spectrometry Group Inc., Bolton, ON, Canada,* [2]*University of Waterloo, Waterloo, ON, Canada*

This work presents the enhancement achieved in limits of detection for a set of prohibited substances and therapeutics by coupling a completely automated thin-film SPME analytical protocol to a powerful LC-MS/MS system developed by IONICS Mass Spectrometry.

LC-MS/MS analyses were performed using a triple quadrupole MS 3Q-320 (IONICS, Bolton, Ontario, Canada). Complete separation was achieved using a pentaflourophenyl column (Phenomenex, Torrance, CA, USA) and a ternary gradient of water, methanol and acetonitrile (0.1% formic acid in all mobile phases). Sample preparation was performed using a Thin Film Microextraction (TFME)-Concept autosampler. The automated procedure consisted of the following steps: TFME pre-conditioning (samples were simultaneously incubated at 30 ºC) (30 min), extraction (75 or 90 min from pooled urine samples spiked at different concentration levels), washing step (10 s), and desorption in a proper solvent for 20 or 60 min. HLB and C18 coatings in thin-film SPME configuration were prepared in-house following a protocol developed in our laboratory.

In order to preserve sporting ideal and ensure fair play game, the World Anti Doping Agency (WADA) has banned performance-enhancing substances in competitive sports. In most cases, despite the use of extensive and cumbersome sample preparation protocols, the extraction and detection of these compounds in complex matrices such plasma, urine, and blood, can be a challenge. Solid phase microextraction (SPME), a green chemistry technique that combines sampling/sample preparation in a single step, has shown to be a powerful tool for the determination of multiple prohibited drugs in complex matrices. However, in some circumstances the quantification capabilities of SPME are constrained by the instrumental limits of detection and quantification of the LC-MS/MS systems.

This work presents a SPME method coupled to an effective MS/MS system towards the quantification of compounds with low minimum required performance level (MRPL) values set by WADA. Analytes selected in this study (boldenone, cannabidiol, cannabinol, dihydrotestosterone, fentanyl, fluoxymestrone, methyl-testosterone, nandrolone, testosterone and 19-norandrosterone) were of particular interests here as they did not meet the MRPL levels with the SPME-LC-MS protocol previously developed in our group. Analysis of neat standards (prepared in methanol at a concentration range of 0.05 to 100 pg/µL) performed in a triple quadrupole MS IONICS 3Q-320 demonstrated allowed meeting the MRPL values required by WADA, and selected compounds displayed outstanding limits of quantification. For instance, instrumental LOQs of 33 and 200 ag/µL were achieved for fentanyl and testosterone, respectively, with a correlation coefficient equal or better than 0.999. In addition, therapeutics such as pancuronium and rocuronium exhibited similar instrumental LOQs using the same MS analyzer. These findings provide an opportunity to expand the applicability of SPME to quantify low concentrations not only for ex-vivo analysis but also for in-vivo applications in which the temporal resolution, sensitivity and accuracy provided by SPME is highly desired.

A-377

Enhanced Resolution and Matrix Interference Reduction for the Analysis of Vitamin D Metabolites

C. Aurand, D. Bell, T. Ascah-Ross. *Supelco, Bellefonte, PA*

Analysis of Vitamin D metabolites has continued to be a topic of interest in recent publications, primarily as biomarkers for possible disease states and vitamin sufficiency. While Vitamin D is present in two forms, current ELISA methods cannot distinguish D2 and D3 forms of the vitamin metabolites resulting in a reporting of total 25-hydroxyvitamin D. In this study, an LC/MS method for the analysis of Vitamin D metabolites is expanded to include dihydroxy metabolites along with the epi-homologs. Chromatographic resolution is utilized for the quantitation of hydroxy and dihydroxy Vitamin D2 and D3 metabolites including the isobaric epimers. In addition, sample preparation techniques are evaluated for the impact of biological matrix ionization effects.

Chromatographic method development consisted of screening C18, Cyano, Phenyl Hexyl and pentyl fluorophenyl (F5) stationary phase. Method development experminets resulted in conditions for the direct quantitation of isobaric metabolites 25 hydroxyvitamin D3, 3-epi-25 hydroxyvitamin D3 1-α hydroxyvitamin D3 along with 25 hydroxyvitamin D2, 3-epi-25 hydroxyvitamin D2. In addition, human serum samples were processed using standard protein precipitation techniques along with novel phospholipid depletion plates for the comparison of matrix interference impact. . The unique combination of the selectivity of the F5 separation along with the novel sample preparation technique allow of a robust and accurate LC/MS method for quantitation of all the associated Vitamin D metabolites

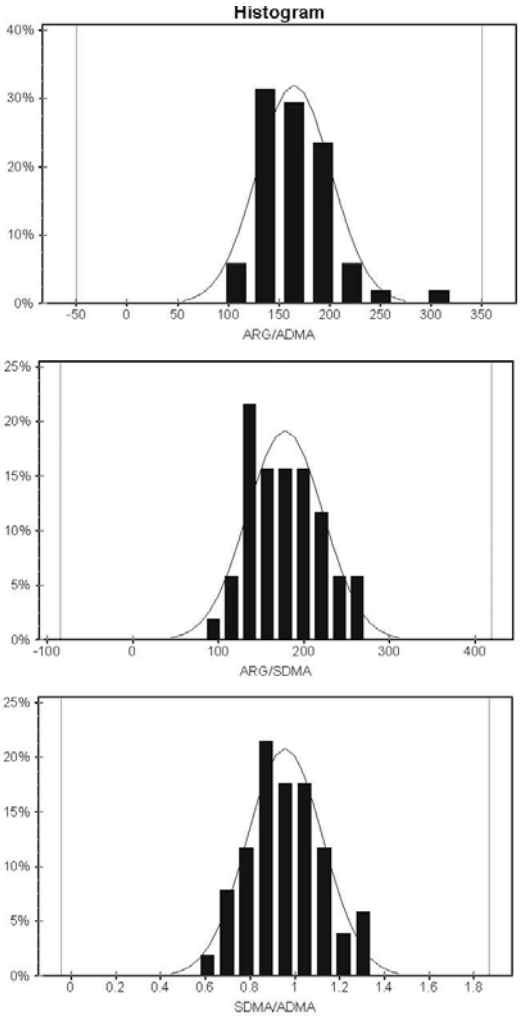
A-378

Reference interval determination for the ratios of L-arginine (ARG), symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA)

J. M. El-Khoury, D. R. Bunch, B. Hu, S. Wang. *Cleveland Clinic, Cleveland, OH*

Background: Symmetric dimethylarginine (SDMA) and asymmetric dimethylarginine (ADMA), metabolic products of methylated L-arginine (ARG) containing proteins, play an important role in regulating nitric oxide production. Recently, SDMA and ADMA have been extensively evaluated as biomarkers of renal and/or cardiovascular diseases with indication of potential use of the ratios of ARG/ADMA and SDMA/ADMA. More specifically, the ratio ARG/ADMA has been shown to be an independent predictor of mortality in patients with dilated cardiomyopathy, while the ratio SDMA/ADMA has been investigated as a biomarker of hypertension in rats and was found to be a better predictor for disease activity and progression than the individual parameters alone. However, reference intervals (RIs) for the ratios ARG/ADMA, ARG/SDMA and SDMA/ADMA have not been reported in the literature. Our objective in this study was to determine RIs for these ratios in a healthy adult population using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. **Methods:** Collection of blood samples for RI determination was approved

by the Institutional Review Board. In brief, EDTA whole blood samples (n = 51) were collected from healthy adults (39 females) identified via a questionnaire, aged 19-64 y (38.8 ± 12.6), after a minimum of 8 hour fasting. A published LC-MS/MS assay was used for the analysis of ARG, SDMA and ADMA. EP Evaluator release 10 was used for statistical analysis. **Results:** The central 95% RI for ARG/ADMA, ARG/SDMA and SDMA/ADMA were 108 to 247, 95 to 261, and 0.64 to 1.27, respectively. Based on EP evaluator's determination a transformed parametric method for ARG/ADMA and a parametric method for ARG/SDMA and SDMA/ADMA were used. Histograms are shown in the figure. **Conclusion:** RIs for ARG/ADMA, ARG/SDMA and SDMA/ADMA were determined using a well-defined healthy population by an LC-MS/MS method. These values are important in defining the clinical utility of these parameters.



A-379

A unique brain lipidome and metabolome biosignature in Alzheimer's Disease

G. Astarita[1], G. Paglia[2], J. Langridge[3], S. Lai[3]. *[1]Georgetown University/ Waters Corporation, Milford, MA, [2]Center for Systems Biology, University of Iceland, Reykjavik, Iceland, [3]Waters Corporation, Milford, MA*

Alzheimer's disease (AD) is the most common cause of adult dementia, but the cause of this inexorable neurodegenerative disease remains still elusive. Alterations in both lipid and polar metabolites biochemical pathways have been associated with AD. Here we conducted an unbiased investigation of the underlying biochemical alterations in AD human tissues. We used an integrated lipidomics and metabolomics approach to survey frozen brain tissue samples from clinically characterized AD patients and age-matched controls. Lipids and polar metabolites were extracted using a biphasic, liquid-liquid extraction procedure. Polar metabolites were separated using a hydrophobic interaction liquid chromatography (HILIC), whereas lipids using an integrated microfluidic device packed with reversed phase C18. Travelling-Wave ion mobility mass spectrometry was used to improve peak capacity and CID fragmentation specificity. Moreover, ion mobility-derived collision cross sections provided orthogonal physicochemical data that were used with retention time, accurate mass and MS/MS data to increase confidence of metabolite identification. Data was collected using both negative and positive ionization mode in the data-independent acquisition mode with an alternate low and elevated collision energy method to acquire both precursor and product ion information in a single analytical run. Lipidome and metabolome data were fused and mined using multivariate statistical and pattern-recognition tools. Initial observations were confirmed using more targeted approaches for quantification. Pathway analysis was then used to incorporate the novel molecular information into the known biochemical pathways. The results obtained were further integrated with clinical data to generate testable hypotheses on the functional significance of the abnormalities observed in AD. Our preliminary results reveal novel molecular alterations in AD and a unique lipidome and metabolome biosignature that differentiates the brains from individuals with AD compared from those from control subjects.

A-380

Pain Management Drug Monitoring in Urine using HPLC-MS/MS

J. K. Wolken, T. Doran, P. Smith, A. Iwanski, D. Wiebe. *University of Wisconsin Hospital and Clinics, Madison, WI*

Background: With prescription drug abuse reaching epidmic proportions, clinicians are seeking solutions to monitor aptients on long-term pain management prescriptions. These monitoring programs need to be sensitive to low level amounts of pain management therapeutics in a patient population correctly adhering to physicians orders while screening for non-prescribed pain medication and drugs of abuse. The University of Wisconsin Hospital and Clinics (UWHC) Toxicology group has developed a clinical test which will monitor 35 drug substances with minimal sample preparation in urine in a 10 minute HPLC-MS/MS run. **Methods:** The following compounds are monitored in the following catagories: **Drugs of abuse**: 6-MAM (heroin metabolite), amphetamine, benzoylecgonine (cocaine metabolite), MDA, MDMA (ecstasy), and methamphetamine. Benzodiazepines: alprazolam, hydroxyalprazolam, 7-aminoclonazepam, diazepam, nordiazepam, lorazepam, midazolam, oxazepam, and temazepam. Opioids: buprenorphine, norbuprenorphine, codeine, fentanyl, norfentanyl, hydrocodone, hydromorphone, meperidine, normeperidine, methadone, EDDP (methadone metabolite), morphine, naloxone, naltrexone, oxycodone, oxymorphone, tapentadol, N-desmethyltapentadol, tramadol, and N-desmethyltramadol. Patient urine is mixed 1:1 with an internal standard which consists of 8 deuterium labeled compounds (a subset of the 35). This is injected onto a BiPh Restek column, and using a Agilent 1200 HPLC the compounds are eluted into a AB Sciex 4000 triple quadruploe mass spectrometer. Each compound has 2 ion transitions for identification and quantitation. The AMR for most of the compounds are 25-1000 ng/ml with most of the benzodiapizines having an AMR of 10-1000 ng/ml. Results: The within run CV range for the compounds at the low end cutoff (25ng/ml for most compounds) was between 1.5-10.1% where the day to day precision at the same low cutoff was 4.0-15.5%. Carryover has not been shown to be an issue for any drug substance despite some extremely high patient samples being analyzed. Comparison of methods to an in-house GC-MS method showed increased sensitivity for most compounds, most notably 6-MAM, morphine, and oxycodone. Assayed College of American Pathologist (CAP) Toxicology surveys from 2011 and 2012 showed 100% qualitative agreement for all 30 survey samples for the compounds in the Pain Management Profile. Conclusion: The new pain panel method shows very little limitation in 'real world' patient samples. Even with minimal sample preparation steps very few issues have arisen with interferences, ion suppression, and retention time shifts. We advise our clinicians that the panel results are only a snapshot in time. The dose, time of dose, state of hydration, and individual metabolism all play a part in the concentration of drug substances in the urine. Intermittant testing over time is recommended to help compile a clearer picture of the patients' compliance.

A-382

Determination of Tacrolimus and Sirolimus in whole blood by liquid chromatography electrospray ionization tandem mass spectrometry

M. E. R. Diniz, L. S. Vilhena, T. M. C. C. Barbosa, B. P. Paulo, E. C. C. Mateo, A. C. S. Ferreira. *Instituto Hermes Pardini, Vespasiano, Brazil*

Tacrolimus and Sirolimus are immunesuppressive drugs used in organ transplantation, exhibit narrow therapeutic ranges and adverse effects are common. Methods based on Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) are considered

the gold standard in therapeutic monitoring of these drugs. So a simple, rapid and sensitive LC/MS/MS method has been developed and validated for the determination of Tacrolimus and Sirolimus in whole blood using Ascomycin as the internal standard (IS). In this process, 100 µL of whole blood samples containing the internal standard were treated by liquid-liquid extraction using ethyl acetate and subjected to LC-MS/MS analysis using positive electrospray ionization (ESI+). Chromatographic separation was performed on a Symmetry C18 column (3.5 µm 4.6 x 75 mm) and mobile phase acetonitrile:methanol:water (80:10:10, v/v/v) with 0,1% of formic acid and 0,02% of Ammonium hydroxide 25% at 400 µL/min. The MS/MS detection was conducted by monitoring the fragmentation ions of 821.7→768.5 (m/z) for Tacrolimus, 931.5→864.5 (m/z) for Sirolimus and 809.7→756.7 (m/z) for Ascomycin. Ammoniated adducts of protonated molecules were used as precursor ions for all analytes. The method had a chromatographic running time of approximately 4 min. The linear analytical range of the procedure was between 1.0 and 51.0 ng/mL for Tacrolimus and 2.0 and 52.0 ng/mL for Sirolimus. The medium range of recovery for the Tacrolimus was 98.1-103.2% over a interval of 2.0-37.5 ng/mL and for the Sirolimus 97.1-106.1% over a range of 2.0-39.0 ng/mL. The intra and inter-day precision was less than 6.8% for Tacrolimus and 10.8% for Sirolimus. In conclusion, the LC-MS/MS method has been developed successfully for the quantitative analysis and therapeutic monitoring of these immunosuppressive drugs.

A-383

Evaluation of high performance liquid chromatography and liquid chromatography-tandem mass spectrometry methods for 25 (OH) D_3 assay

B. Omer, F. A. Aydin, P. Mikailova, S. Genc. *Istanbul University Istanbul Medical Faculty, Istanbul, Turkey*

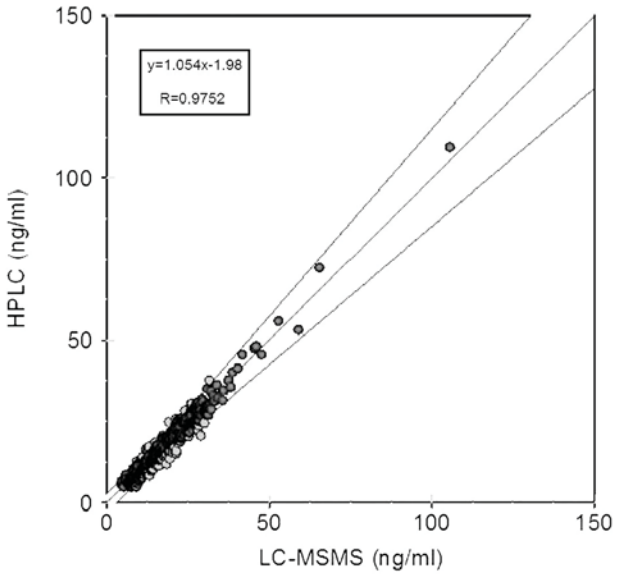
Background: Growing evidence about the role of vitamin D on health in different fields of medicine emerged the necessity of establishing more reliable and accurate 25 (OH) vitamin D_3 assessment methods. This study was designed to compare performance characteristics of two different 25 (OH) vitamin D_3 assessment methods.

Methods: 25 (OH) D_3 vitamin levels were quantified using two methods as follows: high-performance liquid chromatography (HPLC) with Thermo Finnigan TSP (Florida-USA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) with Zivak Technologies ZinMass-200 LC-MS/MS System with APCI ionization source (Istanbul-TURKEY) as the reference method. Assay performance characteristics were performed according to the National Committee for Clinical Laboratory Standards (NCCLS) .The comparison studies were done using randomly chosen 306 plasma samples from routine clinical samples submitted for 25 (OH) D_3 vitamin measurement.

Results: The LC-MS/MS assay had within-run coefficient of variation (CV) 7.3% and between-day CV 6.0% for low control (22.4±3.3 ng/mL), for high control (82.3 ±11.8 ng/mL) within-run CV 7.2% and between-run CV 9.6%. HPLC method had within-run CV 6.9% and between-day CV 12.5% for low control, and for high control, within-run and between-run CVs were 5.6 and 8.7 respectively. The linearity studies showed good correlations between expected and obtained values for both methods. When the relationship between the results obtained from HPLC and LC-MS/MS assays was investigated in 306 subjects, the HPLC assay showed an acceptable correlation with the LC-MS/MS (y=1.054x-1.98, R=0.9752).

Conclusion: With good precision and accuracy, HPLC system revealed an acceptable correlation with LC-MS/MS for 25 (OH) vitamin D_3 assay.

Figure 1: Comparison of plasma 25 (OH) vitamin D_3 levels measured by HPLC vs LC-MS/MS.



A-384

Elimination of false positives and false negatives for the screening of amphetamines, PCP, and benzoylecgonine by Agilent RapidFire

D. Ames. *IQ Laboratories, Fort Wayne, IN*

This research shows a more accurate alternative to using an immunoassay for the screening of amphetamines, PCP and benzoylecgonine. Immunoassays are the typical method used to screen for amphetamines, PCP, and benzoylecgonine. However, immunoassays are known to produce false positives, false negatives and require the use of expensive reagents. By utilizing an Agilent RapidFire 300 High-throughput System we eliminated false positives and false negatives. False positives and false negatives were eliminated because RapidFire uses an Agilent QQQ triple quad mass spectrophotometer as the detection method rather than enzymes or antibodies. The use of a triple quad mass spectrophotometer as the method of detection also allows for selectivity that is not possible with immunoassays. In this study on RapidFire PCP, benzoylecgonine, amphetamine, methamphetamine, MDMA, MDA, MDEA, methylphenidate and ritalinic acid were screened for and on the DxC 600i the reagents from Beckman Coulter for amphetamines, PCP, MDMA and benzoylecgonine were used. Patients were screened on a Beckman Coulter DxC 600i and then on an Agilent RapidFire 300 High-throughput System connected to an Agilent 6460 QQQ triple quad mass spectrophotometer. Those patients found to be positive on either system were then analyzed by LC-MS/MS for further confirmation. The use of RapidFire allowed us to eliminate false positives and false negatives and increase selectivity.

A-386

Determination of urinary ethyl glucuronide and ethyl sulfate by LC/MS/MS for clinical research

L. Cote. *Agilent Technologies, St-Laurent, QC, Canada*

Background: Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited for the rapid analysis of multiple analytes. A highly sensitive and specific LC/MS/MS analytical method has been developed for the quantitation of urinary ethyl glucuronide (EtG) and ethyl sulfate (EtS). A dilution procedure and a solid phase extraction (SPE) procedure are evaluated and compared based on ease of use, analyte recovery and post-extraction cleanliness.

Methods: A dilution procedure and a solid phase extraction (SPE) sample preparation procedure were developed and compared for the simultaneous extraction of ethyl glucuronide and ethyl sulfate in urine. Calibrators were created by spiking synthetic urine (Surine-Cerilliant) with various concentrations of EtG and EtS standards (Cerilliant). The chromatographic system consists of a Polaris 3 C18-Ether column coupled with a guard column and a mobile phase comprised of acetonitrile and water containing 0.1% formic acid. Quantifier and qualifier transitions were monitored. EtG-D5 and EtS-D5 internal standards (Cerilliant) were included to ensure accurate and reproducible quantitation. Urine controls (UTAK Laboratories) were used and samples were kindly supplied by collaborators.

Results: The separation of EtG and EtS from isobaric interferences is especially critical; without proper separation by retention time, impurities present in both compounds can cause interferences with one another and lead to inaccurate quantitation. The described method achieves the required functional sensitivity and is capable of quantitating analytes over a sufficiently wide dynamic range with a single injection. All analytes displayed excellent linearity from 25 to 10000 ng/ml. All calibration curves displayed an R2 > 0.9993. Back calculated accuracies for all calibrators ranged from 93% to 120% for the dilution procedure and from 96 to 113% for the SPE procedure. Commercially available quality control material was used to test the reproducibility of this method. Measurements were repeated on three separate days to assess interday reproducibility and CVs were found to be below 6%.

Conclusion: A method has been developed for quantifying ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine for clinical research. Two sample preparation procedures consisting of a simple dilution from urine and SPE are shown. Chromatographic separation of all analytes and interferences with conditions compatible with LC/MS/MS have been developed. Typical analytical method performance results are well within acceptable criteria.

A-387

A Quantitative Determination of Methadone and its Metabolite (EDDP)in Dry Blood Spot by LC-MS/MS

J. Ye, H. Qiao, E. Majdi, L. Cousins. *IONICS Mass Spectrometry Group Inc., Bolton, ON, Canada*

Background: Critically ill children routinely receive opioids for analgesia and sedation to reduce pain and stress, facilitate ventilation, and avoid secondary complications. The typical course of treatment often induces tolerance, and withdrawal symptoms may be precipitated if the drugs are discontinued abruptly. Withdrawal symptoms are not only unpleasant but can be life-threatening and may prolong the need for mechanical ventilation and potentially extend hospitalization. Methadone is a synthetic opioid receptor agonist widely used in the treatment of severe pain and in maintenance treatment of opioid addicts. It is approved by the U.S. Food and Drug Administration for detoxification treatment of opioid addiction in adults, but does not have a label for pediatric use. Therefore it is necessary to monitor the dosage and to avoid the abuse. The present study provides a much simplified approach whereby a novel, highly sensitive LC-MS/MS triple quadrupole mass spectrometer is used to measure directly the methadone and EDDP concentrations through the dried blood spot (DBS) samples.

Methods: Fresh human whole blood was spiked with different concentrations of methadone and EDDP. 30 ul of the blood were spotted onto Whatman DMPK-C cards. Cards were air dried for about 2 hours. 6 mm spot were punched placed into a vial containing 100ul IS (0.01ng/mL) working solution. Samples were vortexed for 3 minutes and centrifuged for 5 minutes at 4000 rpm. The supernatants were transferred into HPLC vials for analysis. An IONICS 3Q 220 mass spectrometer was used. This instrument is equipped with heated coaxial flow ion source and "Hot Source-Induced Desolvation" interface, with a multi-orthogonal channel and laminar flow sampling. The samples were injected using Shimadzu UFLC XR system. Sample was loaded to a Chromolith-RP18E column (100X3 mm, 3 um) with a gradient method at 0.5 mL/min. Mobile Phases were A (0.1% formic acid 100% H2O) and B (0.1% formic acid in 100% ACN). The total LC run time is 3.5 minutes.

Results: Calibration curve of the neat methadone and EDDP solution showed good linearity over a range of 0.0025-10 ng/mL with correlation values of R^2=0.9997 and 0,9998, respectively. In DBS extraction the calibration curves for methadone and EDDP over a range of 0.1 to 100 ng/mL were created. The curves also showed good linearity with weighting factor of 1/x for both analytes. The correlation values were R^2=0.997 and 0.998 for methadone and EDDP, respectively. At LLOQ of 0.1 ng/mL, a good accuracy of 108% and 99% and CVs of 9% and 8.3% were obtained for methadone and EDDP, respectively.

Conclusion: The results in this study show a fast, accurate, and precise LC-MS/MS method with IONICS 3Q 220 mass spectrometer for quantifying methadone and EDDP in DBS samples. The LLOQs for both samples are 0.1 ng/mL with good precision and accuracy. The sample preparation procedure is simple and rapid without SPE and LLE extraction. Therefore, the LC-MS/MS method in this study has confirmed its clinical applicability and can be used in routine bioanalysis, especially for methadone level monitoring.

A-388

Increased Throughput for the Analysis of delta-9-THC in Oral Fluids using Triple Quadrupole Mass Spectrometry coupled to Automated Dual-Channel HPLC

K. McCann, A. Szczesniewski. *Agilent, Santa Clara, CA*

Background: Many forensic labs are interested in improved sample throughput to get better utilization of the testing instrumentation. Through this work we demonstrated the ability to increase mass spectrometer productivity through the automated use of a dual channel high performance liquid chromatography (HPLC) system. A newly developed software interface intelligently determines the timing of all HPLC components and coordinating the analytical utilization of the mass spectrometer.

Methods: The integrated LC/MS/MS system is comprised of a triple quadrupole mass spectrometer coupled to a configurable HPLC system, all controlled by a single software application. For the purposes of this work, the HPLC system consists of a high-capacity autosampler, two binary pumps, two HPLC columns, two temperature-controlled column compartments and one switching valve. To operate the system, a standard data file collected by LC/MS/MS is loaded into the software. The data analysis method is extracted from the data file and a window of interest is specified using the data file's chromatogram. Based on that information, the software automatically coordinates all timing related to running the HPLC system.

Results: The analysis of delta-9-THC is performed in many forensic toxicology labs analyzed by LC/MS/MS where sample throughput is a major concern. An established LC/MS/MS method for the analysis of this analyte from oral fluids was used for testing the capabilities of this new instrument. The standard method uses an autosampler, binary pumps, HPLC column and temperature-controlled column compartment. With a runtime of 5 minutes, the analytes of interest reach the mass spectrometer between approximately 2 minutes to 4 minutes. Hence, more than 50% of the data collected by the mass spectrometer is of no interest.

The standard method utilizes what is considered a single HPLC stream. The new HPLC system mirrors certain components of this single stream system to provide a second stream, operating in parallel to the first stream. By loading the standard method and window of interest into the automation software, the software is able to determine the most efficient method of injecting and analyzing a list of delta-9-THC samples without any user configuration necessary. By staggering injections on parallel streams and switching between the two streams at the appropriate time, throughput of the integrated expanded system can double the throughput achieved with the standard method, without any sacrifice to the quality of quantitation.

Conclusion: Fully automated software controlling a completely integrated LC/MS system consisting of two parallel LC streams has been developed and implemented in the analysis of Δ9-THC. No special method development is required; the user supplies a standard method and defines a window of interest, allowing the software to determine all necessary timing and coordination of the analysis. Throughput for this method has been doubled through the use of an Automated Dual-Channel HPLC.

A-389

Quantitative Analysis of Acetaminophen and Salicylic Acid in Urine by Rapidfire Coupled with Triple Quadrupole Mass Spectrometry

F. Mbeunkui, S. Sullivan, R. B. Dixon. *Physicians Choice Laboratory Services, Rock Hill, SC*

Background: A rapid and quantitative method for the direct quantitation of acetaminophen and salicylic acid in biological matrices is warranted in clinical and toxicological chemistry. Compliance monitoring and therapeutic drug monitoring of these compounds can inform clinicians when assessing potential side-effects of the drugs including hepatocellular damage or tinnitus. The predominate method for measuring acetaminophen and/or salicylate in biological matrixes is by enzyme immunoassay (EIA). EIA techniques and reagents are labeled and limited to use for serum testing. The purpose of this assay is to provide specific drug and metabolite quantitative data in alternative biological matrices.

Methods: Rapidfire is an automated sampling instrument that injects the sample onto a small cartridge packed with stationary phase. The instrument is programmed to load the sample onto a cartridge then rinse with aqueous mobile phase. After rinsing the sample, the Rapidfire diverter valve switches the flow path to the mass spectrometer. A highly organic mobile phase then elutes the purified compounds which are ionized by electrospray ionization prior to detection by the triple quadrupole mass spectrometer. The panel detects acetaminophen, acetaminophen-glucuronide, and salicylic acid. Deuterium labeled internal standards for acetaminophen and salicylic acid are utilized for isotope dilution. Quantitation is enabled by calibration of the system with 7 levels

of matrix spiked standards constructing a standard curve prior to sample analysis.

Results: We have developed a quantitative Rapidfire mass spectrometry (RFMS) method for the analysis of acetaminophen and salicylic acid in urine. The sample preparation method is simple. Samples are hydrolyzed, then diluted with an internal standard solution prior to loading on the RFMS. The analytical procedure is approximately 10 seconds from sample to sample. Due to the high variability of matrix affects in patients undergoing chronic pain or mental health treatment, it is necessary to have a robust analytical method. To that end, we have investigated a wide range of samples with variable creatinine concentrations. The calibration range of the method is 0.5-200 µg/mL for acetaminophen and 1-400 µg/mL for salicylic acid providing a wide analytical measurement range. External QC materials were also tested with acceptable imprecision and accuracy (<15%). Real patient samples have been analyzed and this procedure demonstrates a robust and reproducible method for this assay.

Conclusion: This technique provides rapid and quantitative analysis of acetaminophen and salicylic acid from biological specimens with minimal sample preparation.

A-390

Determination of Insulin-Like Growth Factor-1 in serum by HRAM LC-MS for clinical research

L. Cote[1], K. McCann[2], C. Chu[2]. *[1]Agilent Technologies, St-Laurent, QC, Canada, [2]Agilent Technologies, Santa Clara, CA*

Background: High Resolution Accurate Mass (HRAM) Liquid Chromatography Mass Spectrometry (LC-MS) is ideally suited for the rapid analysis of biomolecules. A highly sensitive and specific HRAM LC-MS method has been developed for the quantitation of Insulin-Like Growth Factor-1 (IGF-1) in serum. This method uses a simple sample preparation combined with an online sample cleanup procedure coupled to a high resolution accurate mass quadrupole time-of-flight mass spectrometer.

Methods: An efficient sample preparation procedure was developed for the extraction of IGF-1 in serum. Calibrators were created by spiking clean serum with various concentrations of IGF-1. The chromatographic system consists of a C8 extraction column coupled with a high resolution, 300 angstrom pore size analytical column and a mobile phase comprised of acetonitrile and water containing 0.2% formic acid. Quantifier and qualifier transitions were monitored and Rat IGF-1 internal standard was included to ensure accurate and reproducible quantitation.

Results: Online sample cleanup and chromatographic separation of a sample is achieved in less than three minutes. The separation of Rat IGF-1 and Human IGF-1 is especially critical since these compounds share common interferences. Without proper separation by retention time, impurities present in both compounds can cause interferences with one another and lead to inaccurate quantitation. The described method achieves the required functional sensitivity and is capable of quantitating IGF-1 over a sufficiently wide dynamic range. This method displayed excellent linearity from 5.63-990 ng/mL. All calibration curves displayed an R2 > 0.999. Back calculated accuracies for all calibrators ranged from 84% to 105% and showed intra-day CVs below 8%. Separately prepared incurred samples were used to test the accuracy and reproducibility of this method. Measurements were repeated in triplicates and on three separate extractions to assess intra- and inter- day reproducibility and CVs were found to be below 10%.

Conclusion: A robust method for quantifying Insulin-Like Growth Factor-1 in serum with excellent reproducibility and accuracy has been developed.

A-391

Fast Determination of Serum Methylated Arginines By Liquid Chromatography Tandem Mass Spectrometry

A. Unlu, S. Abusoglu, F. Akyurek. *Selcuk University, Konya, Turkey*

Background: NG,NG-dimethyl-l-arginine, or asymmetric dimethylarginine is a naturally occurring amino acid that circulates in plasma. Free ADMA, and related amino acids, NG-monomethyl-l-arginine, as well NG,N_G-dimethyl-l-arginine are produced normally by all cells from hydrolysis of proteins containing methylated l-arginine residues. SDMA shares the pathway for cell entry with arginine. Endogenous methylarginines are important potentially modifiable molecules that may be associated with impaired synthesis of nitric oxide. The aim of this study was to implement a fast, accurate and simple mass spectrometric method for serum methylated arginine.

Methods: 100 µL of internal standard in methanol were added to 200 µL of serum and centrifuged at 13.000 rpm for 10 minutes. Supernatant was evaporised with N2 flow at 65 ◦C. Derivatisation step was performed dissolving the dried extract in 200 µL of a freshly prepared butanol solution containing 5% (v v^{-1}) acetyl chloride and kept at 65 ◦C for 30 min. The solvent was removed by evaporation under nitrogen flow at 65 ◦C. The samples were dissolved in 200 µL of water–methanol (90:10, v v^{-1}) containing 0.1% (v v^{-1}) formic acid and 40 µL injected into system. Multiple reaction monitoring was performed with a continuous infusion of a 50 µM solution of each analyte. Recovery test was calculated as average of "measured value/expected value" ratio (%). Limit of detection and quantification were determined by a signal to noise ratio of 3:1 and 10:1, respectively. Inter- and intra-assay precision were evaluated by analysis of ten replicates of C1, C2 and C3, daily for 3 days and expressed as mean, SD and CV%.

Results: This method's intra-assay CV and % bias values were 15.6,10.2; 9.72,7.88 and 6.45,6.02 for 0.4, 0.8 and 1.6 µmol/L ADMA, respectively. Calibration curves in serum were obtained using concentrations of ADMA, SDMA, NMMA at 0.2, 0.4, 0.8, 1.6, 3.2, 32 µM and of Arg and Cit at 1, 25, 50, 100, 250 µM. The linearity of calibration curves in plasma was estimated by the coefficients of correlation (r^2), which ranged from 0.987 to 0.999. The standard curves for serum asymmetric dimethylarginine was linear within the range of 0.2-32 µmol/L. The equation for calibration was y=0.943x + 7.469 and R^2=0.992. Total run time was 5 minutes. Recovery was found to be between 90-105%. Limit of detection and limit of quantification were 0.1 and 0.25 µmol/L for ADMA, respectively. The intra- and inter-assay CV values were below 20% for SDMA; LNMMA, arginine and citrulline.

Conclusion: Satisfactory characterization, stability of the label during chromatography as well as mass spectrometry, standardization of commercially available as well as of self-synthesized stable-isotope labeled analogs of analytes, and final added concentration of the internal standard in the matrices being analyzed is essential and crucial for reliable

quantitative analysis. Data from calibration curves and method validation reveal that the method is accurate and precise. The short and fast run time, the feasibility of high sample throughput and the small amount of sample required make this method very suitable for routine analysis in the clinical setting.

A-392

Comparison of Q-TOF Acquisition Modes for Quantitative Analysis of Tetrahydrocannabinol Metabolite

H. Park[1], N. Chindarkar[2], R. Fitzgerald[2]. *[1]Sungkyunkwan University School of Medicine, Seoul, Korea, Republic of, [2]University of California-San Diego, San Diego, CA*

Background & Objectives:

In clinical chemistry laboratories, unit resolution tandem mass spectrometry (MS) platform is considered the gold standard for quantitative analysis of small molecules. High resolution mass spectrometry (HRMS) is also a viable option with higher specificity and retrospective full scan analysis capability. The previously limited quantitative capability of HRMS has improved, such that both high performance screening and acceptable quantitation may now be feasible. We evaluated the quantitative performance of a quadrupole-time of flight (Q-TOF) analyzer for urine 11-nor-9-carboxy tetrahydrocannabinol (THC-COOH). Three Q-TOF acquisition modes MS^E, Q-TOF, and Q-TOF with enhanced product ion sensitivity (EPIS) were optimized and compared to unit resolution tandem MS.

In the Q-TOF mode, the quadrupole selects a precursor mass followed by collision cell fragmentation and TOF full scan analysis of product ions. Q-TOF-EPIS mode is the same except product ion sensitivity is increased by limiting the TOF scan range around a set m/z value. MS^E works in dual scan mode to collect TOF full scan data with low collision energy (precursor ion scan) and high collision energy/ramp (fragment ion scan). The quadrupole is RF only in MS^E mode.

Experimental: Waters Acquity UPLC with 1)Xevo G2 Q-TOF and 2)Xevo TQ-S were used with a Phenomenex (Kinetex, C8, 2.6µm, 50x2.1mm) column. Mobile phases A/B were 5mM ammonium formate (pH 3)/0.1% formic acid in water/acetonitrile respectively (flow 0.4mL/min). The gradient was 30-90% B in 4min. During solid phase extraction 2mL of urine, internal standard (THC-COOH-d3) and diluent were applied on Phenomenex Strata-X-Drug B cartridges. Eluates were dried and reconstituted in 100uL of mobile phase of which 10uL was injected on UPLC. Calibration was carried out by using 5 calibrators over a range of 12.5-200ng/mL spiked in drug free urine. All MS were used in negative mode, with optimized settings of cone voltage (40V), capillary voltage (2.5V), desolvation temperature (500°C) and desolvation gas (1000L/h).

Results and Conclusion:

Each Q-TOF mode was first optimized for ionization of THC-COOH. We compared the signal intensity of THC-COOH in each mode by acquiring data by infusing pure

THC-COOH in methanol and by analyzing patient urine specimens that screened positive for THC metabolites by immunoassay. These patient specimens and a CAP PT sample were analyzed for this study. The concentration range of 13.2-175.8ng/mL was observed among patient specimens. We found excellent correlation of THC-COOH concentrations among all TOF modes and the unit resolution tandem MS. Q-TOF-EPIS was 2.4 fold more sensitive than Q-TOF mode. Of the TOF modes, MS^E provided the maximum sensitivity for quantitation (m/z 343.2 for MS^E and 343.2/299.2 for Q-TOF-EPIS). MS^E mode was approximately 2.5 fold more sensitive than the Q-TOF-EPIS mode but was less sensitive than unit resolution tandem-MS. Our data indicate that quantitative analysis on the Xevo G2 Q-TOF compares favorably with traditional unit resolution MS but is less sensitive. Future experiments will determine if the high mass resolution achievable with the TOF can be used to shorten chromatographic run times without sacrificing specificity.

A-394

Ultrafast, high-throughput quantitative analysis of creatinine in serum by laser diode thermal desorption coupled to tandem mass spectrometry

J. Lacoursiere, P. Picard, A. Birsan. *Phytronix, Quebec, QC, Canada*

Background: Serum cretatinine is used as an important indicator of renal health. Elevated level of creatinine in serum is a key clinical biomarker of impaired kidney function in humans. In some clinical or pre-clinical studies, only small amounts (µL) of serum are available for creatinine analysis. Several analytical methods are available for creatinine detection and quantification, but require large serum volume or take more than 3 minutes per sample analysis. An alternative approach to the analysis of creatinine enabling high- throughput analysis and needing low serum volumes was developed, using the laser diode thermal desorption (LDTD) coupled to a tandem mass spectrometer (MS/MS).

The objective of this work is to analyze multiple real patient samples using the LDTD-MS/MS and cross validate the results against known traditional methods for the detection and quantification of creatinine in serum.

Methods: Sample preparation consisted of a protein precipitation extraction by adding 190 µL of IS solution (2000 ng/mL d3-Creatinine) in acetonitrile to 10 µl of serum. After vortex-mixing and centrifugation (2 min at 14000 rpm), a 2 µL aliquot was deposited in a LazWell plate, and allowed to dry at room temperature. Creatinine is an endogenous compound and calibration curve cannot be made into the serum matrix. Standards were prepared into water and treated similarly to real sample. A 2µl aliquot of the final extracts was deposited into Lazwell plate and dried completely before analysis. The LDTD laser power was ramped from 0 to 65% of maximum power in 6 seconds and maintained 1 second at 65%. The mass spectrometer is operated in negative ionization MRM mode, monitoring transitions 112 -> 41 for creatinine.

Results: A simple high-throughput protein precipitation method for creatinine analysis in serum was developed and validated. The optimization of instrumental parameters and a method application will be presented. The method demonstrated a linear dynamic range over two orders of magnitude, between 0.04 and 4 mg/dL. Standard curves of creatinine spiked serum extracted using this method shows good linearity (R2 between 0.9995 to 0.9992 over the quantification range. Three levels of QC samples were prepared by spiking known creatinine levels in serum for the validation tests. The endogenous concentration of creatinine was determined before the QC spiking addition. Sum of endogenous concentration and spiked concentration were used as nominal concentration. The accuracy measured of QC samples ranged from 92.9 to 99.9%. The quality controls had a precision error (% CV) of less than 15 % for inter- and intra-day assay. The use of negative ionization mode gave better signal intensities and eliminated interferences from the serum extract giving no false positives .The cross validation study with a traditional method for the analysis of creatinine confirmed the effectiveness of this new analytical approach using the LDTD-MS/MS. Analysis time (8 sec per sample) as well as sample throughput are significantly improved.

Conclusion: The LDTD-MS/MS method is an effective tool for the quantitation of creatinine at a rate of 8 seconds per sample to support preclinical and clinical studies.

A-396

Analysis of Pain Panel Medications in Urine on Raptor™ Biphenyl by LC-MS/MS

F. Carroll. *Restek Corporation, Bellefonte, PA*

The Restek™ Biphenyl has been the column of choice for clinical diagnostic and Pain Management drug screening testing because of its ability to provide highly retentive, selective, and rugged reversed-phase separations of drugs and metabolites. By bringing the speed of Superficially Porous Particles to the Biphenyl family, Restek's Raptor™ Biphenyl provides clinical labs with an even faster option for a wide variety of clinical assays. Drug screening applications can be difficult to optimize and reproduce due to the limited selectivity and ruggedness of the analytical column. The Raptor™ Biphenyl has been engineered to be rugged and selective with a pain management analyses that can be performed with a 5-minute cycle time and complete isobaric resolution. The Raptor™ Biphenyl beats popular competitor methods in both selectivity and performance.

Comparison analyses were performed on the Raptor™ Biphenyl 2.7µm, 50 x 3.0mm and competitor phenyl hexyl and C18 columns. Each manufacturer's own optimized method conditions were used in this evaluation. A pain panel drug standard was prepared in diluted urine and injected for assessment of retention and resolution. The ruggedness of the Raptor™ Biphenyl column was tested by performing a minimum of 2500 injections of a minimally diluted urine standard on a single column with the guard cartridge changed every 1000 injections. Retention time and response were monitored throughout the experiment. Analysis for both experiments were performed on a Shimadzu UFLC-XR HPLC equipped with an ABSCIEX 4000 LC-MS/MS using electrospray ionization in positive ion mode.

The Raptor™ Biphenyl displayed increased retention over the competitor phenyl hexyl and C18 columns. By improving the separation of the early-eluting compounds such as morphine, oxymorphone, and hydromorphone from hydrophilic matrix interferences, ion-suppression was decreased resulting in an increase in sensitivity. In addition, isobaric compounds such as morphine and hydromorphone display increased resolution and response. The Raptor™ Biphenyl has also proven to be rugged under high through-put conditions. In the first lifetime experiment a pain panel drug standard was diluted in urine 6x and filtered through 0.2 µm PVDF Thomson filter vials. The filtered matrix standards were injected on a single column with the replacement of the guard cartridge every 1000 injections. Under these conditions, the column lasted through 3000 injections when the study was ended. A second lifetime study was executed for 2500 injections using a new column and the same interval for guard cartridge replacement however the matrix standards did not receive any filtration. All 2500 injections were completed without a drastic change in response or peak shape. The Raptor™ Biphenyl 2.7µm, 50 x 3.0mm column has proven to withstand over 2000 injections of matrix samples regardless of filtration. It is the recommendation of Restek that with guard cartridge changes every 500 injections the column can last up to 3000 injections or beyond.

A-397

A reduced workflow SPE- LC-ESI-MS/MS method to distinguish healthy from elevated concentrations of metanephrine and normetanephrine in patient plasma samples

F. A. Kero[1], J. Ye[2], T. Enzweiler[1], V. Vandell[1], E. Gairloch[1], E. Majdi[2], H. Qiao[2], L. Cousins[2]. *[1]Biotage, Charlotte, NC, [2]Ionics, Bolton, ON, Canada*

Background: Adrenal neuroendocrine tumors known as pheochromocytomas induce excessive production of catecholamines in mammalian blood and urine. The salient metabolites, metanephrine and normetanephrine, are routinely screened as biomarkers for this condition in both matrices. The bottleneck of these analytical methods has traditionally been laborious sample preparation methods that mitigate the variability in matrix inherent with patient samples. Additional issues include the complexity of the measurement that challenges detectors that lack sensitivity and robustness. This report details a "load, wash, elute" weak cation exchange solid phase extraction procedure amenable to both plasma and urine samples. The extracts are subsequently injected into an LC-MS/MS system. The preliminary sample preparation method was developed at the Biotage US Applications lab (Charlotte, NC).The method was then transferred to Ionics (Bolton, ON, Canada) to facilitate the nmole/L measurements of the selected biomarkers by laminar flow tandem mass spectrometry. The SPE-LC-ESI-MS/MS method parameters were first optimized using pooled mixed gender plasma. A set of patient samples (n=32) was later supplied by the Mayo Clinic (Rochester, MN) that had previously been analyzed by a validated referee method. The population represented measured values across a range of clinical relevance.

Methods: Plasma samples were processed using a Biotage PPM96 positive pressure manifold. Plasma samples (100 microliters) were diluted with 50 mM ammonium acetate (300 microliters). The plasma samples were then loaded onto a Biotage EVOLUTE EXPRESS 30mg WCX 96 well plate. The plates did not require conditioning or equilibration steps. The samples were sequentially washed with 50/50% MeCN/MeOH and H2O. The analytes were eluted with 47.5/47.5/5% MeCN/MeOH/formic acid. Samples were then dried down using a Biotage SPE Dry nitrogen evaporator. The reconstituted extracts were analyzed using a Shimadzu LC system in tandem with an Ionics 3Q 220 triple quadrupole mass spectrometer.

Results: Pooled plasma samples yielded >80% recovery for both analytes. Analyte

suppression was determined to be <10%. The patient data obtained by this reduced workflow method compared well to historical data obtained by the validated referee method. The method LOQ was 0.1 nmole/L for normetanephrine and 0.05 nmole/L for metanephrine.

Conclusion: It is anticipated that this time saving and sensitive SPE-LC-ESI-MS/MS method will have significant impact in population screening strategies for these metabolites.

A-399

Sensitive LC-MS/MS assay for detecting testosterone in female, pediatric and male serum

C. Grote, S. Erfurth. *PeaceHealth Laboratories, Springfield, OR*

Introduction: Testosterone (Te) measurements are widely used to assess steroid hormone status in children and adults of both sexes. Immunoassays are often not sensitive enough to determine the lower Te concentrations found in children and females. An LC-MS/MS method was developed that provides a single assay with the necessary accuracy ($R^2 \geq 0.97$), precision (CV<10%) and sensitivity (LOQ<5 ng/dL) to routinely measure low concentrations of Te in children and females and higher Te concentrations in males.

Method: Serum (200 µL) extraction involves protein precipitation with 0.1 M zinc sulfate, SPE with Bond Elut Plexa columns and derivatization with 25% hydroxylamine. The chromatographic system consists of a Zorbax Eclipse Plus-C18 guard column, Zorbax Eclipse XDB-C18, 2.1x30 mm, 1.8 micron analytical column and a mobile phase comprised of A: 0.1% formic acid and 1 mM ammonium formate in water and B: 0.1% formic acid in acetonitrile. MRM transitions for qualifier and quantifier ions were monitored and a deuterated Te internal standard was added to each calibrator and specimen. Calibrators (1.0 to 2000 ng/dl Te) were prepared in 2% BSA. LC-MS/MS instrument included an Agilent 1260 HPLC Series SL binary pump, vial plate auto sampler with thermostat, temperature controlled column compartment and an Agilent 6460 QQQ with JetStream Technology and ESI source.

Validation: Ion suppression was evaluated by injecting extracted serum from two low (13 and 53 ng/dL) serum pools while monitoring sixteen phospholipid/lysophospholipids and Te MRM transitions. No phospholipid interference was observed at the Te retention time. Structurally related compounds (androstenedione, nandrolone, cortisol, corticosterone, 11-deoxycortisol, DHEA, progesterone, 5-alpha-dihydrotestosterone, 17-alpha-hydroxyprogesterone, 17-alpha-methyltestosterone and aldosterone) were evaluated for interference. An isotopic molecular ion of androstenedione monooxime, an isobar of Te, increased the concentration of Te by ≈ 0.8%. DHEA, an isomer of Te, produced a product ion transition of Te at a retention time of 0.81 minutes and does not interfere at physiologic concentrations. The average absolute and extraction recovery determined with pooled serum at 13 and 53 ng/dL was 100.4% and 78.2% respectively. Intraday and interday imprecision (CV) using 5 serum pools between 14 and 1031 ng/dL were < 4% and < 6% respectively. The LOQ determined by analyzing Te calibrators was 1 ng/dL. Extracted Te calibration curves from 1.0 to 100 ng/dL using a linear curve fit and 1.0 to 2000 ng/dL using a quadratic curve fit showed correlation coefficients of R^2=0.9996 and R^2=0.9998 respectively. Correlation of Te serum levels for females and children (n=88) with a national laboratory showed an R^2=0.974 and y=0.975x - 1.342 and combined serum levels for children and adults of both sexes (n=171) showed R^2=0.984 and y=1.069x - 1.495.

Conclusions: The method described is a highly sensitive (LOQ=1 ng/dL) and specific LC-MS/MS method suitable for analysis of serum Te in females, children and males. The formation of a Te oxime derivative allows use of a short 30 mm C18 column with a total run time of 3.0 minutes. The analytical protocol is free of cross reactivity, interference from structurally related steroids and phospholipids.

A-400

Application of an Immunocapture-LC-MS/MS Insulin Analogue Method to Clinical and Postmortem Insulin Investigations

S. L. Wong[1], G. Van Der Gugten[2], D. T. Holmes[2]. *[1]University of British Columbia, Vancouver, BC, Canada, [2]St. Paul's Hospital, Vancouver, BC, Canada*

Background: Synthetic insulins, or insulin analogues, are routinely prescribed in type 1 and advanced type 2 diabetes mellitus. Detection and quantitation of synthetic insulins are useful in confirming accidental or intentional overdoses of insulin analogues, as well as in the workup of aberrant insulin results.

In most laboratories, insulin concentration is determined by immunoassay. Insulin immunoassays exhibit variable cross-reactivities to synthetic insulins, are prone to interferences from insulin precursors/degradation products/autoantibodies, and fail to provide information on the type(s) of analogue(s) present. Given these limitations, we have developed an LC-MS/MS method for the identification of 5 commonly prescribed pharmaceutical insulins.

Methods: Twenty-five microliters of 500 µU/mL of bovine insulin (internal standard), and 5 µL of 5 g/L dextran sulfate + 0.5 M $MgCl_2$, were added to 1 mL of patient serum or calibrator (insulin analogues in 20% acetonitrile) in Eppendorf® LoBind tubes. The mixtures were incubated with 500 µL of paramagnetic beads coated with monoclonal mouse anti-insulin antibodies at room temperature for 1 hour. The beads were washed 3 times with 1 mL 0.01M PBS, and the insulin analogues extracted with 2 x 100 µL 1% acetic acid into a BSA-treated 96-well plate. Chromatographic separation was achieved with an ACE 300 C18 column (5 x 2.1 mm, 5 µm ID) with a run-time of 8.5 minutes. The samples were analyzed on an AB SCIEX QTRAP® 5500 system in positive ESI mode, with MRM transitions monitored for regular insulin (qualifiers m/z 1162/226, 1162/652; quantifier m/z 1162/345), lispro (qualifier 969/217; quantifier 1162/217), aspart (qualifiers 972/226, 1166/219; quantifier 972/136), detemir (qualifiers 987/454, 1184/357; quantifier 1184/454), and glargine (qualifiers 1011/1164, 1011/1179; quantifier 867/136). Calibration curves were constructed with linear regression using 1/x weighting.

Results: The method demonstrated good linearity over a concentration range of 5-200 µU/mL, with R >0.997 for all insulin analogues. Analytical recoveries were between 90.3% and 113.4%. Approximate LLOD and LLOQ were 3.5 µU/mL and 5 µU/mL, respectively. Within-run CVs ranged from 3.2% to 14.8%. The utility of the method was shown in a series of case studies: (a) postmortem investigations of deaths secondary to suspected insulin overdose (b) clinical workup of a type 1 diabetic patient who presented with hypoglycemia, and questions of whether excess synthetic insulin was administered deliberately, and (c) confirmation of insulin concentration in an insulinoma patient with discrepant insulin results from 4 commercial immunoassays.

Conclusion: We have developed a robust LC-MS/MS assay for the quantitation of 5 popular insulin analogues. It is valuable in the workup of insulin-related clinical and forensic cases, and has overcome some of the limitations exhibited by current commercial insulin assays.

A-401

Ultra-sensitive simultaneous LC-MS/MS quantification of human insulin, glargine, lispro, aspart, detemir and glulisine in human plasma using 2D-LC and a novel high efficiency column: method development and application in an overdose case

E. E. Chambers[1], K. Fountain[1], T. Rosano[2], C. Kim[2], J. Desemone[2]. *[1]Waters Corporation, Milford, MA, [2]Albany Medical College, Albany, NY*

Background: Recombinant human insulin and its analogs represent the primary treatment for insulin dependent Type I and Type II diabetes patients. Many these insulin formulations have or will be coming off patent shortly, generating a tremendous interest in quantitative methods for pharmacokinetic and bioequivalence assessments. In addition, there is interest in insulin quantification from an anti-doping and forensic perspective. Historically, insulins have been analyzed using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). LC-MS analysis of insulins is needed due to the many shortcomings of these ligand-binding assays, chiefly: lack of standardization, cross-reactivity, limited linear dynamic range, and sample preparation time. Hybrid assays (affinity capture + LC-MS) have been the most effective though they lack the simplicity and throughput required for routine testing and bioanalysis. This work provides single method for quantification of intact human insulin and 5 insulin analogs in human plasma. This investigation solves both the selectivity and sensitivity problems encountered for accurate quantification of insulins in plasma since the former is not possible with conventional assays and the latter with conventional LC-MS/MS. We then retrospectively apply the method to a unique case of insulin glargine and aspart overdose which required prolonged dextrose infusion to prevent hypoglycaemia.
Methods: Blood samples were collected when the patient was admitted and approximately every 5 hours thereafter until symptoms no longer presented.Plasma samples are prepared using protein precipitation to remove high abundance proteins, followed by mixed-mode strong-anion exchange SPE to selectively eliminate closely related interferences and provide orthogonal ity. The multidimensional LC system includes at-column-dilution and trap and back elute components which improve sensitivity (through increased loading) and selectivity (clean-up achieved during the trapping phase.) Insulins are separated with formic acid in water (A) and ACN (B) on a superficially-porous, charged surface column packed with sub-2 µm particles using a linear gradient from 15 to 40% B.
Results: Method LOD's of 50-200 pg/mL were achieved for each insulin. Average

accuracy for standard curve points was 99-100%. Average inter-and intra-day accuracies for QC's samples were 98 and 94 %, respectively. Average inter- and intra-day precisions were 7.5 and 5.3 %, respectively. Matrix factors were calculated in 6 sources of human plasma and CV's of matrix factors for all analogs were <15 %. In addition, the presence of artificially high human insulin did not affect quantification of any of the analogs. Samples from two over-dose incidents were quantified. Dextrose infusion was required for 110 and 96 hours in the two cases.M1 metabolite and aspart were detected up to 90 and 22-29 hours, respectively, after admission. Higher levels of glargine M1 metabolite correlated to higher rates of dextrose infusion. **Conclusion:** This method represents a single, simple method for the simultaneous, direct quantification of intact human insulin and analogs in human plasma which achieves detection limits in the 50 pg/mL (8.6 fmol/mL) range. This assay was successfully applied to quantify glargine, its metabolite, and aspart in samples from two overdose cases. Disclaimer: This method is intended for clinical research use only, not for use in diagnostic procedures

A-402

Analytical and Clinical Validation of an LC-MS/MS Method for Urine Leukotriene E_4: a Marker of Systemic Mastocytosis

J. W. Meeusen, A. Lueke, A. Gray, L. J. Donato, J. H. Butterfield, A. K. Saenger. *Mayo Clinic, Rochester, MN*

Background: Systemic mastocytosis (SM) is a disorder that results in excessive accumulation of clonally derived mast cells in various tissues. When triggered, these mast cells release large amounts of histamine, prostaglandins and leukotrienes. This release of signal molecules causes intermittent "spells" with symptoms of itching, flushing, lightheadedness, tachycardia, gastrointestinal distress, or even loss of consciousness. Diagnostic criteria include the presence of mast cells in tryptase-stained biopsy sections (typically bone-marrow) plus one of the following: abnormal mast cell morphology; KIT Asp816Val mutation, CD25 positive mast cells, or serum tryptase > 20 ng/ml. Urine concentrations of N-methyl histamine (NMH) and 11-beta prostaglandin $F_{2\alpha}$ (BPG), the primary metabolites of mast cell derived histamine and prostaglandin, can aid in screening, reduce unnecessary biopsies and guide therapy. However, NMH and BPG lack sensitivity. Leukotriene E_4 (LTE_4) is the primary stable metabolite of total cysteinyl leukotrienes. We hypothesized that secretion of LTE_4 would be increased in SM and could be used alone or in combination with NMH and BPG to optimize screening for SM. Here we describe a novel liquid chromatography-tandem mass spectrometry assay to accurately and precisely quantitate LTE_4 in urine and outline its clinical utility in SM screening.

Methods: D_5-labeled internal standard was added to urine specimens and followed by an acetonitrile precipitation before injection into a Turboflow MAX mixed-mode anion exchange column with subsequent chromatographic separation using a C8 2.5-µm analytical column. LTE_4 was measured in negative ion mode using an AB Sciex API 5000. All LTE_4 concentrations were normalized to urine creatinine (enzymatic method, Roche).

Results: Intra-assay precision (%CV) determined in pooled urine specimens ranged from 2.6% to 5.0% at mean LTE_4 concentrations of 41, 631, and 1452 pg/mL (n=20). Inter-assay %CV determined over 20 days ranged from 6.9% to 8.2% at mean LTE_4 concentrations of 44, 445, and 1380 pg/mL. Linearity was determined between 0-2000 pg/mL and the mean recovery from mixing studies performed in triplicate was 100% ($y=1.01x-2.28$, $r^2=0.9985$). Limits of detection and quantitation were determined at 2 and 8 pg/mL, respectively. The normal reference range of <104pg/mg creatinine was determined based on the 95th percentile in a cohort of 128 healthy individuals.

Clinical performance was determined in 409 patients referred for clinical evaluation. SM was diagnosed in 66 (16%) patients according to World Health Organization criteria. Clinical sensitivity was 53% for BPG (>1000ng/24h) and 71% for NMH (>200µg/g creatinine) in our cohort of 409 symptomatic patients. Sensitivity improved to 86% with a specificity of 68% when BPG or NMH were both considered. Including LTE_4 (>104pg/mg creatinine) improved the SM diagnostic sensitivity to 97%, with minimal change in specificity (61%).

Conclusion: We have developed a sensitive and precise LC-MS/MS assay for quantitation of LTE_4 in urine. This assay has significant potential utility as a useful screening marker of SM, greatly improving screening sensitivity when used in combination with other biomarkers of mast cell activation. Incorporating LTE_4 into a panel including BPG and NMH provides a much needed screening tool for a complicated disease with non-specific symptoms and invasive confirmatory testing.

A-403

Addition of solid phase extraction to opiate sample preparation for UPLC-MS/MS

H. F. Mohammad[1], P. J. Bates[1], C. A. Hammett-Stabler[2]. *[1]University of North Carolina Hospitals, Chapel Hill, NC, [2]Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC*

Background: Opiate testing has increased in parallel with the rise in patients seeking pain management care. These often prescribed drugs must be monitored due to their potential toxicity. Since qualitative immunoassay screening methods fail to distinguish specific opiates/opioids, clinical laboratories have turned to liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Sample preparation is a critical step in LC/MS/MS analysis. Currently our samples are prepared using "dilute and shoot (D/S)" after digestion with β-glucuronidase. The goal of this study was to assess the inclusion of an additional step involving solid phase extraction (SPE) in the sample preparation.

Methods: Drug free urine spiked with buprenorphine, norpubrenorphine, codeine, hydroxycodone, oxycodone, morphine, hydromorphone, oxymorphone, and 6-monoacetylmorphine (Cerilliant Corp) at known concentrations and previously tested patient samples were used in this study. In brief: 100 µl of urine was incubated with β-glucuronidase at 50° C for 1 h, and centrifuged. For the D/S method, 100 µl of the enzyme-digested sample was diluted 1:1 with water prior to analysis. For the SPE method, Oasis MCX µElution plates (Waters Corp, Milford, USA) were pre-conditioned with 200 µl methanol and 200 µl 5% formic acid. 100 µl of the enzyme-digested sample was applied and washed with 5% methanol in 5% formic acid solution. Samples were then eluted with 100 µl of 5:90 methanol:acetonitrile containing 5% of NH4OH solution. The elutes were dried under N2 and reconstituted with 100 µl water and analyzed. Opiate measurements were obtained using UPLC/MS/MS (ACQUITY UPLC/MS, Waters Corp) as described previously (1). Data were analyzed using EP Evaluator software (Data Innovations, LLC).

Results: The addition of the SPE step increased total analysis time by 10-15 minutes. We did not achieve a significant change in assay performance in terms of the analytical measuring range and linearity. The response curves for each of the nine drugs and metabolites were linear from 50-1000 ng/ml. However, recovery improved for all analytes; but particularly for 6-monacetylmorphine, hydroxymorphone, and norbuprenorphine at the lower concentrations where we saw improvement from 71-87% to >94% with the addition of SPE. Method comparison data using linear regression demonstrated good agreement with all correlation coefficients (R2) > 0.95.

Conclusion: The addition of SPE to our sample preparation increases cost by ~$3/sample and turnaround time by 10-15 minutes. However, we saw improved recovery across all analytes which may be attributed to a reduction in interferents from the sample matrices. This in itself is desirable as cleaner samples extend column life and reduce maintenance. Thus we believe the benefits of this additional step outweigh the costs and time.

1. Bates PJ, et al. Simultaneous detection of nine opiates, including buprenorphine and norbuprenorphine in urine using UPLC-MSMS. www.msacl.org

A-404

Using intact immunoglobulin light chains to quantitate rituximab by mass spectrometry

J. R. Mills, D. R. Barnidge, A. B. Girtman, P. M. Ladwig, D. L. Murray, M. R. Snyder. *Mayo Clinic, Rochester, MN*

Background: The therapeutic monoclonal immunoglobulin rituximab is used to deplete $CD20^+$ B-cells in the treatment of lymphoproliferative and autoimmune disorders. In autoimmune diseases, the exact mechanism by which B-cell depletion modulates disease activity is unknown and instances of clinical relapses can occur in the absence of detectable B-cells. The lack of methods to measure rituximab has restricted clinical studies aiming to better understand how drug levels relate to disease relapse.

Objective: Recently we demonstrated that microLC-ESI-Q-TOF mass spectrometry is capable of detecting residual levels of monoclonal light chains in human serum. We hypothesize this technology would be able to quantitate rituximab light chains in patient sera as a diagnostic tool, eliminating the need for tryptic digestion. Here we compare intact light chain quantification using microLC-ESI-Q-TOF MS to our current method using proteotypic SRM quantification on a triple quadrupole MS.

Methods: Rituximab intact light chain (iLC) quantification was performed on immunoglobulin-enriched serum reduced with DTT, separated on an Eksigent Ekspert liquid chromatography system, and analyzed on an ABSciex 5600 Triple TOF® mass

spectrometer. The peak area for the rituximab iLC (molecular mass - 23,034 Da) was found by integrating the molecular mass peak observed after deconvolution of the summed mass spectra from the rituximab elution time. The therapeutic mAb infliximab was added to each sample prior to Ig-enrichment as an internal standard. For the proteotypic quantitation, peptides unique to rituximab heavy chain (HC) and light chain (LC) variable regions were quantified by SRM from ammonium sulfate-crashed serum that was reduced, alkylated and digested with trypsin at 37°C for 12h. A proteotypic peptide from horse IgG was used as an internal standard and stable isotope labeled peptides were added to monitor retention times. Tryptic peptides were separated using a Thermo TLX-2 system then analyzed on an ABSciex API 5000 triple quadrupole mass spectrometer. Linearity, LOD, LOQ, intra-assay precision were assessed for both assays using rituximab spiked into human serum. For both methods, 6-point standard curves were generated [0-100 ug/mL] by spiking known amounts of rituximab into pooled human serum.

Results: Linearity was established by performing serial dilutions in human serum (100-1.0ug/mL; R^2=0.99). The rituximab iLC molecular mass peak area was detectable above the polyclonal immunoglobulin background with an LOD of 1.2 ug/mL and an LOQ of 2 ug/mL. Intra-assay precision was 6.7% at 100 ug/mL and 16.7% at 2ug/mL. We have established rituximab HC and LC proteotypic peptides have an LOQ ~2-fold lower. A method comparison using weighted linear regression between Intact and light or heavy chain peptides were comparable ([slope =0.99, y-intercept =-0.04 and R^2>0.99] and [slope =0.99, y-intercept =-0.03 and R^2>0.99], respectively).

Conclusion: Measurement of rituximab iLCs based on accurate mass assessment is a viable analytical approach. Quantitation of iLCs compares well to a proteotypic peptide approach, although differences in the analytical sensitivity of the methods may exist. Further studies using this methodology are warranted to understand how rituximab levels correlate with disease relapse.

A-405

Optimization and evaluation of an isotope dilution liquid chromatography tandem mass spectrometry method for the determination of total cholesterol in human serum and a comparison with field methods

H. Lee[1], S. Park[1], E. Ahn[1], S. Park[1], G. Lee[2]. *[1]Korea Research Institute of Standards and Science, Daejeon, Korea, Republic of, [2]Chungnam National University, Daejeon, Korea, Republic of*

BACKGROUND: Abnormal cholesterol levels are strongly associated with cardiovascular disease because these promote atheroma development in arteries. Lowering total cholesterol reduces the risk of coronary heart disease. For the reference intervals of cholesterol levels in blood are narrow, accurate methods of analysis for cholesterol in serum are essential, and the establishment of a reference and a definitive method has been needed to evaluate the field method. Isotope dilution gas chromatography-mass spectrometry has been accepted as a definitive method. Although ID GC-MS is considered to be highly accurate, it contains complex procedures such as derivatization. We describe simple ID LC/MS/MS as another method for the determination of total cholesterol in serum and its optimization conditions here. In order to compare with field method, proficiency testing programs were performed on the base of this method.

METHODS: Human serum samples were obtained from pooling of healthy human serum free from HIV, HCV, and HBV. Cholesterol-d_4 was used as an internal standard. 0.1 mL of serum sample was taken into an amber vial. An appropriate amount of isotope standard solution was spiked into the sample vial to make a 1:1 weight ratio. After adding 0.6 mL of an aqueous 8.6 mol/L KOH solution and 4 mL ethanol, we heated the solution in thermomixer for 3 h at 70 ℃ to hydrolyze the cholesterol esters. After the solution cooled, we added 5 mL of water and 10 mL of hexane, shook the tube for 5 min, separated the hexane phase, evaporated the solvent under vacuum, and dissolved the residue with 1 mL of ethanol. Aliquot of the ethanol solution was filtered and then analyzed by LC/MS/MS. The LC column was C_{18}, and kept at 50 ℃ during the chromatographic run. The mobile phase was methanol containing 0.1% acetic acid, and the flow rate was 0.3 mL/min. Serum samples were distributed to about 180 clinical laboratories in korea for the comparison with field methods.

RESULTS: The optimum volume of KOH solution for hydrolysis of cholesterol ester was about 5% of total sample mixture, and reaction time was 3 h. The optimized ID LC/MS/MS method was verified through the measurement of NIST SRM 909b and the participation in key comparison, and showed good agreement with the SRM values. The pooled serum samples were certified by this method, and used as materials for the proficiency testing programs. Expanded uncertainty of certification was about 2% within the 95% confidence interval. Proficiency testing programs of field laboratories have shown some discrepancies of 6.2% CV and 5.3% CV in total cholesterol results among the laboratories.

CONCLUSION: An optimized ID LC/MS/MS method was proposed as another method for the determination of total cholesterol in serum. This method was verified through comparison with the NIST SRM. We developed the two levels of total cholesterol CRM on the basis of this method and used them as materials for proficiency testing programs. Through the proficiency testings, it was possible to view the state-of-the-art of total cholesterol measurement by field laboratories.

A-406

LC-MS/MS Method For The Detection Of Free Thyroxine And Free Tri-Iodothyronine Using The Ionics 3Q 320 Triple Quadrupole Tandem Mass Spectrometer

V. Gounden, Z. Yuan, S. J. Soldin. *National Institutes of Health, Bethesda, MD*

Background: The majority of routine clinical laboratories perform free thyroxine (FT4) and free tri-iodothyronine (FT3) measurements on immunoassay (IA) platforms. These IA's are affected by changes in binding protein concentrations, have a weak inverse linear log relationship to TSH in hypo- and hyperthyroid individuals and have poor performance at the upper & lower values of the reference intervals. The gold standard for free thyroid hormone analysis involves preparation of sample using equilibrium dialysis. This is a time consuming and technically difficult technique. However liquid chromatography-tandem mass spectrometry (LC-MS/MS) following ultrafiltration of the sample at 37° C (method as previously described by Gu et al. Clin Biochem. 2007;40;1386-1391) has been shown to perform better than IA in the above described circumstances and involves a simpler more convenient sample preparation than equilibrium dialysis. Our objective was to improve on the sensitivity of this initial method. Here we describe our 3rd generation LC-MS/MS method with improved sensitivity over the initial mass spectrometry method.

Method: Sample preparation was performed by ultrafiltration of 500ul of serum using a 30-kDa centrifugal filter (Centrifree YM-30, Millipore). Following addition of sample to filtering device samples were centrifuged in a temperature controlled centrifuge at 1113g for 30 minutes at 37 °C. 150 ul of the ultrafiltrate was added to 450ul of methanol containing deuterium-labeled internal standards (IS) for FT4 and FT3 and centrifuged. 350 uL of the supernatant was further diluted with water, vortexed and 200 uL injected into LC-MS/MS. FT4, FT3 were detected by electrospray ionization in negative mode with the following transitions: FT4 775.6>126.7 and FT3 649.9>126.7. LC-MS/MS setup consisted of a Shimadzu UFLCXR HPLC system interfaced to a Ionics 3Q 320 triple quadrupole tandem MS. Chromatographic separation was performed using a Poroshell 1.7µm C18 column (100mmx2.1mm) with a gradient mobile phase (A: 2% Methanol in water containing 0.01% acetic acid; B: 98% Methanol, at a flow rate of 0.5mL/min. Run time per injection was 13 minutes.

Results: The method described displayed good linearity over a concentration range of 0-25 pg/ml (FT3) and 0-5 ng/dL (FT4) with r2 >0.995. Between day precision CVs for across the concentration range were: FT3 4.8-8.8 %; FT4 7.5-7.8%. Lower limit of Quanititation (LLOQ) at signal to noise ratio(S/N) =10 was 0.2 pg/ml for FT3 and 0.05 ng/dL(S/N=20) for FT4.

MS MS comparison r values with our first generation method were 0.87 and 0.82 for FT4 and FT3 respectively.

Conclusion: The sensitivity of the 3rd generation FT4/FT3 method described above is greatly enhanced due to improvements in mass spectrometer and column technology. LLOQ is now 10 fold lower than that found for previous FT4/FT3 methods.

A-407

A Simple and Robust Targeted Quantitative Method for Insulin and its Therapeutic Analogs

E. E. Niederkofler[1], S. Peterman[2], A. Leber[1], K. Antwi[1], T. Schroeder[3], U. A. Kiernan[1], K. A. Tubbs[1], B. Krastins[2], A. Prakash[2], J. Frahm[1], M. Lopez[2]. *[1]Thermo Fisher Scientific, Tempe, AZ, [2]Thermo Fisher Scientific BRIMS, Cambridge, MA, [3]Thermo Fisher Scientific, Somerset, NJ*

Background – The measurement of Insulin has been identified as a paramount metric in clinical research, drug development, forensic toxicology, and sports doping applications. Conventional insulin assays are plagued by the inability to differentiate endogenous insulin from exogenous insulin analogs. The use of LC-MS can overcome this shortcoming; however, the LC-MS methods to-date lack the analytical sensitivity demanded by the field. Therefore, a highly-analytically selective sample interrogation workflow is required to address the complexity of plasma samples and, ultimately, for accurate and analytically sensitive LC-MS detection and quantification. To meet these

requirements, a Mass Spectrometric Immunoassay (MSIA) method was developed for the high-throughput, analytically-sensitive quantification of insulin and its analogs from human plasma.

Methods – Both neat and plasma samples containing a mix of insulin and its analogs at various concentrations were analyzed. A heavy version of insulin was used as an internal reference standard and spiked into each sample prior to target selection. In a 60-minute protocol (per 96 samples) the Thermo Scientific™ MSIA D.A.R.T.'S™ pipette tips derivatized with a pan-anti insulin antibody were used for insulin target selection. After affinity enrichment, MSIA detection and quantification was achieved in a 10 minute LC-MS method on a Thermo Scientific™ Ultimate™ 3000 LC system coupled to a Thermo Scientific™ Q Exactive™ mass spectrometer. Full MS scans were acquired, thus enabling the full characterization and quantification of multiple insulin analogs from a single sample.

Results - One of the primary limitations to current insulin assays is the inability to distinguish between endogenous and exogenous insulin analogs. The immobilized insulin pan-antibody in the MSIA D.A.R.T.'S pipette tips recognizes a common epitope region in the beta chain that is conserved across all of the analyzed variants, which allows the capture and detection of all variants from the sample. Further, utilizing full scan MS mode in the analysis stage of the MSIA workflow enables simultaneous detection of multiple insulin analogs and the ability to screen for unsuspected insulin analogs post-acquisition. Accurate intact mass and fragmentation of the insulin analogs confirmed the identity of each variant.

An additional limitation to high-throughput targeted quantification of insulin and its analogs are inefficient sample preparation protocols that result in their lack of analytical sensitivity and robustness. Using the MSIA Insulin workflow described above, we achieved an LLOQ of 15 pM (87 pg/mL) and an LOD of < 7.5pM (~47 pg/mL) for the intact variants in plasma. Further, reproducibility studies demonstrated inter- and intra-day CV's of < 3% and spike and recovery resulted in recoveries of 96-100%. In addition to the improved analytical sensitivity, the MSIA workflow significantly reduces the background matrix. The reduced complexity affords shorter LC gradients, and, therefore, shorter LC-MS analysis times. Altogether these results demonstrate the high analytical sensitivity, reproducibility, and robustness of the MSIA Insulin workflow in clinical research methodology.

Conclusion – A robust clinical research methodology incorporating antibody-directed target selection from a complex matrix with highly-analytically sensitive LC-MS detection was developed for the qualitative and quantitative simultaneous analyses of multiple insulin analogs.

A-408

Development and Validation of a High Performance Liquid Chromatography Tandem Mass Spectrometry 9 Steroid Panel using Minimal Sample Volume

B. Stolze[1], V. Gounden[1], J. Gu[1], S. J. Soldin[2]. *[1]National Institutes of Health (Clinical Center), Bethesda, MD, [2]National Institutes of Health (Clinical Center) and Department of Medicine, Georgetown University, Washington DC, Bethesda, MD*

Background and Objectives: Steroid profiles play a critical role in the evaluation of endocrine disorders. High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS), with superior sensitivity, specificity, and simultaneous multi-analyte quantitation capabilities, is the preferred method for steroid analysis. Our first generation steroid profile by HPLC-MS/MS simultaneously measured 9 steroids in 18 minutes using 760µL of serum. Our second generation reduced the sample volume to 200µL with a 1 mL injection. Our objective was to improve on our initial methods whilst reducing sample volume and run time. Here we describe our third generation steroid profile assays, quantifying 9 steroids with a run time of 11.5 minutes using a 50uL sample volume and 100uL injection volume, while providing better sensitivity, specificity, and a ten-fold lower limit of quantitation due to improvements in mass spectrometric and column technology.

Method: An Agilent 6490 triple-quadrupole MS coupled with an Atmospheric Pressure Photoionization (APPI) source and Agilent 1200 Infinity series HPLC were used employing isotope dilution with deuterium labeled internal standard for each analyte. 50uL of serum were deproteinized by adding 75µL of acetonitrile containing internal standards. After centrifugation, 75µL of supernatant was diluted with 250uL of water and a 100µL aliquot was injected onto a Poroshell 120 EC-C18 column. After column washing the steroids were eluted using a methanol gradient as follows: 80% A (methanol: water 2:98, v/v) for 3 minutes, 50% B (methanol: water 98:2, v/v) to 58% B over 3 minutes, 58% B to 90% B over 1 minute, holding at 90% B for 1.5 minutes, and finally 90% B to 20% B in 0.01 minutes. Quantitation for all 9 analytes was performed in positive MRM mode. Instrument parameters were as follows: gas temperature 325 oC, vaporizer 400 oC, gas flow of 11 L/min, nebulizer 60psi, and capillary 4000V.

The MRM for each analyte and compound dependent parameters are listed below:

Cortisol 363.3/121.1 Collision energy (CE) 26; Cortisone 361.2/163 CE 22; 11-deoxycortisol 347.3/97.1 CE 30; Corticosterone 347.2/121.2 CE 22; 17α-hydroxyprogesterone 331.2/109.1 CE 30; Progesterone 315.3/109.1 CE 26; Testosterone 289.1/109 CE 22, Androstenedione 287.1/97.1 CE 18, 21-deoxycortisol 347.4/311.3 CE 13,

Results: Within-day CVs ranged from 2.4-9.5% and between-day CVs from 3.0-9.9%. Method comparison analysis was performed using split sample analysis of 20- 75 serum samples. MS to MS comparison studies yielded r-values between 0.943 and 0.997 with recoveries from 90-105%. Regression analysis slope and intercept values for all steroids in the panel were as follows: slope range 0.89-1.1; intercept range -0.3 to 6.4

Conclusions: Our method measures 9 steroids in 11.5 minutes with minimal sample volume and preparation. This method is advantageous in a clinical environment because of simple sample processing, increased sensitivity, and high-throughput.. The low sample volume used permits assessment of steroid status in neonates and infants thereby optimizing early diagnosis of endocrinopathies. The low limits of quantitation make this method ideal for measurement of androgens and estrogens in women and prepubertal children.

A-409

Quantitation of 1α,25-dihydroxyvitamin D using solid-phase extraction and fixed-charge derivitization in comparison to immunoextraction

N. Chan, E. Kaleta. *Houston Methodist Hospital, Houston, TX*

Background: Quantitation of 1α-25 dihydroxyvitamin D (DHVD) has been a difficult task due to the relative concentration of this metabolite with respect to 25-hydroxyvitamin D, and well known cross-reactivity of immunoassays. Immunoextraction (IE) techniques have been well characterized, but can be costly due to antibodies required for extraction. As an alternative to IE, solid-phase extraction (SPE) coupled to enhanced ionization with fixed-charge derivitization has been made commercially available. This work describes the validation of the SPE method directly comparing against IE with traditional triazole-dione derivitization.

Methods: DHVD was extracted by SPE and IE preparations prior to LC-MS/MS. SPE was performed using Amplifex™ C1000 and S500 cartridges with diisopropyl ether and hexane/isopropanol extraction, and Amplifex™ Diene reagent derivitization (AB Sciex, Framingham, MA). Immunoextraction was performed using ImmunoTube® 1,25(OH)2 Vitamin D extraction kits (ALPCO), with derivitization using 9mmol/L 4-phenyl-1,2,4-triazole-3,5-dione (PTAD, Sigma). LC was performed for quantitation using an Acuity UPLC BEH C18 column (Waters), A: H_2O, 0.1% formic acid and B:acetonitrile, 0.1% formic acid, from 37%B to 51%B over a 3.5 min linear gradient. MS/MS was performed using an AB Sciex 5500 Q-Trap with multiple reaction monitoring (MRM) for DHVD2 and DHVD3. Clinical validation was performed for each laboratory developed test, including accuracy, intra- and inter-assay precision, reportable range, reference range, sensitivity and specificity.

Results: Extraction of DHVD by SPE and Amplifex™ derivitization demonstrated similar assay performance to the traditional IE followed by traditional PTAD derivitization.

	SPE-Amplifex		IE-PTAD	
	DHVD2	DHVD3	DHVD2	DHVD3
Linear Range	4-200 pg/mL	4-200 pg/mL	4-200 pg/mL	4-200 pg/mL
Intra-assay Precision				
12 pg/mL	7.1	5.5	11.4	8
60 pg/mL	11.4	9.2	8.9	6.1
Inter-assay Precision				
12 pg/mL	13.30%	7.20%	8.80%	12.80%
60 pg/mL	4.00%	5.50%	6.10%	8.20%
Limit of Detection	1.9 pg/mL	2.7 pg/mL	2.7 pg/mL	1.7 pg/mL
Limit of Quantitation	4 pg/mL	4 pg/mL	4 pg/mL	4 pg/mL

The SPE method requires more technologist time (1hr vs 30 min), but requires equal derivitization and LC time, making overall assay time comparable. Cost analysis shows the SPE method to be lower cost than IE by avoiding expense associated with antibody extraction.

Conclusion: Work demonstrated that SPE shows comparable analytical performance to IE, showing promise for utility as a clinical method for sensitive measurement of DHVD.

A-410

Development of an Ultra Pressure Liquid Chromatography -Tandem Mass Spectrometry Method for Pain Management Drugs in Urine

A. A. Ejilemele[1], S. Sharma[1], D. Patel[1], J. Petersen[2], R. Patel[1]. *[1]Universal Clinical & Research Laboratories, Webster, TX, [2]University of Texas Medical Branch, Galveston, TX*

Background: Provision of adequate pain relief is an important standard of care in the management of chronic pain and the use of opioids is one of the mainstays of the management plan for this category of patients. These medications provide partial analgesia and maintain or improve function. However, their use must be monitored by regular urine screening in order to monitor compliance, identify diversion as well as the concomitant use of drugs of abuse.

Methods: The UPLC-MS/MS quantitative method detected 17 drugs and 5 metabolites. Calibrators were prepared in drug free urine using certified reference materials from Ceriliant at five levels ranging from 50 -1000 ng/mL for 20 high concentration analytes and 5 - 100 ng/mL for 2 low concentration analytes. Quality control samples were also prepared in drug free urine at three target levels (125 ng/mL, 375 ng/mL and 750 ng/mL for the 20 high analytes and 12.5 ng/mL, 37.5 ng/mL and 75 ng/ml for the two low concentration analytes). Preparation of sample/standards/quality control for analysis required the dilution of 100 µL with 850 µL of diluent (0.1% formic acid in 100% methanol) and 50 µL internal standard. The internal standard contained 22 analytes at a concentration of 1000 ng/mL. The 22 analytes were separated on a Waters Acuity TQD instrument using a BEH C18 1.7µm 2.1 X 50 mm column and binary mobile phase (A: 0.1% formic acid in 5 mM ammonium acetate; B: 0.1% formic acid in 100% Methanol) within 7.5 minutes and a flow rate of 0.3 mL/min. Analytes were detected on the tandem mass spectrometer in a positive ion mode. Chromatographic peaks of each analyte were acquired using quantitating and confirmatory ion transitions at cone voltages and collision energies specific to each compound. Accuracy was tested using recovery experiments.

Results: All analytes had linear calibration curves (r2 >0.950 for the 20 high concentration analytes; r2 > 0.999 for the 2 low concentration analytes). The within run coefficient of variation (CV) for the low, medium and high QC ranged from 1.9 - 12.2% for the high concentration analytes and 1.6 - 12.4% for the low concentration analytes at three levels. The between run CV for the low, medium and high QC ranged from 0.05 - 10.8% for the high concentration analytes and 0.1 - 8.6% for the low concentration analytes. Matrix effect, carryover and interference were minimal. Samples could be diluted a minimum of eight fold and still remain linear. Analysis of 24 positive and 50 negative CAP samples as well as 10 spiked samples gave excellent correlation with expected concentrations (r2 > 0.97 for all analytes).

Conclusion: We developed a rapid, linear, accurate and sensitive UPLC-MS/MS method for the measurement of 17 pain management medications and 5 metabolites which is suitable as a screening and confirmatory method.

A-412

Evaluation of Q-Exactive coupled with liquid chromatography for Total Testosterone and Dehydroepiandrosterone Quantification in Serum

G. Altawallbeh[1], D. R. Bunch[2], J. Gabler[3], S. Wang[2]. *[1]Cleveland State University, Cleveland, OH, [2]Cleveland Clinic, Cleveland, OH, [3]ThermoFisher Scientific, San Jose, CA*

Background: Clinically, total testosterone (TT) and dehydroepiandrosterone (DHEA) are measured in men and women for androgen abnormalities, and in pediatrics for cases of delayed or precocious puberty. Historically, immunoassays were most often used to measure TT. However, studies have shown that immunoassays overestimate the serum TT at the lower concentrations typically found in females and pediatrics. Liquid chromatography tandem mass spectrometry (LC-MS/MS) has been identified as the "gold standard" technology for steroid determination. Most of the published LC-MS/MS methods for TT and DHEA analysis are performed on triple quadrupole MS. Recently, a high resolution quadrupole-Orbitrap (Q-Exactive) MS is available and may offer improved specificity. **Purpose:** To evaluate the bench-top Q-Exactive MS coupled with LC for the quantification of low levels of TT (2.5 ng/dL) and DHEA (20 ng/dL) in serum and to investigate possible interferences from blood collection tubes. **Methods:** Charcoal stripped serum was spiked with testosterone and DHEA then serially diluted at 10 concentrations to demonstrate linearity. Female serum specimens were obtained in BD vacutainer tubes with serum separator (SST) and without serum separator (Non-SST) and compared to check for interference. All samples (200 µL) were spiked with testosterone-d3 internal standard (25 µL; 225 ng/mL) and extracted with methyl tert-butyl ether. The supernatant was evaporated at 40°C under a stream of nitrogen then derivatized with hydroxylamine (50 µL; 100 mg/mL). Methanol (50 µL) was added then it was incubated for 30 min at room temperature before injection of 50 µL. The analysis was carried out on an LC-Q-Exactive system using an Accucore C18 column (50 x 2.1 mm, 2.6 µm). **Results:** The coefficient of variation for the linearity study was <15% for both DHEA and testosterone. DHEA was linear from 4-4000 ng/dL using mass transition 304.2>253 with a correlation of R^2=0.9877. Testosterone was linear from 0.12–120 ng/dL and had an R^2=0.9706 for 304.2>111.6 and R^2=0.9727 for 304.2>123.6. The SST to Non-SST comparison demonstrated interference with testosterone for mass transition 304.23>111.57 in SST which confirms published findings. Accurate mass was unable to eliminate the interference, however there was no interference for mass transition 304.2>123.6. Testosterone and DHEA were separated both chromatographically and with unique mass transitions post-derivatization. **Conclusions:** The Q-Exactive MS coupled with LC can be used to quantify TT and DHEA at very low concentrations.

A-413

A Sensitive and Rapid Liquid Chromatography-Tandem Mass Spectrometry Method for Quantification of Arginine Derivatives

D. A. Payto, C. Heideloff, S. Wang. *Cleveland Clinic, Cleveland, OH*

Background: Arginine (Arg) is the substrate of nitric oxide synthase for the production of nitric oxide. Arginine can be methylated to form asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) through the activity of methyltransferases. ADMA is an endogenous inhibitor of nitric oxide synthase and a biomarker for endothelial function. SDMA is a biomarker for renal function that has been shown to outperform creatinine based equations for determining estimated glomerular filtration rate (GFR) in predicting kidney function when compared to measured GFR (mGFR). **Objective:** To develop a sensitive and rapid liquid chromatography tandem mass spectrometry (LC-MS/MS) method for measurement of Arg, ADMA, and SDMA in plasma. **Methods:** EDTA plasma (50µl) and 50µL of internal standard (IS) solution (1.8µM Arg-IS as L-arginine:HCL [U-^{13}C6, 97-99%], 0.5µM of ADMA-IS as ADMA:HCl:H_2O [2,3,3,4,4,5,5-d_7, 98%] and SDMA-IS as [N^G,N^G-Dimethyl-L-arginine-d_6]) were vortex mixed. 1% ammonium acetate in methanol (300µL) was added to the mixture, vortex mixed and centrifuged. Supernatant (50µL) was mixed with 150µL of 1% formic acid in acetonitrile and 10µL was analyzed on an LC-MS/MS system using a Polaris Si-A analytical column. Total chromatographic run time was 3.5 minutes. Multiple Reaction Monitoring (MRM) transitions were 175.00-70.40, 203.05-46.50, 203.00-172.10 for Arg, ADMA, and SDMA respectively. **Results:** Matrix effects were shown to be compensated by the deuterated internal standards through a mixing study. No carryover was observed up to 822.1µM, 8.9µM, and 9.3µM for Arg, ADMA, and SDMA respectively. Analytical Measurement Range (serial dilution of a spiked patient pool), analytical recovery, and CV (based on CLSI EP10-A3 guidelines) are shown in table 1. **Conclusion:** This validated LC-MS/MS method offers sensitive and rapid quantification of Arg, ADMA, and SDMA in EDTA plasma.

Table 1: Method Validation Data

	Arginine	ADMA	SDMA
Analytical Measurable Range	7.40-1022.3 µM	0.09-9.54 µM	0.09-11.50 µM
Analytical Recovery (%)	87.6-114.9	89.3-114.0	100.2-106.6
Total CV (%)	8.2-10.4	6.8-9.1	6.4-8.8
Intra-Assay CV (%)	5.7-10.3	4.6-8.2	4.4-6.1

A-414

Flow injection-tandem mass spectrometry for inborn error metabolism research using a meta calculation software

J. Lai[1], B. Hart[1], C. De Nardi[2], M. Kozak[1], K. Van Natta[1], B. Duretz[3], D. C. Kasper[4]. *[1]Thermo Fisher Scientific, San Jose, CA, [2]Thermo Fisher Scientific GMBH, Dreieich, Germany, [3]Thermo Fisher Scientific, Villebon, France, [4]Medical University of Vienna, Vienna, Austria*

Background: Since bacterial inhibition method was first developed for research of inborn error metabolism in 1960, the technology has changed drastically from EIA, RIA, FIA, ELISA to LC and Tandem MS over the past 50 years.

Tandem MS allows for higher quality results compared to the old approaches. However, manual data processing in the post-analytical phase still remains a common cause of errors in the total testing processing.

This research describes a method of flow injection-tandem MS in analyzing donor samples for the quantitation of amino acids and acylcarnitines with a meta calculation software.

Methods: Samples were extracted from dried blood spot cards; the internal standards

were added during the extraction procedure and extracted samples were derivatized prior to injection onto an LC-Tandem MS system. QC samples were added to the batch.

The flow injection was conducted using a LC with open-tube providing an automated sample introduction to a Tandem MS (Thermo Scientific) without chromatographic separation. The Tandem MS used Selected Reaction Monitoring scanning for the detection of amino acids and acylcarnitines. This beta version software is developed for an automatic calculation of mass ion ratio and user defined formulas uaing data files generated from Tandem MS.

Results: A total of 41 samples and 779 analytes were processed.

The comparison result of this sample set shows that over 99% of concentration calculations (Analytes and Formulas) are within 10% of bias. Over 87% of Formulas Ratios are within 10% of bias. Table below shows comparison between software calculations (One-Step) and manual calculations (Multiple-Steps).

Conclusion: This offline automated data processing tool shows a good agreement with manual calculation process, and it can process both concentration and user defined formulas. This meta calculation software improves time effectiveness by eliminating manual calculation process and removing transcription errors in post-analytical phase.

Comparison Between Software Calculation and Manual Calculation

Type of Calculations	Analyte/ Formula	Number (N)	Bias%	R2	Linearity Equation	Value Range
Analyte Concentration	C0, C8, C14, C14:1, C16, Cit, Met, Orn, Phe, Tyr	410	< 20%	0.9986	Y = 0.0593 + 0.9993 X	0.81 to 199.15(ng/mL)
		407	< 10%			
		381	< 5%			
Formula Concentration	F1=C0 + C14:1	41	< 5%	0.9992	Y = -0.7703 + 0.9996 X	43.98 to 167.04 (ng/mL)
Formulas Ratios	F2=(Orn - Phe)/ Tyr	82	< 40%	0.9966	Y = 0.0039 + 0.9983 X	-0.98 to 2.25
		80	< 20%			
	F3=(C8 + C14:1 - C16)/ (Orn + Tyr)	72	< 10%			
		61	< 5%			

A-415

Analysis of serum testosterone and androstenedione for clinical research using either manual or automated extraction

D. Foley[1], B. Keevil[2], L. Calton[1]. *[1]Waters Corporation, Wilmslow, United Kingdom, [2]University Hospital of South Manchester, Wythenshawe, United Kingdom*

Background: Here we evaluate a UPLC/MS/MS method used to measure serum testosterone and androstenedione enabling investigation of metabolic dysfunction for clinical research purposes. An analytically selective method was developed using a mixed-mode Solid Phase Extraction (SPE) sorbent in 96-well plate format. Either manual or automated extraction was employed, providing flexibility in sample preparation options depending on the laboratory environment.

Methods: Certified testosterone and androstenedione reference material purchased from Cerilliant (Round Rock, TX) were used to create calibrators and QC material in stripped pooled serum purchased from Golden West Biologicals (Temecula, CA). Hormone Standardization (HoSt) testosterone certification program samples from the CDC (Atlanta, GA) were used to provide an initial assessment of analytical method bias. A set of serum samples (University Hospital of South Manchester, UK) were analyzed using the newly developed method and an independent LC/MS/MS method for testosterone and androstenedione and results were compared. This same set of serum samples was used to show equivalence of the manual and automated extraction techniques. All samples were pre-treated with ammonia, zinc sulphate and methanol. SPE was carried out with a Waters® Oasis® MAX µElution 96 well plate to reduce ion suppression and concentrate the samples without the need for evaporation. Automated extraction was performed using the Waters Offline Automated Sample Preparation Station (OASPS). Using an ACQUITY UPLC® I-Class system, samples were injected onto a 2.1 x 50 mm Waters ACQUITY UPLC HSS C18 SB column using a water/ methanol/ammonium acetate gradient and quantified with a Waters Xevo® TQD Mass Spectrometer.

Results: The method was shown to be linear from 0.05 - 15 ng/mL for testosterone and androstenedione. Coefficients of variation (CV) for total precision and repeatability on 5 separate days for low (0.15 ng/mL), mid (1.0 ng/mL) and high (10 ng/mL) QC samples were all < 6% (n = 30) for both testosterone and androstenedione using manual or automated extraction. Comparison with the values assigned to HoSt testosterone certification program samples analyzed with this method was described by the Deming equation y = 1.07x - 0.03 and Bland Altman mean bias was shown to be < 5% for testosterone. Comparison with samples previously analyzed by the independent LC/MS/MS method were described by the Deming equations y = 1.06x + 0.03 and y = 1.00x - 0.09 for testosterone and androstenedione, respectively. Comparison of the manual and automated extraction techniques within our laboratory was described by the Deming Equations y = 1.01x + 0.01 and y = 0.97x + 0.17 for testosterone and androstenedione, respectively. Bland Altman mean bias between the manual and automated methods was shown to be < 2.5% for both testosterone and androstenedione.

Conclusion: We have successfully quantified serum testosterone and androstenedione using both manual and automated SPE with UPLC/MS/MS for clinical research purposes. The method demonstrates excellent linearity, precision and accuracy.

For Research Use Only, Not for Use in Diagnostic Procedures.

A-416

Alternative Calibration Strategies for LC-MS Based Analysis of Broad Reportable Range Analytes

C. Crutchfield, M. T. Olson, W. Clarke. *The Johns Hopkins University School of Medicine, Baltimore, MD*

Background: Traditional LC-MS calibration strategies are associated with increased cost and time to result relative to response factor (Rf) based calibration strategies. Most reports using Rf based calibration have been applications where the AMR is less than two orders of magnitude. Testosterone requires an AMR of ~3 full orders of magnitude to encompass both male and female reference ranges (Females: 2-45 ng/ dL; Males: 250-1,100 ng/dL). Accurate determination of the analyte:internal standard ratio is problematic as the ratio diverges from unity particularly when A/IS = <0.1, >10. We wanted to examine the response factor performance of using ***two*** non-isobaric isotopically labeled internal standards, Testosterone-$^{13}C_3$ and Testosterone-$^{2}H_5$, placed at different concentrations in the same solution.

Methods: Testosterone calibrators at 2, 5, 10, 20, 50, 100, 200, 500, 1000 & 2000 ng/dL were prepared with internal standard concentrations of 20 ng/dL Testosterone-$^{13}C_3$ and 200 ng/dL Testosterone-$^{2}H_5$. QC material was prepared with testosterone at 2.2 ng/dL, 75 ng/dL, & 1,800 ng/dL. Measurements were performed using a Prelude SPLC coupled to Thermo TSQ Vantage mass spectrometer equipped with a HESI-II probe using reverse phase chromatography. Response factors were generated as n=12 replicate measurements (Testosterone-$^{2}H_5$ Rf = 1.03 $\pm$ 0.09; T-$^{13}C_3$ Rf = 0.71 $\pm$0.17, 95% CI). Complete linear regression included all calibrators in the set with 1/ x^2 weighting; constrained linear regression excluded the bottom 3 and top 3 calibrators for the High IS and Low IS, respectively.

Results: The concentration of the internal standards influenced performance with respect to the recovery and CV of the QC materials. Interestingly, the higher concentration internal standard performed as well as the low concentration internal standard. The table includes a complete comparison.

Conclusion: These data demonstrate the feasibility of applying RF based calibration to testosterone analysis; however, optimization of internal standard placement and resolution of the disparity of IS response factors warrant further investigation.

		Calibration Strategy					
		Complete Linear Regression		Constrained Linear Regression		Response Factor	
		Low IS	High IS	Low IS	High IS	Low IS	High IS
High QC	Bias	18%	19%	28%	8%	39%	20%
	CV	7.0%	4%	7%	4%	7%	4%
Mid QC	Bias	5%	12%	14%	8%	24%	12%
	CV	7%	4%	7%	3.8%	7%	4%
Low QC	Bias	11%	7%	9%	235%	13%	-7%
	CV	10%	6%	11%	2%	7%	7%

A-417

Comparison of Voriconazole Levels Using LC-MS/MS and HPLC.

M. Hinsdale[1], A. Veenis[2], S. Wong[1], E. Palavecino[1]. *[1]Wake Forest Baptist Medical Center, Winston Salem, NC, [2]Bridgewater College, Bridgewater, VA*

Background: Voriconazole is a triazole antifungal agent used for treatment of invasive fungal infections. Large variations in voriconazole pharmacokinetics may be associated with decreased efficacy or with toxicity and therefore monitoring of voriconazole levels is highly recommended. The objective of this study was to investigate the performance of a newly developed method for measuring voriconazole levels using LCMS/MS compared to standard HPLC methodology.

Material and Methods: Serum samples from patients receiving voriconazole therapy were collected according to our institution standards protocols. Aliquots from each sample were tested by HPLC at a reference laboratory and by the LCMSMS method developed on site and validated according to standard recommendations using for

separation an Aria two channel HPLC with a Cyclone (50 x 0.5mm) column from ThermoFisher for online cleanup and a Hypersil Gold (50 x2.5 mm 3um particle size) column from ThermoFisher for the separation. The detection was accomplished by a ThermoFisher Vantage tandem quadrapole mass spectrometer The LCMS/MS protocol was the following: A 100 µl aliquot of each serum sample, control, and calibrator was added to 300ul of extraction mixture (50ng/ml voriconazole D3 in MEOH). The mixture was vortexed for 20 seconds and then centrifuged at 12000 rpm for 5 minutes. The supernatant of this mixture was diluted 1:1 with water and 100 µl of this supernatant was injected into the column. Once the sample was introduced into the LCMS/MS system, automated turbo-flow analysis was followed by LCMS/MS. The mass spectrometer was run in HESI mode with positive polarity. The spray voltage was 4500v and the vaporizer temperature was 400°C and the sheath gas pressure was 20 psi and the N2 gas pressure was 100 psi. We detected fragments in atomic mass units of 281.2 and 224.1 from voriconazole 350.1.The internal standard voriconazole D-3 amu 353.15 yielded fragments 284.2 and 130. Runtime was (6.3 min) with a detection window of 3min/sample. Performance of the LCMS/MS method for detecting voriconazole levels in 21 clinical serum samples was compared with that of the HPLC method.

Results: The LCMS/MS method for voriconazole was linear over the analytical range of 0.25 to 6 mcg/mL and r^2= 0.9958. This study found that the LCMS/MS is precise with an intra- and inter-assays coefficients of variation of <6% and <4% respectively. The correlation between the LCMS/MS method and the standard HPLC was very good with r^2= 0.9711 (y= 0.833x + 0.4724).

Conclusions: The LCMS/MS method is a rapid and accurate method for measuring voriconazole levels and compared well with the values obtained by standard HPLC procedure. This method is an efficient tool for monitoring voriconazole levels in serum samples from patients receiving voriconazole therapy.

A-418

Unified LC-MS/MS Assays for Therapeutic Drug Monitoring and Clinical Trials

D. A. Svinarov, L. Kassabova. *Medical University of Sofia, Sofia, Bulgaria*

Background: Triple quadrupole LC-MS/MS with its unique selectivity and sensitivity for quantification has become unbeatable tool to resolve analytical challenges in the field of therapeutic drug monitoring (TDM), clinical toxicology, steroid analysis, vitamin D, thyroid hormones and many newer biomarkers. Pharmacokinetic assays for clinical trials (CT) are another major application of this technique. This study presents an unified analytical system for TDM and CT based on LC-MS/MS. **Methods:** Twelve assays were combined in a common analytical system via unified sample preparation and chromatography: analytes and internal standards were extracted from 50-100 µl of human whole blood (WB) or plasma (PL) with respective organic solvent, isocratic separation was performed on a C18 analytical column with a mobile phase consisting of 85% aqueous methanol with 0.005 mM ammonium acetate and 0.1% formic acid. Electrospray positive ionization and selected reaction monitoring were used to follow the respective predominant transitions. Mass chromatograms were collected and processed by specialized software, and linear regression was performed to determine analyte concentrations. Validation strategy was strictly adhered to industrial guidance. **Results:** For each analyte selectivity was assessed with 6 individual sources of WB or PL with matrix effect in the range 90÷112%; extraction recoveries of 70÷93%; stability: freeze-thaw was determined for three cycles of 24 h; post-preparative was documented for 36÷72 h at 8°C, short-term - at ambient temperature was proven for 6÷24 h in the dark and for 2÷6 h at daylight; stock solution and long term in WB or PL - for 1÷4 months at -20°C. List of analytes, application profile, and rest of validation characteristics are as follows:

Matrix	Compound	Accuracy	Precision	Linearity Range	Application
PL	Alprazolam	± 11%	6 %	0.1 ÷ 24 µg/L	CT
PL	Amlodipine	± 10 %	9 %	20÷14 400 ng/L	CT
PL	Clarithromycin	± 4 %	5 %	0.4 ÷ 1725 µg/L	CT
PL	Clopidogrel	± 7 %	10 %	5 ÷ 2160 ng/L	CT
WB	Cyclosporine A	± 11 %	7 %	10 ÷ 2000 µg/L	TDM, CT
WB	Everolimus	± 11 %	10 %	1 ÷ 45 µg/L	TDM, CT
PL	Fexofenadine	± 6 %	8 %	0.8 ÷ 322 µg/L	CT
PL	Galantamine	± 10 %	11 %	0.2 ÷ 8 µg/L	CT
WB	Indapamide	± 8 %	8 %	0,2 ÷ 79,0 µg/L	CT
PL	Midazolam	± 12 %	5 %	0,1÷ 100,0 µg/L	CT
PL	Sildenafil	± 4 %	7 %	0.4 ÷ 740 µg/L	CT
WB	Sirolimus	± 11 %	10 %	1 ÷ 40 µg/L	TDM, CT
WB	Tacrolimus	± 11 %	10 %	1 ÷ 42 µg/L	TDM, CT
PL	25-Hydroxyvitamin D	± 11 %	7 %	1 ÷ 150 µg/L	TDM

Conclusion: With validation according to current industrial requirements, a throughput of 100÷200 samples per working day and immediate method switching, this unified system provides convenience and optimal versatility for a single LC-MS/MS instrument.

A-419

Simultaneous Analysis of Multiple Azole Antifungal Drugs in Plasma for Clinical Research using a simple Protein Precipitation Extraction Protocol

B. J. Molloy[1], B. Keevil[2], L. J. Calton[1]. *[1]Waters Corperation, Wilmslow, United Kingdom, [2]University Hospital of South Manchester, Manchester, United Kingdom*

Background: Here we evaluate a UPLC/MS/MS method to simultaneously measure the azole antifungal drugs voriconazole, fluconazole, itraconazole, ketoconazole and posaconazole in plasma for clinical research purposes to determine pharmacodynamic and pharmacokinetic properties to understand possible drug-drug interactions. A simple, sensitive, precise and robust analytical method was developed using a simple protein precipitation protocol and state of the art UPLC/MS/MS technology.

Methods: Voriconazole, fluconazole, itraconazole, ketoconazole and posaconazole were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) and were used to create calibrators and QC material in pooled plasma obtained from Sera Laboratories International (West Sussex, UK). Stable labelled forms of all analytes were used as internal standards and were purchased from Toronto Research Chemicals (Toronto, Canada).

Linearity, precision, analytical sensitivity, carry-over and matrix effects were all assessed. The method was also compared to an independent LC/MS method for the measurement of voriconazole, and the effect of potential interferences on the method was assessed. All samples were prepared by precipitation with a solution of the internal standards in methanol. Using an ACQUITY UPLC® I-Class system, diluted samples were injected onto a 2.1 x 30 mm Waters CORTECS UPLC C18 column employing a water/methanol/ammonium acetate/formic acid gradient for separation and quantified with a Waters Xevo® TQD Mass Spectrometer.

Results: The method was shown to be linear from 0.060 - 9.5 mg/L, 0.062 - 10.2 mg/L, 0.055 - 10.4 mg/L, 0.048 - 8.8 mg/L and 0.067 - 10.2 mg/L for voriconazole, fluconazole, itraconazole, ketoconazole and posaconazole respectively. Total precision and repeatability was assessed over five days with five replicates per day and is expressed as coefficient of variation (CV). For all analytes the CV for the low QC (0.25 mg/L) was ≤ 5.6% and the CV for the mid (3.5 mg/L) and high (7.5 mg/L) QC was ≤ 3%. Comparison of the results with those for the same samples previously analyzed by an independent LC/MS/MS method for voriconazole was described by the Deming equation y = 0.95x + 0.05.

Conclusions: We have successfully quantified voriconazole, fluconazole, itraconazole, ketoconazole and posaconazole in plasma using a simple protein precipitation extraction protocol with UPLC/MS/MS for clinical research purposes. The method demonstrates good linearity, precision, analytical sensitivity and lack of significant matrix effects.

For Research Use Only. Not for use in Diagnostic Procedures

A-420

Development and Validation of a Dried Blood Spot Method for 25-Hydroxyvitamin D

Z. Wu, D. Bass, K. Urek, J. A. Maggiore. *Doctor's Data, Inc., Saint Charles, IL*

Background: With sustained consumer and professional demand for Vitamin D testing, we sought to develop a convenient, precise, and accurate method for 25-hydroxyvitamin D analysis of a dried blood spot (DBS) sample. Single use and self-retracting bloodletting devices enable the self-collection of capillary blood from lay users. Filter paper collection and transport media have become highly standardized and are increasingly used for the analysis of several analytes in the clinical laboratory. The employment of Liquid Chromatography-Tandem Mass Spectroscopy (LC-MS/MS) serves to expand the DBS offerings in clinical laboratories for the reliable analysis of micronutrients. Combining these components provides the basis for the Doctor's Data, Inc. (DDI) DBS method for 25-Hydroxyvitamin D.

Methods: Capillary blood samples are self-collected using SurgiLance™ sterile lancets, and spotted onto PerkinElmer 226 Spot Saver Cards and permitted to dry. Cards are desiccant packaged and shipped via US or International postage to DDI Laboratory.Two 6-millimeter spots are punched from homogeneous blood spots, and extracted using a methanol-rich solvent solution which also contains deuterated internal standards for 25-hydroxyvitamin D2 and D3. Extracts are further processed and purified using solid phase extraction, eluted, and prepared for injection through a C-18 analytical column on an Agilent 6460 LC-MS/MS System. Results are read from a 5-point calibration curve, derived from certified standards for 25-hydroxyvitamin

D2 and D3. Analytical precision, linearity, recovery, accuracy, interference, and stability were assessed.

Results: For 25-hyroxyvitamin D3, intra-assay precision coefficients of variation (CV) (n=24) at 35.3 and 74.8 ng/mL were 2.7% and 1.7%, respectively. Inter-assay CV (n=24) at the same levels were 3.2% and 3.5%, respectively. For 25-hydroxyvitamin D2, the intra-assay CV (n=24) at 15.9 and 76.8 ng/mL were 3.7% and 2.6%, respectively. Inter-assay CV (n=24) at the same levels were 5.0% and 3.2%, respectively. For assay linearity (n=5), 25-hydroxyvitamin D3 was confirmed linear between 2.0 and 224.0 ng/mL, with recovery between 98.9% and 105.1%; 25-hydroxyvitamin D2 was confirmed linear between 0.5 and 76.5 ng/mL, with recovery between 98.9% and 104.2%. Volunteers provided DBS and paired serum samples allowing sample matrix comparison. Least-squares regression analysis comparing Total 25-hydroxyvitamin D values in serum to DBS (n=46, range 7.5 - 92.6 ng/mL) yielded a correlation coefficient (R^2) of 0.972, y = 0.957x + 8.48; standard error of estimate = 3.58. Both forms of vitamin D demonstrated one-year stability when collection cards are stored desiccated in sealed Ziploc™ bags at ambient (25°C) temperatures or lower. No detectable analytical interference from hemoglobin was apparent. Of the first 2000 DBS samples submitted to DDI for Vitamin D testing, 99.1% of DBS cards received contained blood spots of sufficient quantity and quality to permit processing and analysis.

Conclusion: The analytical method developed and validated by DDI for DBS 25-hydroxyvitamin D testing provides a precise and accurate means of determining Vitamin D status. The collection system for this method has proven to be well-accepted by lay users, while the transportation system provides extended stability to preserve sample integrity to facilitate shipping from remote locations to a central laboratory for analysis.

A-421

Development of an Assay for Methotrexate and its Metabolites 7-hydroxymethotrexate and DAMPA in Serum by LC-MS/MS

R. Schofield[1], K. Murata[1], L. V. Ramanathan[1], M. E. Grace[2], M. Fleisher[1], M. S. Pessin[1], D. Carlow[1]. *[1]Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, [2]Department of Pathology, Mount Sinai School of Medicine, New York, NY*

Background: Methotrexate (MTX) is a folic acid antagonist that is widely used as an immunosuppressant and chemotherapeutic agent. After high dose administration of MTX serum levels must be monitored to determine when to administer leucovorin, a folic acid analog that bypasses the enzyme inhibition caused by MTX and reverses its toxicity. Patients in renal failure who are given high-dose MTX are often given carboxypeptidase-G2 ($CPDG_2$) to reverse the effects of MTX. $CPDG_2$ is an enzyme that converts MTX into glutamate and 4-amino-4-deoxy-N-methylpteroic acid (DAMPA) that are much less toxic. DAMPA cross-reacts in immunoassays rendering them unsuitable for monitoring patients given $CPDG_2$ therapy.

Objective: The objective was to develop a very sensitive and specific assay for MTX by LC-MS/MS that had no cross-reactivity with DAMPA or other metabolites, including the major metabolite 7-hydroxymethotrexate (7-OH MTX). The assay needed to be relatively simple to allow its use in a clinical laboratory. In addition, the assay needed to be able to accurately measure the levels of 7-OH MTX and DAMPA to support clinical trials utilizing $CPDG_2$ and related compounds.

Methods: Serum samples were prepared by protein precipitation using methanol containing deuterated MTX as internal standard. LC-MS/MS analyses were performed on a Thermo Scientific TLX-2 HPLC system (TurboFlow® technology) interfaced to a TSQ Quantum Ultra mass spectrometer operated in the positive ion ESI mode. Chromatographic separation was achieved using a Cyclone-P TurboFlow®column and a Hypersil Gold C8 analytical column. The HPLC gradient elution was 20-80% of 10 mM ammonium formate/0.1% formic acid in methanol over 1.8 minutes. Calibrators (7) were prepared in blank human serum.

Results: The LOQs of MTX and DAMPA were 10 nmol/L and for 7-OH MTX it was 20 nmol/L. The analytical measurement ranges for MTX, 7-OH MTX and DAMPA were 0-1000 nmol/L; the calibration curves were linear over the AMR with correlation coefficients $R^2 \geq 0.995$. Dilutions of 10, 100 and 1000-fold were validated giving a clinically reportable range of 0-10^6 nmol/L. The accuracy of MTX was evaluated by comparison to a dihydrofolate reductase (DHFR) enzymatic inhibition assay, Abbott TDx immunoassay (Abbott Laboratories, Abbott Park, IL), and an alternate LC-MS/MS assay. The slopes of the linear regression curves comparing the 4 assays were all +/- 1% with excellent correlation coefficients. MTX recoveries at concentrations spanning the AMR were between 98 and 103%. The accuracy of 7-OH MTX and DAMPA was evaluated using recovery experiments; recoveries of 7-OH MTX and DAMPA at five different concentrations spanning the entire AMR were between 98.8% and 105.1%. Within-day and between-day (N=10) CVs at concentrations spanning the AMR were less than 10% for all three analytes.

Conclusion: We have developed a simple, accurate and sensitive assay to measure MTX levels in serum by LC-MS/MS. Unlike immunoassays this assay shows no cross-reactivity with either DAMPA or 7-OH MTX and can be used in the setting of $CPDG_2$ therapy. In addition, the assay accurately measures the levels of 7-OH MTX and DAMPA to support clinical trials utilizing $CPDG_2$ and related compounds.

A-422

Use of complementary scanning methods by LC-MS/MS in the detection of urinary synthetic glucocorticoids in patients being investigated for Cushing's syndrome

N. K. Djedovic, S. J. Rainbow. *North West London NHS Trust, Middlesex, United Kingdom*

Introduction: Liquid chromatography tandem mass spectrometry methods (LC-MS/MS) are now routinely being used for the analysis of steroids in clinical biochemistry laboratories. The majority of methods use multiple reaction monitoring mode (MRM), which confers the highest specificity. The purpose of this study was to assess the usefulness of complementary LC-MS/MS scan methods such as precursor ion (PI) and neutral loss (NL) used in doping analysis studies for the open detection of synthetic glucocorticoids and their metabolites in patients under investigation for Cushing's syndrome. PI scanning may be used to detect steroids which share a product ion, while NL scanning detects analytes with a common loss irrespective of the parent ion mass.

Method: The ionization and fragmentation behaviour of eight synthetic glucocorticoids (prednisolone, methylprednisolone, betamethasone, dexamethasone, triamcinolone acetonide, fluocinolone acetonide, beclomethasone dipropionate and fluticasone propionate) and two endogenous glucocorticoids (cortisol and cortisone) was assessed in positive and negative electrospray on an API 3000 tandem mass spectrometer equipped with a TurboIonSpray source. Common fragments and neutral losses were identified, and MRM, NL and PI scan methods were developed. In MRM mode, two m/z transitions were monitored for each analyte in positive electrospray: 363.3 > 97.3/121.2 for cortisol, 361.3 > 121.3/162.9 for cortisone, 361.2 > 147.1/279.1 for prednisolone, 375.2 > 161.0/279.0 for methylprednisolone, 393.0 > 147.1/237.1 for betamethasone and dexamethasone, 435.4 > 212.9/339.2 for triamcinolone acetonide, 453.0 > 121.2/337.1 for fluocinolone acetonide, 521.4 > 279.1/337.1 for beclomethasone dipropionate, and 501.2 > 121.3/293.0 for fluticasone propionate. In PI mode, four fragment ions were monitored in positive electrospray: m/z 121.0, 147.0, 275.0 and 279.0. In NL mode, two neutral losses were monitored in negative electrospray: m/z 76.0 and 104.0. Samples were analysed after liquid-liquid extraction with dichloromethane of 500 µL urine spiked with internal standard (d4-cortisol). Chromatographic separation was achieved using an Agilent 1100 system HPLC system and BDS Hypersil C8 column (50 x 2.1 mm, 3 µm).

Results: In order to assess the precision and sensitivity of each method, a urine sample was spiked with a mixture containing the selected corticosteroids at two different concentrations: 5.00 and 50.0 nmol/L. In MRM mode, the limit of detection (LOD) was 5.00 nmol/L, while, in PI and NL modes, the LOD was 50.0 nmol/L. Inter- and intra-assay precision (n = 10) was less than 15% at the LOD. Interference from isobaric compounds was detected using the branching ratios established for each compound. Dexamethasone and betamethasone were resolved mathematically. Patient samples containing synthetic glucocorticoids (methylprednisolone, prednisolone, betamethasone and dexamethasone) were identified by MRM, and metabolites of these compounds were detected using the PI and NL modes. Negative samples from patients were analysed using the established methods to identify endogenous metabolites.

Conclusion: We have developed an MRM method specific for certain synthetic glucocorticoids and scanning methods for the potential detection of other exogenous glucocorticoids based on structural similarities. This additional information should improve patient management.

A-423

Analysis of plasma catecholamines and metanephrines by mixed-mode SPE and HILIC LC/MS/MS

J. Danaceau, E. Chambers, K. Fountain. *Waters Corporation, Milford, MA*

Background: In clinical research, elevated concentrations of urinary catecholamines can be used in conjunction with their O-methylated metabolites (metanephrines) to indicate the presence of conditions such as pheochromocytomas, neuroblastomas,

ganglioblastomas and ganglioneuromas. However, these compounds (in particular, norepinephrine, epinephrine, and dopamine) can be a challenge to analyze via reversed-phase LC/MS/MS due to their high polarity. As a result, many research laboratories still analyze this panel using ion-pairing reagents and ECD detection. While reversed-phase LC/MS/MS has been used successfully, challenges still exist due to ion-suppression from matrix components, insufficient retention, and inadequate separation of normetanephrine and epinephrine. This work describes a single extraction and analysis method for monoamine neurotransmitters and metanephrines from human plasma.

Methods: 250 µL plasma samples were pretreated with 50 mM NH_4CH_3COO, and loaded onto pretreated wells of mixed-mode µElution SPE plates. SPE wells were then washed with 20 mM NH_4CH_3COOH and 50:50 ACN:IPA and eluted with 2 x 25 µL aliquots of 85:15 ACN:H_2O with 2% formic acid. HILIC-based chromatographic separation was achieved using an UHPLC silica-hybrid amide column. MPA and MPB consisted of 30 mM NH_4COO dissolved in 95:5 H_2O: ACN and 15:85 H_2O: ACN, respectively. Compounds were detected by MRM in ESI positive ionization mode.

Results: All compounds eluted within 2.0 minutes, with baseline separation between normetanephrine and epinephrine enabling their unambiguous identification and quantification. Recoveries ranged from 45-90% and averaged 76%. Matrix effects were less that 25% for dopamine and norepinephrine and under 10% for the remaining analytes. Calibration curves were linear from to 10-2000 pg/mL for dopamine, 3-MT, metanephrine and normetanephrine, and from 50-10,000 pg/mL for epinephrine and norepinephrine. Calibration curves for all compounds had R^2 values of 0.999 or greater. %CV and bias values for quality control samples were less than 10% for all analytes at even the lowest QC concentration (40 pg/mL).

Conclusion: This combination of mixed-mode sample preparation and HILIC chromatography results in a rapid, robust method with excellent linearity, accuracy, and precision that is suitable for measuring even the lowest endogenous concentrations of these compounds.

For Research Use Only, Not for Use in Diagnostic Procedures.

A-424

Simultaneous Detection of 60 Pain Management Drugs and Metabolites in Urine with A High Performance Liquid Chromatography - Tandem Mass Spectrometry (HPLC-MS/MS) Method

Z. Cao, E. Kaleta, P. Wang. *Houston Methodist Hospital, Houston, TX*

Background: For chronic pain management, there is a growing need to closely monitor patients taking pain medications for compliance and illicit drug use. In recent years, LC-MS/MS based methods that are highly sensitive, specific and cost-effective have been reported. However, most of these existing methods are limited to common drugs, such as opiates, benzodiazepines, amphetamines or designer drugs. Therefore, a method monitoring multiple drugs and drug classes is necessary, especially for patients with chronic pain that are frequently prescribed multiple medications. The objective of this work was to develop an HPLC-MS/MS method simultaneously detecting 60 drugs and metabolites from the following groups: opiates, synthetic opioids, benzodiazepines, stimulants, anticonvulsants and opioid antagonists.

Methods: Sixty drug standards and 37 deuterated internal standards were monitored using scheduled multiple reaction monitoring (sMRM) on an AB Sciex QTRAP® 5500 mass spectrometer with electrospray ionization in a positive ion mode. Reversed-phase HPLC separation was performed using a Kinetex™ Phenyl-Hexyl column (50x2.6 mm, 2.6 µm particle size) (Phenomenex, CA) with a binary mobile phase (A: 10 mM ammonium formate in water; B: 0.1% formic acid in methanol) by gradient (5-90% mobile phase B) with a 0.6 mL/min flow rate. Four-level calibrators were prepared in drug-free urine (Bio-Rad, CA) in a range of 5-1000 ng/mL (with exceptions of fentanyl: 0.2-25 ng/mL; gabapentin and pregabalin: 10-2000 ng/mL). All internal standards were prepared at 50 ng/mL in mobile phase A. For sample preparation, 50 µL patient urine or calibrator and 50 µl internal standard mixture were diluted with 400 µl mobile phase A before LC injection.

Results: In this lab developed HPLC-MS/MS assay, all analytes were chromatograpically resolved. Without additional sample clean-up, none of the analytes was affected by ion-suppression with this dilute-and-shoot method. All calibration produced linear calibration curves (R^2 >0.940), with the within-run coefficient variations of 2-33%. We tested 21 patient urine samples previously screened positive by the Alere Triage ® TOX Drug Screen assay. Using LC-MS/MS cut-offs consistent with other reference laboratories, our HPLC-MS/MS assay was 35/40 (88%) in agreement with the positive Triage results in the following drug classes: opiates (11/11), amphetamine (7/7), methamphetamine (5/5), cocaine (8/8) and benzodiazepines (4/9). The 5 samples missed by our LC-MS/MS assay were positive for benzodiazepine in the Triage assay: one was prescribed alprazolam; four were prescribed lorazepam. In urine, lorazepam is mostly present as its metabolite lorazepam-glucuronide, which was detected by Triage but not included in the LC-MS/MS assay. The HPLC-MS/MS assay also identified some blinded-spiked drugs not detected by the Triage assay in 21/22 (95%) urine samples. The identities of these drugs were confirmed by comparing to standards.

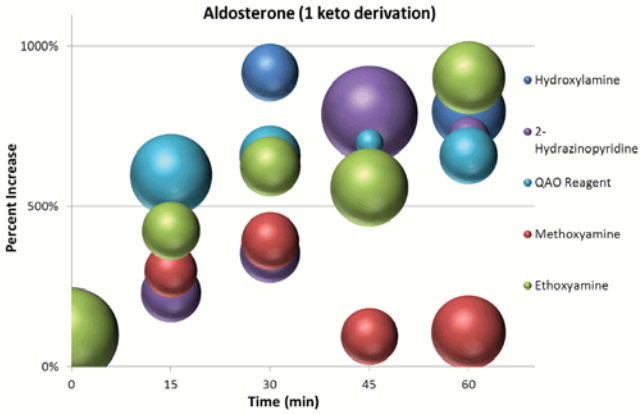
Conclusion: We presented an HPLC-MS/MS method that can simultaneously detect and quantify 60 pain management drugs and metabolites with complete separation. Investigation of the cause of false negative in alprazolam, and further optimization and validation of the assay is ongoing. This work provides a solid foundation for further development of this method into a robust quantitative assay for clinical workflows.

A-425

Steroid Ionization Efficiency as a Function of Derivation using Multiple Derivation Reagents

D. R. Bunch, S. Cahalan, S. Wang. *Cleveland Clinic, Cleveland, OH*

BACKGROUND Ionization plays a crucial role for mass spectrometry measurements. Blood steroid measurements are important for the diagnosis of various endocrinological disorders. Conditions such as congenital adrenal hyperplasia would benefit from simultaneous measurement of multiple steroids with high sensitivity. However, some of these compounds have inefficient LC-MS/MS ionization, which is required to achieve the sensitivity for clinical use. The aim of this study was to explore the impact of derivatization reagents and reaction time on ionization efficiency for multiple steroid compounds. METHODS Steroids (pregnenolone, cortisone, cortisol, aldosterone, testosterone, 17-hydroxylprogesterone, progesterone, 11-deoxycortisol,) at 3.6 µM in methanol were derivatized using 50 L of hydroxylamine, methoxylamine, ethoxyamine, and 2-hydrazinopyridine each at 100 mg/mL at room temperature and QAO reagent (AB Sciex, Framingham, MA) per the manufacture instructions. Aliquots were collected at 0, 15, 30, 45, and 60 min and analyzed in real time. Each time course was performed in triplicate and each sample was spiked with 10 L of reserpine (521 mg/mL; 609 m/z), which was used as an ionization internal standard. The precursor masses were collected for 150 scans (0.1 s each). Ionization was normalized to the reserpine peak. RESULTS Due the large amount of data a representative figure is presented (Figure 1) for aldosterone. The impact of derivation differed significantly using different reagents. The areas of the circles represent the coefficients of variation for the triplicate measurements. The y-axis is the ionization normalized to reserpine and time zero. The x-axis is the time course for the experiments. CONCLUSION Ionization was greatly increased through derivation with these reagents. Hydroxylamine and QAO derivations produced the highest and most consistent responses.



A-426

Identifying four serum peptides as biomarkers for T2DM early diagnosis by MALDI-TOF MS

Q. Meng[1], W. Yan[1], Q. Ma[2], W. Xu[3], X. Liu[4], F. Wang[4], W. Wang[5]. *[1]The PLA 301 Hospital, Beijing, China, [2]Peking University, Beijing, China, [3]Shenyang Pharmaceutical University, Shenyang, China, [4]Bioyong Technology Co. Ltd., Beijing, China, [5]Capital Medical University, Beijing, China*

Background: Currently, there is no ideal serum biomarker for the early diagnosis of type 2 diabetes mellitus (T2DM). Established diagnostics for T2DM include oral glucose tolerance (OGT), fasting blood glucose (FBG) level, and hemoglobin A1c

level, all of which are markers for the late stages of the disease. The aim of this study was to apply magnetic bead fractionation coupled with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) to screen serum samples from patients with T2DM and healthy controls to screen and identify T2DM specific peptides.

Methods: We (1) performed a discovery screen for peptide differences in serum proteomic profiles using magnetic-bead enrichment; (2) used a model based on a genetic algorithm (GA) to distinguish between the serum peptide profiles of patients with T2DM from healthy controls, to establish a training set, and to validate an independent test set; and (3) identified the most promising protein/peptide biomarkers of T2DM using linear ion trap (LTQ)-Orbitrap-MS. Patients selected in the study were pathologically diagnosed with T2DM, with FBG levels > 7.0 mmol/L and 2-h OGT (75 g) > 11.1 mmol/L. A total of 306 patients (141M/165F) and 330 healthy volunteers (83M/247F) were recruited and divided into training sets (206/230) and test set (100/100). Serum samples were collected before meals, prepared, and fractionated using weak cation exchange magnetic beads (MB-WCX) according to the manufacturer's instructions (Bioyong Tech, Beijing, China). The resultant samples were diluted, spotted onto a ClinTOF® target and performed the MALDI-TOF-MS measurements by calibrated ClinTOF® instruments (Bioyong Tech, Beijing, China). All spectra in this research were analyzed using BioExplorer® (Bioyong Tech, Beijing, China) to subtract baseline, normalize spectra, and determine peak m/z values and intensities in the range of 1,000 to 10,000 Da.

Results: Using LTQ-Orbitrap-MS detection, the sequences of seven diagnostic peptides with m/z values of 1691.7, 1778.7, 1865.5, 2022.1, 2210.3, 2929.3, and 4093.2, which were used to establish the GA model, were found to represent four different proteins. Four peaks (1691.7 m/z, 1778.7 m/z, 1865.5 m/z, and 2022.1 m/z) were identified as complement C3f, which is cleaved from C3b by factor I and enters the alternative complement pathway to promote the generation of iC3b. One peak (2210.3 m/z) was identified as the kininogen-1 isoform 1 precursor. Two peaks (2929.3 m/z and 4093.2 m/z) were identified as the fibrinogen alpha chain precursor. An 1473.3 m/z peak was not recognized by this assay, but we identified this peak as transthyretin according to previous results.

Conclusion: A diagnostic model was generated using a genetic algorithm which may discriminate T2DM patients from healthy subjects. Four peptides were derived from complement Cf3 (1691.7 m/z, 1778.7 m/z, 1865.5 m/z, and 2022.1 m/z), kininogen-1 isoform-1 precursor (2210.3 m/z), fibrinogen alpha chain precursor (2929.3 m/z and 4093.2 m/z), and transthyretin (1473.3 m/z). The presence of these peptides at elevated levels and our laboratory findings may provide new biomarkers for the early detection of T2DM.

A-427

A Sensitive and Specific Ultra-High Pressure Liquid Chromatography - Tandem Mass Spectrometry Method for the Quantitation of Hepcidin in Human Serum

D. Chu[1], C. Hedin[1], D. Chollet[2], D. Bertelson[1], E. Ellis[1]. *[1]Covance, Indianapolis, IN, [2]Covance, Geneva, Switzerland*

Background: Hepcidin is a 25-amino acid peptide hormone produced in the liver and is considered to be the central regulator of iron metabolism. It is a promising biomarker for the diagnosis and monitoring of iron metabolism disorders such as anemia, hypoxia and inflammation. Until recently, the assays for measuring hepcidin have lacked precision, accuracy, and specificity. The objective of this study was to develop and validate a sensitive and specific ultra-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the quantitative determination of hepcidin in human serum samples.

Methods: Calibrators were created by spiking charcoal stripped human serum with hepcidin concentrations ranging from 0.2 to 100 ng/mL. Human serum samples (0.2 mL) were combined with labeled internal standard (13C18,15N3-hepcidin), and extracted using a 96-well format solid phase extraction (SPE) plate (Waters Oasis HLB). Hepcidin and its internal standard were analysed using a Waters UPLC system coupled to an AB Sciex QTRAP 5500 mass spectrometer in MRM mode. Chromatographic separation was performed using a Waters Acquity reverse phase column (1.7µM, 2.1x100mm). The mobile phases consisted of 0.5% acetic acid in water (mobile phase A), and 0.05% acetic acid in methanol:acetonitrile (50:50, v:v) (mobile phase B). The linear gradient started at 20% B and ramped up to 100% B over 6 minutes, followed by 1 minute of re-equilibration. Hepcidin and its internal standard were detected by positive electrospray ionization with the following transitions: hepcidin m/z 558.7→693.7 and internal standard m/z 562.8→697.1.

Results: The method described displayed good linearity over a concentration range of 0.2-100 ng/ml with r2 >0.99. Intra-day and inter-day precision for all 3 QC levels showed CVs =<3.5% and =<5.9%, respectively. Accuracy was evaluated using a spike and recovery experiment and yielded recoveries ranging from 97.9-102% for 3 QC levels. The lower level of quantitation was 0.2 ng/mL. Specificity was evaluated and no interference was observed for serum spiked with hepcidin-20 and hepcidin-22 at 200 ng/mL each. Dilution linearity was verified to be acceptable up to 8x. The reference interval was verified to be 3.1-43.5 ng/mL for males, 1.1-25.7 ng/mL for pre-menopausal women, and 2.0-46.9 ng/mL for post-menopausal women. Stability was established for up to 2 days at ambient temperature and up to 4 days at refrigerated temperature (2-8°C). Freeze-thaw stability was established for 4 cycles at both -70°C and -20°C. Long-term frozen stability was established for up to 4 months at both -70°C and -20°C.

Conclusion: We have developed and validated a sensitive and specific UHPLC-MS/MS method for the quantitative measurement of hepcidin in clinical serum samples. The method is capable of quantitating hepcidin from 0.2-100 ng/mL. The method utilizes solid phase extraction for sample preparation. The chromatography is carried out on a reverse phase sub-2 µm particle size column using ultra-high pressure liquid chromatography. The total chromatographic run time is 7 minutes. The mass spectrometry is carried out on an AB Sciex QTRAP 5500 instrument. The predominant precursor ion for hepcidin was determined to be the quintuple charged species, [M+5H]5+.