

Tuesday, August 1, 2017

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

Mass Spectrometry Applications

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**Paper-spray Ionization Analysis of Endogenous Glucose and Cholesterol in Human Serum**

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**Background:** Paper spray ionization mass spectrometry (PSI-MS) is a soft ionization, high-throughput analysis method that can be operated in atmospheric pressure. This technique allows direct detection of chemical compounds in complex mixtures such as metabolites in serum.

**Method:** Serum volume as low as 2  $\mu$ L is required for paper spray analysis. The fragmentation pattern of endogenous glucose match well with that of glucose standard. To improve the sensitivity and specificity of targeted compounds in serum, betaine aldehyde (BA) is used as a highly selective reagent to conduct nucleophilic addition with hydroxyl group of cholesterol and glucose. After reacting with betaine aldehyde, higher sensitivity is achieved by forming ion products with permanent positive charge. Isotope labeled compounds can be spiked into the serum samples as internal standards for quantitative measurement of targeted metabolites through this PSI-MS platform.

**Results:** A calibration curve was obtained by spiking plasma samples containing different amounts of cholesterol (Chol) with known amounts of deuterated cholesterol (d6-Chol) as internal standard, with molar ratios [Chol]/[d6-Chol] in the range from 0 to 2. A quite good linearly was obtained ( $R^2=0.995$ ) and some experiments performed on spiked plasmas samples with known amounts of cholesterol confirmed the validity of this method.

**Conclusion:** A simple protocol of PS-MS described above successfully identify endogenous glucose and cholesterol in human serum. The application of betaine aldehyde reagent improves sensitivity of cholesterol. In the future, this rapid and high-throughput platform can further be applied to the analysis of diverse biomolecules in fluid biopsy.

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**Validation of Serum Voriconazole Assay Using Liquid Chromatography and Tandem Mass Spectrometry for Therapeutic Drug Monitoring in Cancer Patients in a Tertiary Cancer Hospital in India.**

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**Background:** Voriconazole is currently the antifungal of choice for treatment of invasive aspergillosis (IA) in hematological malignancies. A high degree of inter and intra-patient variability has been noted during the administration of this drug. Inter-patient variations result mostly from genetic polymorphisms in cytochrome p450 enzymes whereas intra-patient variations may occur due to disturbances in absorption, distribution, and elimination of the drug in the patient during the course of cancer therapy. This variability may be responsible for toxicity or therapeutic failure which carry a high degree of morbidity and mortality in IA in the backdrop of cancer. In our clinics, in the absence of TDM, toxicity or a breakthrough infection could be perceived as a failure of voriconazole therapy, even though only a dose correction of voriconazole could have rectified the problem. Voriconazole assays based on high-performance liquid chromatography are available only in a handful of centers across India. Here we describe a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the purpose of TDM and pharmacokinetics at our hospital. This study has received ethical approval from the Institutional Review Board.

**Methods:** To prepare the samples, 30 microliters of serum, 600 microliters of posaconazole (as internal standard (IS)) in methanol, and 120 microliters of zinc sulfate (0.1 M) were sequentially added to microfuge tubes. Serum proteins, the principal interferent, was precipitated by adding a combination of methanol (containing IS) and zinc sulfate. The tubes were shaken vigorously and centrifuged at 15,000 rpm for 10 minutes. 300 microliters of the resulting supernatant were then transferred to glass vials for estimation by LC-MS/MS. The same preparation was applied to the serum calibrators and internal quality control (IQC) samples. An ultrahigh-pressure liquid chromatography system was used to perform separation on a diphenyl 50 x 2.0 mm, 2.8-micron column with a step-up gradient of 30% to 95% methanol containing 0.1% formic acid (v/v). The column was held at a constant temperature of 45 degrees Celsius. The run time per sample was 7 minutes. The analytes were monitored on an Agilent 6420 Triple Quadrupole Mass Spectrometer using the following transitions in Multiple Reaction Monitoring mode: Voriconazole (m/z 349.9>280.9) and Posaconazole (m/z 701.7> 683.3).

**Results:** The retention times for voriconazole and posaconazole were 2.7 and 3.7 min respectively. The lower limit of quantification in serum was 0.3 microgram/mL, well below the therapeutic value of Voriconazole (1 - 5.7 micrograms/mL). The method was found to be linear over a range of 0.3 - 10 micrograms/mL with a correlation coefficient better than 0.999. Reproducibility experiments showed good inter-batch and intra-batch coefficient of variations <15% and <5% at 0.3 micrograms/mL and 10.0 micrograms/mL in serum, respectively. The ruggedness of the assay was shown by its stability which was < 10% when calibrator and IQC materials were run over a period of 4 months. A recovery between 87.5% to 102.5% at 0.3, 5.0 and 10.0 micrograms/mL indicated good accuracy for the assays.

**Conclusion:** Following satisfactory validation of the voriconazole, the method has been made available for TDM service and pharmacokinetic measurements in our Institute.

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**State-of-the-Art thyroid hormone (Total Thyroxine, Free Thyroxine) Assays: A Comparison of Automated Immunoassays with Liquid Chromatography-Tandem Mass Spectrometry Method**

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**Background:** Accurate serum thyroid hormone (Total Thyroxine, TT4; Free thyroxine, FT4) is useful in the diagnosis and management of various thyroid disorders. Presently, the testing is mainly performed on automated clinical chemistry instruments in clinical laboratories with proficiency schemes demonstrating a wide dispersion of result. Here, we compared the performance of 6 automated immunoassays and one liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, and investigated the effects of different diseases, TSH and thyroglobulin on these methods.

**Methods:** Aliquots of 154 randomly selected patient samples were collected and the following cohorts were created: hyperthyroidism patients, hypothyroidism patients, patients with postoperative thyroid carcinoma, pregnant women. TT4 and FT4 was measured by automated immunoassays from Abbott(Architect), Siemens(ADVIA Centaur XP), Roche(E601), Beckman(Dxi 800), Autobio(Autolumo A2000), and Mindray(CL-1000i), TT4 also was measured by a candidate reference measurement procedure LC-MS/MS method of National Center of Clinical Laboratory. Within-run and between-run imprecision were evaluated by measurement of 4 replicates of three serum pools on 5 consecutive days.

**Results:** Among the automated immunoassays detecting TT4, the concordance correlation coefficient (CCC) of the performance comparable to LC-MS/MS varied between 0.94 (Siemens, Beckman) to 0.97(Roche, Mindray). The absolute mean bias ranged between -1.3(Abbott) to 1.6(Siemens). For FT4, the CCC of the performance comparable to mean value of the immunoassays varied between 0.69 (Abbott) to 0.99(Beckman). The mean bias ranged between -0.61(Abbott) to 0.55(Roche). All immunoassays demonstrated good intra- and inter-assay precision, with CV% <10% for both TT4 and FT4.

**Conclusion:** All the automated immunoassays for TT4 showed excellent comparable performance, while not all assays had comparable results for FT4, the standardization of FT4 in China is urgent.

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**Development of an LC-MS/MS method for biomarkers of alcohol ingestion for use with post-mortem samples**

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**Background:** Numerous factors can affect post-mortem ethanol concentrations generating uncertainty relating them accurately to ante-mortem levels. There has been increased interest in the quantification of direct ethanol metabolites, such as ethyl glucuronide (EtG) and ethyl sulfate (EtS), in order to better corroborate post-mortem levels to ante-mortem ethanol consumption.

**Objective:** To develop and compare 3 co-extraction methods for EtG and EtS with quantification by LC-MS/MS to Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines.

**Methods:** Drug free whole blood was spiked with various concentrations of EtG and EtS to create IQC material and calibrators to cover the expect post-mortem range of 0-20 mg/L (EtG) and 1-6 mg/L (EtS). EtG-d5 and EtS-d5 were used as internal standards. IQC material and calibrators were subjected to liquid extraction, extraction from dried blood spots (DBS) or solid phase extraction prior to quantification with LC-MS/MS. Each method was assessed to SWGTOX standards including linearity, precision, stability, carry over, matrix effects, recovery, stability.

**Results:** All extraction methods were linear across the analytical range ( $r^2 > 0.95$ ) and had minimal carryover. The most promising method was liquid extraction as it met all criteria outlined by SWGTOX. However, the DBS method was imprecise at low EtG concentrations and demonstrated poor stability while the SPE method failed the majority of the criteria.

**Conclusions:** Liquid extraction combined with LC-MS/MS was the optimal method for quantification of EtG and EtS.

	EtG			EtS		
	Liquid	DBS	SPE	Liquid	DBS	SPE
<b>Linearity (r<sup>2</sup>)</b>	0.999	0.997	0.997	0.998	0.997	0.993
<b>Sensitivity (mg/L)</b>	LLOD	0.007	0.023	0.257	0.003	0.002
	LLOQ	0.014	0.055	0.642	0.007	0.004
<b>Inter-assay Precision (% CV)</b>	Low IQC	6.93	9.39	39.51	5.27	7.37
	Mid IQC	5.46	8.21	22.59	9.09	7.71
	High IQC	5.73	7.38	17.12	9.03	6.4
<b>Intra-assay Precision (% CV)</b>	Low IQC	6.15	5.69	21.99	2.96	3.47
	Mid IQC	2.66	5.37	15.79	1.99	1.67
	High IQC	2.53	3.97	10.35	1.43	2.6
<b>Recovery (%)</b>	Low IQC	96.78	95.05	238.3	97.39	75.85
	Mid IQC	108.76	106.91	252.3	110.08	107.85
	High IQC	96.98	116.59	74.91	95.24	90
<b>Matrix Effects (%)</b>	3.22	-1.61	3.8	-2.3	-0.75	-1.34
<b>Stability (% Remaining)</b>	Low IQC	99.8	106.54	89.79	99.79	90.71
	Mid IQC	94.54	108.13	96.81	94.53	107.73
	High IQC	94.54	108.13	96.81	94.53	107.73
<b>Carry Over (%)</b>	0.01	0.027	0.171	0.003	0.009	0.094

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**Quantitation of 5-Hydroxyindoleacetic Acid (5-HIAA) and Vanillylmandelic Acid (VMA) in Urine using Liquid Chromatography Electrospray Tandem Mass Spectrometry**

T. D. Hawley, D. M. Goertz, K. Yaraei. *Pathology Associates Medical Laboratories, Spokane, WA*

**Background:** 5-Hydroxyindoleacetic Acid (5-HIAA) and Vanillylmandelic Acid (VMA) are metabolites of serotonin and catecholamines, respectively. Urinary 5-HIAA is used to detect, diagnose, and monitor carcinoid tumors. While there are many factors that can cause 5-HIAA concentrations to increase in urine including medications and various foods, patients with a carcinoid tumor will show highly elevated levels of 5-HIAA. Moreover, urinary VMA is used to detect and monitor pheochromocytomas and catecholamine producing tumors. In the case of these tumors, urine VMA levels will be significantly elevated. For both 5-HIAA and VMA, a 24-hour urine collection is the preferred method as levels can vary throughout the day.

**Methods:** Aliquots of 50 µL of patient sample, calibrators, and controls and 50 µL of internal standard (20 mg/L 5-HIAA-D6 and 30 mg/L VMA-D3) were added together. The samples were vortexed and then diluted with 400 µL of 0.05% formic acid. Once the samples were mixed, they were centrifuged at 15,000 g for 10 minutes. The supernatant was removed and placed into a 96 deep well plate. The samples were analyzed on an API 3200 LC-MS/MS (AB Sciex) using negative electrospray and Multiple Reaction Monitoring (MRM). A Phenomenex Gemini® 3 µm C18 110 Å,

LC Column 100 x 3 mm column was used and 10 µL of supernatant was injected at a flow rate of 0.6 ml/min. Total run time was 6 minutes.

**Results:** For VMA, validation was done by running 59 samples previously run by HPLC and 25 samples previously run by LC-MS/MS from an independent clinical lab. Correlation for the HPLC and LC-MS/MS samples showed high agreement with a slope of 0.995 and 1.033 and an R value of 0.9967 and 0.9993, respectively. For 5-HIAA, the validation was performed by analyzing 91 samples previously run by LC-MS/MS from an independent clinical laboratory and showed good agreement with a slope of 1.012 and an R value of 0.9968. The overall CV for VMA and 5-HIAA was less than 6% and 8%, respectively. The linearity for both analytes was 0.5 mg/L to 60 mg/L. Matrix effect was observed, but the deuterated internal standard was shown to compensate. No interfering substances were observed.

**Conclusion:** We have developed and validated a simple and effective method for quantitating 5-HIAA and VMA levels in urine.

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**Multiomics Analysis of a Drop of Blood for Clinical Diagnosis**

D. Wang, P. Mao, G. Gil, M. Li. *Newomics Inc., Emeryville, CA*

**Background:** Rapid, sensitive, and specific, biofluids-based clinical diagnosis will greatly advance precision medicine. However, there is no current technology that could analyze thousands of molecules including proteins, metabolites, lipids, and exogenous environmental toxins using small volumes of blood samples. We here report the development and proof-of-principle applications of a silicon-microfluidic-chip platform that can perform highly-sensitive, multiplex, and multiomics analysis of a drop of blood for biomonitoring and disease diagnosis.

**Methods:** We developed various types of silicon microfluidic chips for LC-MS analysis of a drop of blood. For analysis of full-length proteins including their posttranslational modifications, we incorporated C4 columns on the chip. For analysis of small molecules, we monolithically integrated on-chip solid-phase extraction (SPE) with online nanoflow liquid chromatography-electrospray ionization-mass spectrometry (nanoLC-ESI-MS) detection. The chip contains several key functional modules including a SPE column, a herringbone mixer, a trap column, a LC column, an emitter, and multiple inlet/outlet holes for sample and solvent input and output. We next developed multiomics assays of a drop of blood. We used Waters Q-TOF MS interfaced with a Waters CapLC system, and Thermo Fisher Q-Exactive plus interfaced with Dionex LC system, for top-down proteomics, bottom-up proteomics, and metabolomics. We used Thermo TSQ Quantiva Triple Quadrupole MS, interfaced with an UltiMate 3000 nanoUPLC system for selected reaction monitoring (SRM) assay of peptides and small molecules such as perfluorinated compounds (PFCs) and pesticides, as well as for shotgun lipidomics. We then analyzed the molecules in small volumes of blood for diabetes monitoring and biomonitoring of PFCs.

**Results:** We demonstrated proof-of-principle applications using our platform. We have developed a new assay that is able to measure glucose, HbA1c, glycated HSA, and glycated apolipoprotein AI (apoA-I) for monitoring of individual blood glycemia, as well as cysteinylated HSA, S-nitrosylated HbA, and methionine oxidized apoA-I for gauging oxidative stress and cardiovascular risks, all in 5 microliters of blood. We have also developed a new microassay for analyzing PFCs in small volumes (less than five microliters) of blood. We demonstrated high sample recovery, excellent interday and intraday accuracy and precision, and a limit of detection down to 50 femtogram of PFCs, in one microliter of human plasma. We validated our assay performance using pooled human plasma and NIST SRM 1950 samples. Our ongoing efforts are towards integrating the multiplex assays for multiple classes of molecules on a single chip, as well as targeting clinical diagnosis of other diseases including Alzheimer's disease and cancer.

**Conclusion:** Our silicon microfluidic chip platform may enable ultrahigh-sensitivity LC-MS analysis of thousands of endogenous and exogenous molecules in small volumes of biological fluids for clinical diagnosis.

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## Renal metabolomic LC-MS/MS analysis for 7 metabolites

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**Background:** Targeted metabolomic analysis allows different biochemical pathways to be analyzed simultaneously in a single assay to monitor organ function, damage, and/or prognostic markers. We developed and validated a multiplexed LC-MS/MS assay of urine to monitor 7 renal metabolites including kidney function marker (creatinine), Krebs cycle intermediates (citrate, succinate, and oxoglutarate), oxidative stress (trimethylamine oxide, TMAO), reabsorption (sorbitol), and active kidney secretion and amino acylase activity (hippurate). **Methods:** De-identified urine samples from 24 pain management patients were used to detect seven metabolites. Six stable isotopes labeled metabolites as internal standards were added, followed by scheduled multiple reaction monitoring by ABSciex 6500 Triple Quadrupole mass spectrometer with electrospray ionization in a positive and negative ion switching mode. A Shimadzu Nexera LC with a Fusion-RP (4x2 mm) guard column and a Synergi 4 µm Fusion-RP 80Å (100 x 2mm) column were used for the analyte separation. Mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Starting mobile phase consisted of 20% B at 0.7mL/min flow rate, transitioned to 60% B from 0.3 to 0.6 min, 100% B at 0.7 min, and reverted to 20% B at 5.1 min. For sample preparation, 1: 50 diluted urines, calibrators, and quality controls (QC) were centrifuged at 20000 rcf for 15 min. Then 480 µl aliquots of supernatants were mixed well with 20 µl internal standards solution. Aliquots of 5 µl samples were analyzed LC-MS/MS. **Results:** Calibration was linear except oxoglutarate which was established with the quadratic fit. Correlation coefficients (R<sup>2</sup>) were greater than 0.99 with a range of 0.1 - 100 µm for hippurate, oxoglutarate, sorbitol, succinate, and TMAO, 0.2 - 200 µm for citric acid, and 1 - 1000 µm for creatinine. Coefficients of variation for low, middle, and high QCs were less than 8% for intra-day assays and less than 10% for inter-day assay. Method validation also did not show significant matrix effects and carry-over. Correlation of urinary creatinine concentrations by the LC-MS/MS method and AU5800 in our clinical lab was acceptable with R<sup>2</sup> = 0.9947 (y = 0.8925x + 8.1852). The other 6 metabolites level in the urines were also comparable with the published references from Human Metabolome Database. **Conclusions:** The developed multiplexed LC-MS/MS method may be used to quantify 7 renal metabolites in urine with minimal sample preparation and accepted precision and accuracy.

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## Urine Purine and Pyrimidine metabolite determination by LC-MS/MS for research use

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**Background:** Liquid chromatography triple quadrupole (QQQ) mass spectrometry (LC-MS/MS) is suited for rapid analysis of multiple analytes. Inborn errors of purine and pyrimidine metabolism lead to a wide spectrum of biological presentations either due to deficiencies of synthesis, degradation, salvage and metabolic pathways. The purine and pyrimidine metabolites analyzed included succinyladenosine, succinyladenosine monophosphate, beta-alanine, ureidopropionate, beta-aminoisobutyric acid, AICAR, uric acid, orotic acid, cytosine, dihydrouracil, uracil, 5-hydroxy-methyluracil, pseudouridine, cytidine, xanthine, hypoxanthine, guanine, uridine, AICar, dihydrothymine, thymine, deoxyuridine, inosine, deoxyinosine, guanosine, deoxyguanosine, adenine, thymidine, adenosine, and deoxyadenosine. A highly sensitive and specific LC-MS/MS analytical method has been developed for the determination of purine and pyrimidine nucleoside and base concentrations in urine. A simple sample preparation technique that involved sample dilution was utilized along with a one (1D) dimensional liquid chromatographic configuration. The described method achieves the required sensitivity and is capable of determining the purine and pyrimidine metabolites over their dynamic range. Therefore, a simple and accurate quantitative analytical method was developed for the quantitatively measurement of Purine and Pyrimidine metabolites in urine.

**Method:** A Thermo Scientific™ Endura™ tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system were utilized for this analysis. 100 µl of urine were used

for the analysis of the purine and pyrimidine metabolites. Various columns were evaluated and an Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 3 µm with a water:methanol mixture containing 2 mM Ammonium Acetate and 0.1% Formic Acid achieved baseline chromatographic separation for all the purine and pyrimidine metabolites in less than 8 minute run time. Quantitative analysis was performed using selective reaction monitoring (SRM) with transition pairs for each analyte and internal standard in negative mode and the precision and accuracy of the method was verified using pooled quality control materials and urine samples.

**Result:** Good linearity and reproducibility were obtained with the concentration range of 0.5 to 100 µmol/L for the respective purine and pyrimidine metabolites with a coefficient of determination >0.95 for the sample preparation technique and the LC-MS/MS configuration used. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to be range from 0.25 to 1 µmol/L and excellent reproducibility was observed for all compounds (CV < 10%).

**Conclusion:** A sensitive, simple, specific and accurate liquid chromatography tandem mass spectrometry method was developed and verified for the simultaneous determination and screen of purine and pyrimidine metabolites in urine. The sample preparation technique is quick and easily applied for high throughput analysis.

## A-394

## Analysis of Immunosuppressive Drugs from Whole Blood by LC/MS/MS

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**Background:** Immunosuppressive drugs are used to suppress the body's immune response and are typically administered to prevent the rejection of transplanted organs or tissues. Cyclosporin A, tacrolimus, sirolimus, and everolimus are four of the most commonly used drugs in the therapy of organ transplantation. Cyclosporin A and tacrolimus are classified as calcineurin inhibitors, and sirolimus and everolimus are grouped as mTOR inhibitors. These two classes of drugs can be used in combination for synergistic blocking of T cell activation and proliferation. Due to their pharmacokinetic variabilities and narrow therapeutic indexes, time-sensitive and highly accurate therapeutic drug monitoring is necessary, not only to prevent rejection but also minimize toxic side effects. Therefore, a fast and accurate measurement of drug concentration is critical to assist the clinicians for timely and proper treatment of the patients. By combining a simple sample preparation step and a fast chromatographic elution with a Raptor™ Biphenyl column, a high-throughput analysis was established for simultaneous measurement of these four drugs in human whole blood. **Methods:** Human whole blood was fortified with 4 analytes to prepare the calibration standards and QC samples. For quantitation, cyclosporin D was used as the internal standard for cyclosporin A and ascomycin was used as the internal standard for tacrolimus, sirolimus, and everolimus. The blood sample (100 µl) was mixed with 200 µl of precipitation solution (1:4 v/v 0.2M ZnSO<sub>4</sub>:methanol) containing 50 ng/mL of cyclosporin D and 5ng/mL of ascomycin. After centrifugation, the supernatant was directly injected (5 µl) onto the Raptor™ Biphenyl 2.7 µm, 50 mm x 2.1 mm column for analysis using Waters Acquity UPLC® System coupled with Xevo® TQ-S mass spectrometer.

**Results:** Linearity was evaluated in the range of 10-1000 ng/mL for cyclosporin A and 1-100 ng/mL for tacrolimus, sirolimus, and everolimus. Using 1/x<sup>2</sup> weighted linear regression for cyclosporin A and 1/x weighted linear regression for tacrolimus, sirolimus, and everolimus, all 4 compounds showed good linearity with r<sup>2</sup> values of 0.999 or greater, and the % deviations were less than 10%. The signal-to-noise ratios of the lowest standard samples were from 100 to 300 indicating that this method could be used for the detection of much lower concentrations if necessary. Three QC levels were prepared at 15, 150, and 800 ng/mL for cyclosporin A; 5, 15, and 80 ng/mL for tacrolimus, sirolimus, and everolimus. Precision and accuracy analyses were performed on three different days. The method accuracy was demonstrated with %recovery of less than 10% of the nominal concentration for all QC levels. The %RSD was 0.2-4.0% and 1.2-5.4% for intra-day and inter-day, respectively, indicating good method precision. No chromatographic interferences were observed from the analysis of blank blood samples.

**Conclusion:** It was demonstrated that the Raptor™ Biphenyl column is excellent for rapid and accurate analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in human whole blood. With a fast and simple sample preparation procedure and 3 minutes of chromatographic analysis time, the established method provides high-throughput therapeutic drug monitoring for these commonly used immunosuppressive drugs.

**A-395****Targeted Benzodiazepines Screening Assay for Pain Management Using High Resolution Mass Spectrometry**D. Danso. *Mayo Clinic, Rochester, MN*

**Background:** Benzodiazepines represent a large family of medications used to treat a wide range of disorders from anxiety to seizures and they are also used in pain management. With a high risk for abuse/diversion, professional practice guidelines recommend compliance monitoring for these medications using urine drug tests. However, traditional benzodiazepine immunoassays suffer from a lack of cross-reactivity with all the benzodiazepines, so many compliant patients taking clonazepam (Klonopin®) or lorazepam (Ativan®) may screen negative by immunoassay but are positive when confirmatory testing is done. This new targeted benzodiazepine screening test provides a more sensitive and specific test to check for compliance to all the commonly prescribed benzodiazepines and looks for both parent and metabolites in the urine.

**Aims:** The aim of this study was to validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method using high-resolution, accurate-mass Orbitrap detection for the qualitative identification of twenty-six benzodiazepines and metabolites (aprazolam, alpha-hydroxyalprazolam, chlordiazepoxide, clonazepam, 7-amino clonazepam, diazepam, nordiazepam, oxazepam, oxazepam glucuronide, temazepam, temazepam glucuronide, flunitrazepam, 7-amino flunitrazepam, flurazepam, a-hydroxyethylflurazepam, lorazepam, lorazepam glucuronide, midazolam, alpha-hydroxymidazolam, triazolam, alpha-hydroxytriazolam, zolpidem, zolpidem phenyl-4-carboxylic acid, clobazam, norclobazam, and prazepam.

**Methods:** A simple dilute and shoot method was developed in which urine samples (100 µL) were diluted (1:10) with internal standard in clinical laboratory reagent water (CLRW) and analyzed by LC-MS/MS using high-resolution, accurate-mass Orbitrap detection with heated electrospray ionization in positive mode. Mass spectrometer method was Full MS/ddMS<sup>2</sup>. A step gradient elution off of an Ultra Biphenyl (3 µM 50 x 3.0 mm) column achieved complete chromatographic separation of isobaric compounds with a total run time of 8 minutes, a flow rate of 0.5 mL/min, and the ability to multiplex samples. Mobile phase A contained 10mM ammonium formate with 0.1% formic acid in CLRW, while mobile phase B contained 0.1% formic acid in acetonitrile. Compounds were identified by retention time; exact masses (m/z) at (<5ppm), and spectra library match.

**Results:** A LC-MS/MS targeted screening method was validated. The cutoffs for the twenty-six benzodiazepines ranged from 10 ng/mL for (alprazolam, lorazepam, temazepam, clonazepam, etc), 50 ng/mL for the glucuronides (lorazepam, temazepam, and oxazepam), and 200 ng/mL for norclobazam. The intra- and inter-assay precision coefficients of variation for all compounds were <10% at concentrations 50% below the cutoff, at the cutoff, and at 50% above the cutoff concentrations for each analyte. Analytical accuracy was determined by comparing patient and proficiency testing samples for

each analyte against quantitative confirmatory LC-MS/MS tests and spiked recovery experiments. The Orbitrap method showed 100% concordance with the confirmatory method for all analytes based on the individual drugs' cutoff concentrations. Absence of interferences from the common prescribed drugs, over-the-counter drugs, therapeutic drugs, and common drugs of abuse tested at ≥ 100µg/mL was observed. No significant carryover was seen at concentrations up to 100µg/mL, and no matrix effect observed.

**Conclusions:** The laboratory was able to successfully validate a highly specific and sensitive LC-MS/MS targeted screening assay using a high-resolution, accurate-mass method for the comprehensive detection of benzodiazepines in urine.

**Keywords:** Benzodiazepines, Pain Management, Liquid Chromatography Tandem Mass Spectrometry, High-resolution, Accurate-mass

**A-396****Development and validation of a quantitative method for plasma folates**J. A. Maggiore, Z. Wu, K. Urek, E. Roth, A. Gruszecki, D. Quig. *Doctor's Data, Inc., Saint Charles, IL*

**Background:** An increased interest in folate analysis has arisen from an enhanced ability to detect single nucleotide polymorphisms (SNPs) in the genes that transcribe the enzymes of folate metabolism. Defects in folate metabolism affect the remethylation of homocysteine to methionine. The C677T and T677T methyltetrahydrofolate reductase (MTHFR) SNPs occur at a frequency of 32.4% and 7.4%, respectively, in

the general US population<sup>(1)</sup>. When consuming folate-rich foods or supplements, the majority of individuals may experience excessive plasma levels of unmodified folic acid (UMFA), which has been associated with cellular hyperplasia and neoplasia<sup>(2)</sup>. Folinic acid and 5-methyltetrahydrofolate (5-MTHF) are synthetic folate supplements recommended by clinicians for those with specific MTHFR C677T heterozygous (C/T) and homozygous (T/T) genotypes to provide a source of folates while minimizing undesired levels of UMFA in circulation. Immunoassays that determine total folates in serum or red blood cells cannot differentiate between UMFA and the other folates. To aid in the monitoring of the folate status, we developed a method for measuring UMFA and the folate metabolites folinic acid, 5-MTHF, and tetrahydrofolate (THF) using liquid chromatography with tandem mass-spectrometry (LC-MS/MS).

**Methods:** Plasma samples prepared in 100 mM ascorbic acid were mixed with internal standards and extracted by polymeric reversed phase solid phase extraction cartridges. Certified standards traceable to the National Institute of Standards and Technology (NIST) were acquired to derive a 5-point calibration curve for each analyte. Calibrators, NIST standard reference materials (SRM 1955), and plasma extracts were analyzed on an Agilent 6490 LC-MS/MS with chromatographic separation achieved on a C18 analytical column, permitting identification and quantitation of the folate compounds of interest. Analytical performance characteristics evaluated include precision, linearity, recovery, and stability.

**Results:** The intra-assay and total imprecision coefficients of variation (CV<sub>w</sub> and CV<sub>p</sub>) (n=42) in plasma samples was determined for UMFA: 4.0% and 7.0% at 6.5 nmol/L, and 3.7% and 7.7% at 9.6 nmol/L; folinic acid: 4.5% and 10.9% at 45.2 nmol/L, and 4.1% and 11.6% at 51.7 nmol/L; 5-MTHF: 1.0% and 4.9% at 46.7 nmol/L, and 1.1% and 5.6% at 57.2 nmol/L; and THF: 5.3% and 14.6% at 2.7 nmol/L, and 3.9% and 12.9% at 5.0 nmol/L. Linearity range (n=11) and percent recovery was confirmed in spiked plasma samples for UMFA: 1.0-425.0 nmol/L, 98.3%-107.6%; folinic acid: 0.15- 402.4 nmol/L, 95.7%-108.6%; 5-MTHF: 1.2-632.8 nmol/L, 99.7%-104.1%; and THF: 0.25-405.2 nmol/L, 98.6%-109.0%. Stability of all analytes in acidified plasma samples was demonstrated for 8-days stored at -20°C, and 4-days stored at 2-8°C.

**Conclusion:** This LC-MS/MS method developed for assessing plasma folate status has been validated to be analytically precise, accurate, and sensitive for the measurement of UMFA, folinic acid, 5-MTHF, and THF. Sample suitability has been established for the expedited temperature-controlled transportation of acidified plasma aliquots from remote locations to a central laboratory for analysis. Studies are underway to use this method to assess reference intervals for the folate analytes in those with previously determined MTHFR C677T genotypes.

**References:** 1) *J Med Genet.* 2003;40:601-605. 2) *J Nutr.* 2015; 145(3):520-531.

**A-397****Simultaneous LC/MS/MS Quantitation of 20 Antiepileptic Drugs in Human Serum**L. E. Frick<sup>1</sup>, C. J. Adler<sup>2</sup>, V. P. Miller<sup>1</sup>. <sup>1</sup>*Agilent Technologies, Lexington, MA*, <sup>2</sup>*Agilent Technologies, Santa Clara, CA*

**Background:** One major strength of liquid chromatography-mass spectrometry (LC/MS/MS) as a detection method is that it allows the concurrent monitoring of multiple analytes in a single injection. Here, a method has been developed to quantify a panel of 20 antiepileptic drugs in human serum. Compounds included were: Acetyltretigabine, Carbamazepine-10,11-Epoxy, Carbamazepine, 10,11-Dihydro-10-Hydroxy-Carbamazepine, Felbamate, Gabapentin, Lacosamide, Lamotrigine, Levetiracetam, Oxcarbazepine, Phenobarbital, Phenytoin, Pregabalin, Retigabine, Rufinamide, Tiagabine, Topiramate, Valproic Acid, Vigabatrin, Zonisamide. The method further utilized the ability of LC/MS/MS to detect compounds over a wide range of concentrations simultaneously, as the calibration concentrations ranged from 12 ng/mL to 200,000 ng/mL for the various analytes. Top concentrations ranged from 1.5 to 200 µg/mL.

**Methods:** MS/MS transitions were obtained using MassHunter Acquisition's Optimizer software to determine optimal parent and fragment ions, fragmentor voltages, and collision energies upon injection of a neat solution of each individual compound or internal standard. Samples were prepared by spiking compounds into clean human serum to create an 8-point serially-diluted calibration curve. Each calibrator or blank control was then combined with an internal standard solution and extracted through a protein precipitation using methanol before vortexing and centrifugation. An aliquot of supernatant was then diluted into water and injected onto the LC system. Compounds were separated from each other and from regions of phospholipid suppression on an Agilent Poroshell 120 EC-C18 analytical column (2.1 x 100 mm, 2.7 µm) paired with an Agilent Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm). Water supplemented with 2 mM ammonium acetate was employed as mobile phase A and methanol with 2 mM ammonium acetate as mobile phase B. A

6460 triple quad mass spectrometer was used to detect the 20 compounds in dynamic MRM mode. Positive/negative switching was utilized to monitor compounds of both polarities in a single injection. Compounds were separated over 7.5 minutes, followed by a 1.5 minute column reequilibration at starting mobile phase conditions, resulting in a total cycle time of ~9.5 minutes injection to injection. Data were analyzed using MassHunter Quantitative Analysis B.07.01 and Qualitative Analysis B.07.00.

**Results:** Calibration curve accuracies were within 20% of the expected concentration at the lowest level, and well within 15% at all higher levels. Reproducibility was good, with all CVs <15% and most well under 10%. R<sup>2</sup> values were all >0.995, with some compounds displaying a linear response across their concentration range, and others requiring a quadratic fit.

**Conclusion:** An accurate, reproducible, and robust LC/MS/MS analytical method has been developed to quantitate 20 antiepileptic drugs simultaneously in human serum. Future work will include testing multiple sources of human serum for interferences that would impact the quantitation of any of the members of the panel. Additionally, different sources of mobile phase and samples from an alternate source will be analyzed. For Research Use Only. Not for use in diagnostic procedures.

### A-398

#### Urinary Biomarkers of Idiopathic Membranous Nephropathy Identified by High Resolution Mass Spectrometry Coupled with Liquid Chromatography

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**Objectives:** Idiopathic membranous nephropathy (iMN) is an important cause of nephrotic syndrome in adults. Recently, M-type phospholipase A2 receptor (PLA2R) and thrombospondin type 1 domain containing 7A (THSD7A) have been identified as target antigens in iMN. However, it is likely that these are not the only proteins involved in the pathogenesis of iMN. Urine may provide important clues of pathophysiological mechanisms in iMN because it directly reflects the physiological state of the kidney. In the current study, we analyzed and compared the proteome of urine from patients with iMN and normal controls.

**Methods:** Second morning urine samples were collected from patients with biopsy proven iMN with serum anti-PLA2R antibody (group A, n = 5), iMN without serum anti-PLA2R antibody (group B, n = 5) and healthy volunteers (n = 5). Trichloroacetic acid (TCA) precipitation and enzymatic digestion were performed to prepare peptides. Peptides were processed strictly according to the manufacturer's protocol for 6-plex Tandem Mass Tags (TMT). Nanoscale liquid chromatography tandem mass spectrometry analyses were performed for protein identification. Gene ontology (GO) enrichment analysis was performed to analyse general characterization of the proteins. The proteins were also matched against the database of well-known metabolic pathways (KEGG). The increase of candidates was validated by Western blot.

**Results:** In this study, we identified 509 proteins in the iMN urinary proteome. There are 187 and 177 proteins up- or down-regulated in group A and group B with a fold change of 2, respectively. GO enrichment analysis revealed immune response (16 proteins) and complement activation (13 proteins) as the dominant biological process in group A and group B, respectively. Immune system is the major classification in the pathway analysis using the KEGG database in both groups. Intersecting the set of up- or down-regulated proteins in the group A and group B proteomes with a fold change of 10, we identified 5 proteins may provide targets for iMN. Western blot analysis confirmed our proteomics findings that alpha-1-antitrypsin (A1AT) and crkl-like (CRKL) were up-regulated in the patients with iMN but not in normal controls.

**Conclusions:** In conclusion, our data show the important role of immunologic mechanism in the development of iMN, and the value of urinary A1AT and CRKL in biomarker discovery of patients with iMN. The discovery of the up-regulation of A1AT and CRKL in the urine can help to further elucidate pathogenetic mechanisms involved in this disease.

### A-399

#### Comparison of human serum 17β-estradiol quantification using the ID-LC-MS/MS assay with the chemiluminescent immunoassays

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**Background:** 17β-Estradiol is routinely analyzed in clinical laboratories of the assessment of female reproductive function and has expanding roles in other fields.

However, due to low concentration levels as well as the presence of the metabolites or structural analogues which having molecular masses close to 17β-Estradiol that can cross react with the immunoassay, measurements of 17β-Estradiol in human serum are complicated.

**Methods:** [2,3,4-<sup>13</sup>C<sub>3</sub>] 17β-estradiol was used as an internal standard. The estradiol and its internal standard were extracted from serum matrix using liquid-liquid extraction prior to reversed-phase LC-MS/MS and require no derivatization. The analysis was carried out with electrospray ionization in the negative ion mode monitoring the m/z 271→145 m/z as the quantifier, 271→183 m/z as the qualifier, and 274→148 m/z for [2,3,4-<sup>13</sup>C<sub>3</sub>] 17β-estradiol. Bracketing calibrators was used for quantification. The accuracy of the measurement was evaluated by a comparison of results of this reference method on lyophilized human serum reference materials for estradiol [2015 IFCC external quality assessment scheme for Reference Laboratories in Laboratory Medicine] with the certified values determined by six reference laboratory from different countries and by a recovery study for the added E2. We evaluated intra-assay and inter-assay imprecision. The method procedure was validated against the JCTLM-certified reference method and used in measuring 17β-estradiol of 60 patient serum samples for evaluating 3 immunoassays, that are commonly used in China, i.e., Siemens IMMULITE 1000 (Siemens), ARCHITECT i2000<sub>SR</sub> (Abbott), and Cobas 6000 (Roche).

**Results:** The LC-MS/MS method was validated and showed limit of detection 5 pg/mL; limit of quantification 10 pg/mL; linearity of response to 14.82 ng/mL; The intra-assay precision CVs (n = 15) were 3.69%, 1.92%, and 1.84%; and the inter-assay precision CVs (9 runs/day, over 5 days) were 4.21%, 2.54%, and 2.74%, respectively. And analytical recoveries were from 98.73 to 100.77%. The linear regression equation showed r<sup>2</sup>=0.9395 (Siemens IMMULITE 1000 = 0.9429 LC-MS/MS+28.1300, 95% CI for the slope 0.8800 to 1.0060, 95% CI for the intercept: -1.8790 to 58.1400 pg/mL, S<sub>y,x</sub>=88.64, P < 0.0001). r<sup>2</sup>=0.9797 (Cobas 6000 = 1.0390 LC-MS/MS+16.5300, 95% CI for the slope 1.0000 to 1.0790, 95% CI for the intercept:-2.2490 to 35.3000 pg/mL, S<sub>y,x</sub>=55.46, P < 0.0001). r<sup>2</sup>=0.9962 (ARCHITECT i2000<sub>SR</sub> = 1.0630 LC-MS/MS-12.9200, 95% CI for the slope 1.0460 to 1.0810, 95% CI for the intercept: -21.1700 to -4.6740 pg/mL, S<sub>y,x</sub>=24.36, P < 0.0001). Bland-Altman plots were achieved by MedCalc and showed: By Siemens IMMULITE 1000, there are 16.7% of samples showed exceed ±30% biases from the mean of difference (2.4%). And there are only 5.0% and 6.7% of samples showed exceed ±30% biases in Cobas 6000 and ARCHITECT i2000<sub>SR</sub> systems.

**Conclusions:** We report a direct comparison of the ID-LC-MS/MS assay with the chemiluminescent immunoassays for human serum 17β-estradiol. Linear regression revealed good overall correlation with the LC-MS/MS and chemiluminescent immunoassays, and Bland-Altman plots showed that the differences were concentration dependent.

### A-400

#### Improvement of DNA methylation quantitation method with LC-MS/MS by optimization reaching a breakthrough

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**Background:** DNA methylation is one of the typical phenomena of epigenetics. Many methods for detecting global DNA methylation have been developed, among which the method using LC-MS/MS is an excellent method from the viewpoint of sensitivity, reproducibility, and cost. Several studies have proved that the LC-MS/MS method can detect a slight change of global DNA methylation. However, the potential problems seem to lie in the absence of studies on the stability and standardization of measurement methods. The aim of the present study is to establish a robust assay that guarantees high accuracy.

**Methods:** For the standard materials, ribonucleoside (Adenosine, Guanosine, 5-Methyluridine, Uridine, and Cytidine), deoxynucleoside (2'-Deoxyadenosine, 2'-Deoxyguanosine, 2'-Deoxythymidine, 2'-Deoxyuridine, 2'-Deoxycytidine, 2'-Deoxy-5-methylcytidine and 2'-Deoxy-5-(hydroxymethyl) cytidine), 5-Azacytidine, and 5-Aza-2-deoxycytidine were used. The LC-MS/MS apparatus, Acquity UPLC and TQD (Waters) was used. To separate the substances shown above, we utilized a column distinct from the C18 column reported in previous studies. To degrade DNA, DNA Degradase Plus Kit (Zymo Research) was used, together with some degrading enzymes. For preparing a calibration curve, a mixture of cytosine DNA standard and 5-methylcytosine DNA standard (Zymo Research), which are linear dsDNA (897bp), were used. The ratio of

methylated cytosine (%mC) of cytosine DNA standard and 5-methylcytosine DNA standard were 0% and 100%, respectively. The typical linear regression equations with the use of no weighting factor of the calibration curves were: y = ax + b with

y as %mDC and x (%) as %mDC<sub>nominal</sub>. As a result, the DNA methylation rate was determined. For the QC samples, Human genomic DNA (Clontech Laboratory) (Middle QC), EpiScope Unmethylated HCT116 DKO gDNA (Low QC) and methylated HCT116 DKO gDNA (Takara Bio) (HighQC) were used.

**Results & Discussion:** By improving the eluent, it was possible to remove sodium ion adducts observed by nucleoside ionization. In addition, the chromatograms showed good separation by selection of column. Intra assay precision CV = 2.6%, 2.3% and 2.3% (Low, Middle, HighQC) (n=10), inter assay CV = 4.7, 4.2, 4.1% (Low, Middle, HighQC) (n=15). The accuracy (CV) were 94.9% (3.7%), 99.9% (3.6%), 101.9% (4.5%) (n=4) (%mDC<sub>nominal</sub> were 0.96%, 4.80% and 9.60%), respectively.

We developed a new method with favorable conditions for the measurement of very small amount of methylated nucleoside. Three points should be discussed. First, our ionization conditions resulted in improvement of detectable sensitivity limited by sodium adduct ions. It has been found that ionization of nucleosides was different depending on the composition of the eluent. Second, the advantageous column selection

made proper separation of various nucleosides without unsuitable overlapping of chromatography peaks. Third, the reduction of errors by the improved calibration method solved the problematic limitations given by the matrix effects on assays and the errors due to two-step quantification for individual nucleosides. These advantageous points are essential to the successful solution of the problems of LC-MS/MS-based assays reported previously.

**Conclusion:** Our developed method is robust and easy to standardize for quantitative assays of DNA methylation based on LC-MS/MS.

#### A-401

##### Primary Q1/Q3 ion pair positive interference for internal standard in a 25-OH-Vitamin D assay by LC-MS/MS among patients receiving the antiemetic ondansetron (Zofran)

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**BACKGROUND:** Monitoring of the consistency of counts of internal standard (IS) across samples (often referred to as the metric plot) is an essential aspect of quality assurance in MS/MS assays. Aberrant IS cps for an individual sample can flag improper processing, or the presence of an interferent affecting ionization by enhancement or suppression, or, rarely, the presence of interference by an isobaric compound. Soon after adoption of LC-MS/MS for measurement of 25OH-vitamin D (25-OH-D) at our institution, we noted regular occurrences of samples having distinct apparent elevations of IS (d6-25OH-D3). We describe our investigation to determine that this interference was associated with patients receiving the antiemetic ondansetron (Zofran). **METHODS:** Our method for measurement of 25OH-D by LC-MS/MS is based on that of Garg et al. [PMID: 23001980] using positive-ion APCI and performed on an AB Sciex 3200MD instrument, with IS = d6-25OH-D3. In our first encounter with this interference, there was an approximately 2-fold elevation of IS (m/z Q1/Q3 = 389.3/211.3). It was determined immediately that this primary Q1/Q3 signal was not derived from d6-25OH-D3, as the interference lacked qualifier Q1/Q3 ion pairs characteristic of d6-25OH-D3 (389.3/263.3, 389.3/229.2). Among the first 6 patients encountered, there were no known medications in common. For the 7th patient, an inpatient, records review of medications and analysis of samples predating and postdating the original interference sample indicated onset of interference immediately after administration of the antiemetic ondansetron, with a rise and fall of interference occurring over an interval of 4 days. Ondansetron itself was tested in samples and produced no interference, indicating that interference was likely due to a metabolite of ondansetron. We then identified in-house patients with known time of administration of ondansetron, and tested existing specimens from these patients for the presence of the interference. **RESULTS:** Using a database of a four-day interval of inpatient pharmacy records for administration of ondansetron, we were able to retrieve existing post-administration plasma or serum specimens for 20 patients from this interval. Samples for 3 patients (15%) exhibited IS interference, being positive for the primary Q1/Q3 signal for IS and negative for the qualifier Q1/Q3 signals for IS. Alteration of LC parameters (e.g., lengthening of column) could shift the retention time of the interferent away from coelution with d6-25OH-D3. **CONCLUSIONS:** Results indicated that a low-incidence metabolite of ondansetron was the likely cause of a positive interferent in the primary Q1/Q3 measurement of d6-25OH-D3 in our 25-OH vitamin D assay. Low incidence may very plausibly reflect conditions of inherited or induced variations in CYP2D activity known to affect ondansetron metabolism. As yet, however, we are unable to deduce a plausible structural identification of the interference derived from ondansetron or its known metabolites. Given longstanding and widespread use of LC-MS/MS for measurement of 25-OH-D using this IS,

discovery of an IS interferent was surprising. The findings are a case study in the importance of a review of the metric plot (monitoring of IS across samples) as quality assurance for MS/MS assays.

#### A-402

##### Dried urine and blood spot analysis of essential and toxic elements by ICP-DRC-MS with an emphasis on inter-assay stability of samples kept at room temperature

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##### Background:

Elemental analysis plays a key role in monitoring the health of individuals and populations around the world. The sensitivity and specificity of inductively coupled plasma mass spectrometry (ICP-MS) has allowed precise quantification of a broad range of elements using various sample types. The collection, storage, and transport of samples without preservatives at room temperature while maintaining accuracy would be advantageous for health surveys, specifically in remote areas without refrigeration. We aimed to validate an assay for, and show the stability of, essential and toxic elements in urine and whole blood collected on filter paper.

##### Materials and Methods:

Our laboratory developed dried urine and blood spot elemental assays using Whatman 903 filter paper for sample collection and a Perkin Elmer NexION 300D ICP-MS with Dynamic Reaction Cell Technology for analysis. We focused on elements that have shown clinical utility in population health surveys and wellness assessments. Analytes selected were iodine, bromine, selenium, arsenic, cadmium, and mercury in dried urine, and zinc, copper, magnesium, selenium, cadmium, lead, and mercury in blood spot.

Dried urine and blood spot samples were extracted in 96-well fritted filter blocks using dilute ammonium hydroxide, L-cysteine, ethylenediaminetetraacetic acid (EDTA), triton-X, and internal standards. Six 6-mm punches and 1.2 mL of extraction solution were used for dried urine analysis while two 6-mm punches and 0.55 mL of extraction solution were used for blood spot analysis. Dried urine and blood spot assays were run separately on the ICP-MS in kinetic gas mode using helium, and employing a micro-flow pump and nebulizer to make effective use of the small sample volume.

##### Results:

Accuracy of the method was assessed by spotting onto filter paper available proficiency samples from the Centers for Disease Control and Prevention (CDC) and College of American Pathologists (CAP), which demonstrated excellent agreement with expected concentrations ( $R^2 > 0.96$ ). Elements for which CDC or CAP samples were not available were validated against Seronorm and ClinChek Trace Elements Urine and Whole Blood controls. Recovery was demonstrated by spiking blood and urine samples with a known concentration of analyte; acceptable recoveries of 80-120% were obtained. Linearity was assessed by diluting samples and comparing results to expected concentrations, and found to be acceptable. Limits of quantification were based on analysis of blank and low level samples, and were found to be acceptable. Intra-assay precision was based on 20 sample replicates, and the coefficient of variation was <8.3% for all analytes. Inter-assay precision was tested during 14 sample runs over 1 month keeping samples at room temperature to replicate conditions of collection and transport in areas without refrigeration. The coefficient of variation for inter-assay precision was <15.1% for all analytes.

##### Conclusions:

Dried urine and blood spot element analysis using ICP-MS was successfully validated. Demonstrated stability of elements in samples dried on filter paper allows accurate elemental analysis in population studies in remote areas without access to refrigeration, as samples can easily be collected, transported, and stored without the use of preservatives for at least a month.

**A-403****Adapting High-Resolution Mass Spectrometry for Clinical Toxicology: Comparison and Optimization of SWATH to Data-Dependent Acquisition for Drug Screening**

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**Background:**

The field of clinical toxicology is overrun with ever-changing synthetic drugs, adulterants, and unregulated supplements. Tandem mass spectrometry (LC-MS/MS) is not readily adaptable to these changes considering it relies on targeting expected ion fragments within a sample. High-resolution mass spectrometry (HRMS) has shown great success in unknown screening by allowing for compound detection through database comparison. The current standard for HRMS scanning is data-dependent acquisition (DDA), which acquires fragment ion spectra from the 10-20 most abundant precursor ions. This has come to show weaknesses in clinical and forensic toxicology where compounds of interest may be in low abundance compared to polypharmacy background or physiologic matrix.

Sequential Window Acquisition of All Theoretical Fragment-Ion Spectra (SWATH) is an emerging method of data acquisition that partitions all ions within a small mass window (e.g. 20 Da) for fragmentation so that no spectral data is lost. Additionally the size of these windows can be fixed (fSWATH) or varied (vSWATH) in order to separate similar compounds (e.g. amphetamine and methamphetamine). This study seeks to optimize and compare SWATH to DDA in clinical drug screening to assess and validate the fidelity of this emerging method and provide a model for adoption in the clinical laboratory.

**Methods:**

Urine samples were diluted 1:10 and separated with a Kinetex C18 column (50x3mm, 2.6 $\mu$ m) (Phenomenex). Data was acquired on a TripleTOF<sup>®</sup>5600 (SCIEX) in positive-ion mode. Analyst TF<sup>®</sup> software (SCIEX) was used to create three acquisition modalities; DDA: TOF-MS survey scan (100-650 Da) with triggered-collection of 20 product ion scans; fSWATH: SWATH acquisition (100-650 Da) with 30 fixed 18 Da windows; vSWATH: SWATH acquisition (100-650 Da) with 30 variable mass windows (6-59 Da) created to specifically separate similar drugs.

These methods were compared by limit of detection of 88 drug-spiked urine samples (5ng/mL-100ng/mL; duplicate runs) and drug/metabolite detection in 50 clinical samples previously characterized by LC-MS/MS.

**Results:**

Drug/metabolite limit of detection (LOD) was the lowest in vSWATH compared to fSWATH and DDA. vSWATH had the lowest LOD for 37(43%) of the drugs/metabolites as compared to 20(22%) for DDA; however in many cases the difference in LOD was 5-15 ng/mL. Thirty-five(31%) drugs/metabolites had equal LODs between vSWATH and DDA.

Detection of drugs/metabolites in clinical urine samples was similar for DDA and vSWATH (275 and 274, respectively). vSWATH detected 92% of compounds previously found where DDA and targeted LC-MS/MS confirmed 90%.

Additionally, 5 low-concentration compounds were confirmed by vSWATH that were negative by DDA (not triggered as abundant fragments), but could be observed in the extracted ion chromatogram.

**Conclusion:**

This study demonstrates that HRMS acquisition by vSWATH was optimal to DDA and fSWATH for clinically-relevant drug detection in both spiked urine and clinical samples. Furthermore, we demonstrated that vSWATH was able to accurately detect a number of low-abundance compounds that DDA missed due to biased acquisition of abundant ions. These findings are novel and clinically relevant considering they are the first to compare these four acquisition methods (LC-MS/MS, DDA, vSWATH, and fSWATH) and provide a model for optimizing HRMS platforms already in use.

**A-404****Analytical and Clinical Validation of a Novel Metabolite-based Serum Test to Precisely Determine the Glomerular Filtration Rate (GFR)**

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**Background:** Traditionally, glomerular filtration rate (GFR), an indicator of kidney function, is estimated using equations based on creatinine combined with age, sex,

and race (eGFR) because the gold standard methodology, measured GFR (mGFR), is complex, time-consuming, invasive, and expensive. The commonly used eGFR equations show limited precision and accuracy against mGFR. We have developed and validated a method to estimate GFR from a single serum sample using LC-MS/MS, using no demographics. The test measures four analytes: pseudouridine, acetylthreonine, phenylacetylglutamine, and tryptophan. The clinical performance of the assay has been validated against mGFR and eGFR.

**Methods:** The four analytes were measured using LC-MS/MS on an Agilent 1290-Sciex Qtrap 5500 UPLC/MS/MS system. Creatinine was also included and validated to use for clinical comparisons. All analytes were assayed in one run, with a run time of less than 4 minutes. The precision in serum was validated at the low, mid, and high points of the calibration range for each analyte. Linearity, precision, accuracy, LLOQ, recovery, specificity, interference, matrix effect, carryover, method comparison, ruggedness, and stability validation experiments were performed. Analyte results were used in a logistic regression analysis against mGFR data to generate a 4-term equation to calculate GFR. The clinical performance of the metabolite-based equation was compared to that of eGFR estimated by the CKD-EPI equation. Clinical performance of the method was evaluated against mGFR in 1,618 patient samples, equations developed, then validated in an additional 811 samples drawn randomly from four different cohorts (AASK, MDRD, CRISP, AGES-kidney). The sample cohorts included normal or reduced GFR patients with and without known kidney disease, of varying age and race; average mGFR result was 55 (26 SD) and ranged from 6-169 in the overall cohort.

**Results:** The assay for pseudouridine, acetylthreonine, phenylacetylglutamine, tryptophan, and creatinine was validated according to CLSI guidelines in a 20-day validation with two runs per day. All five of the analytes performed robustly over the 20-days with %CVs ranging from 3.7% to 6.9%. All other validation tests met acceptance criteria. Clinically, concordance with mGFR was better for the four metabolite-based test vs. for CKD-EPI and MDRD Study eGFR (0.68 vs. 0.55 and 0.54 in development, and 0.68 vs. 0.58 and 0.56 in validation,  $p < 0.001$ ). The rate of large errors expressed as  $1-P_{30}$  was 16.3% for CKD-EPI, 16.6% for MDRD but only 9.4% for our four metabolite-based test in development (14.3% and 14.2% vs. 10.0% in validation), equivalent to a 30-40% reduction in the rate of large errors ( $p < 0.001$ ). Added precision translates into more accurate diagnosis at the threshold of 60 ml/min/1.73m<sup>2</sup>, the cutpoint used to diagnose kidney disease. At 60 ml/min/1.73m<sup>2</sup>, the four metabolite-based test led to a reduction in the rate of misclassification of more than 30%.

**Conclusion:** In summary, we developed and validated a robust metabolite assay which substantially improves precision and accuracy of GFR estimation, thus having the potential to improve clinical care when greater GFR accuracy is needed.

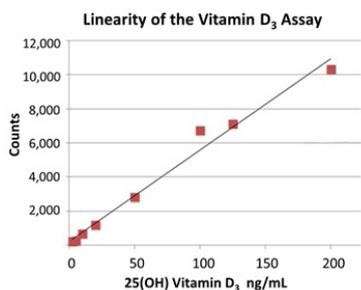
**A-405****Fast, New Derivatization Method for Vitamin D Analysis by LCMS**

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**Background:** Vitamin D analysis has become a major clinical test with millions of tests performed annually. Liquid Chromatography/Mass Spectrometry (LCMS) is the gold standard for the determination of vitamin D. Native vitamin D does not produce a strong signal in LCMS because it lacks a positive charge. Derivatization agents for vitamin D have addressed this problem but these reactions are slow. This new derivatization agent forms a vitamin D derivative in just 2 minutes.

**Methods:** Vitamin D plasma reference standards were obtained from NIST (Gaithersburg, MD) from 2014 to 2016. Human blood was collected in accordance with our IRB protocol. A portion was used to generate plasma by centrifugation. An internal standard (d6-vitamin D) from Medical Isotopes (Pelham, NH) was added and then plasma was extracted using methyl tert-butyl ether. The ether layer containing vitamin D was separated and evaporated to dryness. Reagents were obtained from a vitamin D kit developed by Novilytic. An aliquot was injected onto a C18 column followed by gradient elution. Vitamin D was determined with an AB Sciex 4000 mass spectrometer.

**Results:** The Vitamin D derivatization method was evaluated with plasma reference standards obtained from the NIST Quality Assurance Program (VitDQAP) from 2014 to 2016. The vitamin D results agreed within 5% of the specified target levels for these NIST samples. The within-run precision was 6% or better and the average CV was 3.1%. These results were in the top 20% of all LCMS submissions. The linearity of the Vitamin D assay was measured by spiking plasma with varying amounts of the heavy isotope form of the vitamin D<sub>3</sub> from 5 to 200 ng/mL. The coefficient of determination (R<sup>2</sup>) was 0.982 over the assay range. **Conclusion:** A new derivatization reagent reduces sample preparation time and improves the analysis of Vitamin D by LCMS.

**A-407****Implementation and Validation of Liquid Chromatography Mass Spectrometry Method for the Quantitation of Immunosuppressive Drugs**

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**Background:** Measurement of immunosuppressive drug concentration in blood is an important application of the Therapeutic Drug Monitoring (TDM) concept. Therapeutic drug monitoring of immunosuppressive drugs in organ-transplanted patients is crucial to prevent intoxication or transplant rejection due to inadequate dosage. After transplantation, there is a need for immunosuppressive treatment with individualized drug dosing and continuous life-long monitoring. Recently, LC MS/MS was installed in the laboratory for analysis of tacrolimus, cyclosporine and sirolimus as LC MS/MS is the preferred technique for the assessment of immunosuppressive drugs. The commonly used immunoassays have been gradually undergoing replacement by mass spectrometry, since this physical method offers both a higher sensitivity and specificity and low cost of analysis as well. The objective of this study is to evaluate the performance of Shimadzu LC MS/MS using Recipe® reagents for the analysis of tacrolimus, cyclosporine, and sirolimus.

**Methods:** The validation was performed using the on-line SPE LCMS/MS method for immunosuppressant therapeutic drug monitoring (Recipe GmbH, Germany) on analytical platform Shimadzu HPLC NEXERA X2 – LCMS-8050. A total of 100 samples were evaluated in this study. Samples were collected in 5 ml EDTA tubes from adult renal transplant, bone marrow transplant and pediatric liver transplant patients. Precision study was verified according to CLSI EP05-A2 using Recipe Clincheck controls in three concentration levels: 48.3 nmol/L, 98.1 nmol/L and 189 nmol/L for Cyclosporine A; 4.37 ng/ml, 13.2 ng/ml and 21.5 ng/ml for Sirolimus; 3.93 ng/ml, 8.13 ng/ml and 15.9 ng/ml for Tacrolimus and coefficient of variations (CV's) were calculated. Verification of Linearity was verified according to CLSI EP06-A using ClinCal®-Calibrator with six (6) different concentration spanning the analytical range of each assay.

**Results:** All % CVs were consistent with those claimed by the manufacturer for all tests. The method was found to be linear over the range of 0.017 - 1455 nmol/L for cyclosporine; 0.014 - 65 ng/mL for tacrolimus and 0.24 - 72 ng/mL for sirolimus. Analytical precision revealed a coefficient of variation of less than 10% on all assay. Analytical sensitivity was 0.017 nmol/L, 0.014 ng/mL, 0.24 ng/mL for cyclosporine, tacrolimus and sirolimus respectively. Recovery ranged from 82.5 to 92.0% for Cyclosporine A; 70.8 to 106.0% for Sirolimus and 89.1 to 115.5% for Tacrolimus. Matrix effects were well compensated by deuterated internal standards. Furthermore, the obtained results from the proficiency testing program (College of American Pathologists) were within the target value.

**Conclusion:** Overall performance of the immunosuppressive drugs on Shimadzu LC MS/MS using Recipe Reagents was acceptable. It provides reliable results for the required tests for all transplant services. Verification of the method has confirmed that all set criteria are achieved and suitable for routine use in clinical laboratory as the LC MS/MS technique provides a viable platform for the analytical routine service for Therapeutic Drug Monitoring of immunosuppressive drugs that meet clinical needs of reporting timely and reliable results.

**A-409****Clinical validation and comparison of serotonin analysis in various blood fractions for the follow-up of neuroendocrine tumor patients**

H. van Rossum<sup>1</sup>, A. C. Heijboer<sup>2</sup>, D. van den Broek<sup>1</sup>, M. E. T. Tesselaar<sup>1</sup>, O. van Tellingen<sup>1</sup>, C. M. Korse<sup>1</sup>. <sup>1</sup>NKI-AvL, Amsterdam, Netherlands, <sup>2</sup>VUMC, Amsterdam, Netherlands

**Background:** Serotonin, an endogenous neurotransmitter and paracrine agent, is used in the diagnosis and follow-up of neuroendocrine tumors (NET). We recently developed a LC-MSMS based method for serotonin analysis in serum and platelet-rich plasma (PRP). Here, we study the clinical application of various blood fractions for serotonin analysis used for the follow-up of NET patients. **Methods:** 94 patient samples obtained from 78 patients visiting our NET outpatient clinic were collected. Furthermore blood samples of 112 healthy volunteers were used for determination of the upper limits of normal for serum and PRP serotonin. Serum and PRP serotonin concentrations were determined using the LC-MSMS method and whole blood serotonin analysis was performed using the Chromsystems HPLC-ECD method. Method comparisons were performed using Passing-Bablok regression and Spearman's correlation analysis. Furthermore serotonin concentrations of the healthy volunteers, 14 NET patients without evidence of disease and 51 NET patients with evidence of disease were compared. **Results:** All obtained correlation coefficients were 0.98 and the slope of the whole blood versus serum regression was not significantly different from 1. The slopes obtained when comparing whole blood and serum serotonin with PRP serotonin were 0.74 and 0.71 respectively. NET patients with confirmed evidence of disease had significantly higher whole blood, serum and PRP serotonin concentrations when compared to NET patients without evidence of disease and healthy volunteers. **Conclusion:** Our results suggest that as long as serotonin is expressed per platelet, serotonin results obtained from whole blood, serum and PRP seem to be interchangeable and a similar clinical performance can be expected.

**A-410****A Clinical Research UPLC-MS/MS Method for the Quantitative Analysis of Urinary Free Cortisol**

R. Sanig, H. Brown, G. Hammond, L. Calton. *Waters Corporation, Wilmslow, United Kingdom*

**Background:** Cortisol is a glucocorticoid (steroid hormone) produced in the adrenal glands and is connected with stress response in humans. A clinical research method has been developed to aid hypercortisolism research using UPLC-MS/MS.

**Methods:** Samples (50 µL) were prepared with cortisol-<sup>2</sup>H<sub>3</sub> internal standard in methanol, diluted with aqueous trichloroacetic acid (0.5%), vortex mixed, centrifuged and analysed directly (10 µL). Chromatographic separation was achieved, in less than 3 minutes, using a Waters® ACQUITY UPLC® CSH C18 column (2.1 x 30 mm, 1.7 µm) with a water/methanol/ammonium acetate/formic acid gradient on the Waters ACQUITY UPLC I-Class system. Cortisol was detected using electrospray positive ionization with multiple reaction monitoring on the XEVO® TQD mass spectrometer. The precursor to product ion transitions used for the detection of cortisol were *m/z* 363.2 > 121.0 (quantifier ion) and 97.0 (qualifier ion) and *m/z* 366.2 > 124.0 for the detection of cortisol-<sup>2</sup>H<sub>3</sub>. In-house calibrators (13.8-1103.0 nmol/L) and quality controls (20.7, 41.4, 206.9, and 827.7 nmol/L) were prepared using cortisol reference material from Cerilliant (Round Rock, TX) in phosphate-buffered saline.

**Results:** The method was shown to be linear from 4.0 to 1340.0 nmol/L, with total precision and repeatability coefficients of variation (CV) for the four quality control levels all ≤6.20% (n = 5, days = 5). Analytical sensitivity of the method allows for cortisol quantification at 1.38 nmol/L (≤20%, inter-day CV). The method demonstrated no interferences from analogous steroids. Carryover following a 1655.4 nmol/L extracted sample was below the limit of quantification. Minimal matrix effects were observed and recovery was unaffected (mean 100.5%, range 93.9-105.6%) when six urine pools were spiked with 20.7, 41.4, 206.9, and 827.7 nmol/L cortisol. The accuracy of the method was determined by analysing samples (years 2015-2016, n=48) from the UK NEQAS Scheme (Birmingham, UK); good agreement was obtained with a mean method bias of ≤4.1% for all samples (Altman-Bland).

**Conclusions:** A rapid, simple and cost-effective UPLC-MS/MS procedure for the determination of urinary free cortisol concentrations has been developed for clinical research. The method has shown good analytical sensitivity, selectivity, linearity, precision and accuracy.

For Research Use Only, Not for use in diagnostic procedures.

Keywords: Cortisol, Urine, Mass Spectrometry, LC-MS/MS

## A-411

**Mucopolysaccharides quantitation in dried blood spots by liquid chromatography-tandem mass spectrometry**

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**Background:** Mucopolysaccharides (MPS) are sulfated polysaccharides that contain repetitive disaccharide units attached to a protein core. The various mucopolysaccharidoses exhibit different patterns of excretion of four mucopolysaccharides: dermatan (DS), heparan (HS), keratan (KS S1 and KS S2) and chondroitin sulfate (CS). We describe a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for routine determination of DS, HS, KS S1, KS S2 and CS in dried blood spots (DBS), as an efficient and effective screening test for MPS-I/MPS-II, MPS-III, MPS-IV and MPS-VI.

**Method:** Four 1/8" disks from a DBS are utilized for DS, HS and KS determination (two disks) and CS determination (two disks). DS, HS and KS are enzymatically digested to disaccharides by the addition of heparinase I, II, III and chondroitinase B, while CS is enzymatically digested to disaccharides by chondroitinase AC. Elution mixture rotates at 30°C for 120 minutes. The reaction is stopped by the addition of EDTA buffer including internal standard GICNCoEt-6S. The reaction mixture is then centrifuged and subjected to LC-MS/MS analysis. The MS/MS is operated in the multiple reaction monitoring negative mode to follow the precursor to product species transitions for DS ( $m/z$  458.0 to  $m/z$  300.0), HS ( $m/z$  378.0 to  $m/z$  175.0), KS S1 ( $m/z$  462.0 to  $m/z$  361.0), KS S2 ( $m/z$  542.0 to  $m/z$  462.0), CS ( $m/z$  458.0 to  $m/z$  282.0) and the internal standard GICNCoEt-6S ( $m/z$  472.0 to  $m/z$  97.0).

**Results:** Inter-assay calibration curves (N=3) were linear and reproducible over the concentration range 0-600 nmol/L for DS, HS and KS S2, 0-1200 nmol/L for KS S1 and 0-1800 nmol/L for CS. Intra- and inter-assay precision were assessed using three DBS samples of varying concentrations (DS = 118, 219 and 318 nmol/L; HS = 96, 217 and 290 nmol/L; KS S1 = 411, 603 and 640 nmol/L; KS S2 = 140, 227 and 265 nmol/L; CS = 408, 862 and 760 nmol/L). Intra-assay precision CVs were 13.0, 10.4 and 11.5% for DS; 16.9, 13.5 and 8.1% for HS; 11.1, 16.3 and 9.2% for KS S1; 12.0, 14.0 and 11.3% for KS S2; 14.1, 20.6 and 17.2% for CS, respectively (N=20). Inter-assay precision CVs were 14.4, 10.5 and 17.1% for DS; 20.9, 18.7 and 18.1% for HS; 16.9, 13.0 and 16.7% for KS S1; 16.3, 13.8 and 14.3% for KS S2; 35.2, 19.5 and 20.6% for CS, respectively in the same specimens (N=20). Two DBS specimens spiked with DS, HS and KS S2 (100 and 400 nmol/L), KS S1 (200 and 800 nmol/L) and CS (300 and 1200 nmol/L) standard solutions exhibited recoveries ranging from 87% - 113%. Newborn DBS (N=208), pediatric DBS (age > 2 week - 18 years) (N=97) and adult DBS controls (N=125) were analyzed for reference range determination. Clinical sensitivity was 100% for MPS-I (N=10), MPS-II (N=6), MPS-III (N=5), MPS-IVA (N=4) and MPS-VI (N=3). Low molecular weight heparin and sodium or lithium heparin interfere with HS determination.

**Conclusions:** Preliminary data show that our test is a rapid and specific method for timely identification of patients with MPS-I, MPS-II, MPS-III, MPS-IV and MPS-VI.

## A-413

**Simple sample preparation technique for the determination of Metanephrines, Catecholamines and their metabolites in Urine by LCMSMS for research use**

R. M. Doyle. *Thermo Scientific, Inc, Somerset, NJ*

**Background:** Liquid chromatography triple quadrupole (QQQ) mass spectrometry (LC-MS/MS) is suited for rapid analysis of multiple analytes. The metanephrines and catecholamines are compounds involved neuromodulation and act as hormones. The Metanephrines, Catecholamines and their metabolites analyzed included metanephrine, normetanephrine, dopamine, epinephrine, norepinephrine, 3-methoxytyramine, vanillylmandelic acid, homovanillic acid and 5-Hydroxyindole Acetic Acid. A highly sensitive and specific LC-MS/MS analytical method has been developed for the determination of Metanephrines, Catecholamines and their metabolites in urine. A simple sample preparation technique that involved liquid-liquid extraction and diphenyl-boronate complexing was utilized along with a one (1D) dimensional liquid chromatographic configuration. The described method achieves the required sensitivity and is capable of determining the purine and pyrimidine metabolites over their dynamic range. Therefore, a simple and accurate quantitative analytical method was developed for the quantitatively measurement of Metanephrines, Catecholamines and their metabolites in urine.

**Method:** A Thermo Scientific™ Endura™ tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Scientific™ Dionex™ Vanquish™

Horizon HPLC system were utilized for this analysis. 500 µl of urine were used for the analysis of the Metanephrines, Catecholamines and their metabolites in urine. Various columns were evaluated and an Thermo Scientific™ Accucore™ PFP 100 x 2.1 mm, 3 µm with a water:methanol mixture containing 0.2% Formic Acid achieved baseline chromatographic separation for all the Metanephrines, Catecholamines and their metabolites in urine in less than 8 minute run time. Quantitative analysis was performed using selective reaction monitoring (SRM) with transition pairs for each analyte and internal standard in positive and negative mode and the precision and accuracy of the method was verified using pooled quality control materials and urine samples.

**Result:** Good linearity and reproducibility were obtained with the concentration range of 5 to 1000 ng/ml for the respective Metanephrines, Catecholamines and their metabolites in urine with a coefficient of determination >0.95 for the sample preparation technique and the LC-MS/MS configuration used. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to be range from 2 to 5 ng/ml and excellent reproducibility was observed for all compounds (CV < 10%).

**Conclusion:** A sensitive, simple, specific and accurate liquid chromatography tandem mass spectrometry method was developed and verified for the simultaneous determination and screen of Metanephrines, Catecholamines and their metabolites in urine. The sample preparation technique is quick and easily applied for high throughput analysis.

## A-414

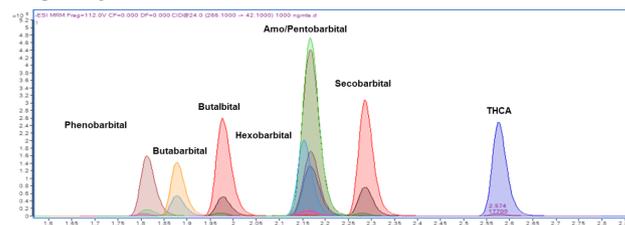
**A Fast LC/MS/MS Method for the Simultaneous Analysis of Barbiturates and 11-nor-9-Carboxy- $\Delta^9$ -Tetrahydrocannabinol (THCA) in Urine Using Negative ESI Ionization Mode and Alternate Column Regeneration (ACR)**

A. Szczesniowski. *Agilent Technologies, Wood Dale, IL*

**Background:** Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is suited for rapid, simultaneous analysis of multiple analytes. A fast, highly sensitive and specific LC/MS/MS analytical method has been developed for the quantitation of Barbiturates and 11-nor-9-Carboxy- $\Delta^9$ -Tetrahydrocannabinol (THCA) in urine using the negative ESI ionization mode. Barbiturates included; Amobarbital, Butalbital, Butobarbital, Hexobarbital, Pentobarbital, Phenobarbital and Secobarbital. Simple sample preparation techniques such as dilute and shoot and a short chromatographic configuration achieved good analytical sensitivity and was capable of quantitating all analytes over a wide dynamic range. **Methods:** An Agilent 6470 tandem mass spectrometer with Jet Stream technology in negative electrospray (ESI) mode and an Agilent Infinity II 1290 UHPLC system were utilized for this analysis. Use of the ACR was achieved by addition of second pump and 2 position 10 port switching valve for the reduction of method runtime that excluded column equilibration time. A 100 µl aliquot of urine was used for the analysis of Barbiturates and THCA. The Agilent Polaris C18-Ether, 100 x 2.0 mm, 3.0 µm column with a water:acetonitrile mixture containing a 5 mM Ammonium Acetate gradient achieved chromatographic separation in less than 3 minutes. Quantitative analysis was performed using multiple reaction monitoring (MRM) transition pairs for each analyte and an internal standard in the negative mode.

**Results:** The isobaric pair, Amobarbital and Pentobarbital, were not separated under these chromatographic conditions. Good linearity and reproducibility were obtained for the concentration range from 5 to 1000 ng/ml with a coefficient of determination >0.995 for all analytes. Excellent reproducibility was observed for all analytes (CV < 15%).

**Conclusion:** A fast, specific and accurate quantitative liquid chromatography mass spectrometry (LC/MS/MS) method was developed and verified for the simultaneous measurement of Barbiturates and THCA in urine. For Research Use Only. Not for use in diagnostic procedures.



**A-416****Impact of Matrix-assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry for Rapid Microbial Identification in a Community Hospital**

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In an effort to accelerate organism identification in positive blood cultures, and facilitate targeted antibiotic treatment sooner, our community hospital implemented the use of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS<sup>®</sup>).

**Goal:** The goal of this study was to determine if the implementation of MALDI reduced organism identification times, and subsequently resulted in any reductions in patient length of stay (LOS) or charges.

**Method:** All positive blood cultures from January-June 2014 (pre-MALDI group) were compared to those from January-June 2016 (MALDI group). Time from “positive bottle to organism ID” (PBID), LOS, and charges were determined for all specimens.

**Results:** The table below shows the average PBID time and range, the average LOS and the average charges.

	n	Avg PBID(hr)	Time range PBID (hr)	Avg LOS days	Avg Charges
Gram Pos (GP) 2014 - ALL	70	22.90	9.1-70.8	8.44	\$123,188
Gram Pos (GP) 2016 - ALL	71	12.46	3.3-49.40	8.3	\$95,958
Gram Neg (GN) 2014 - ALL	92	43.54	21.8-92.6	7.63	\$88,292
Gram Neg (GN) 2016 - ALL	71	9.76	3.73-22.1	6.95	\$79,758
Gram Pos (GP)2014 – uncomplicated sepsis	30	22.44	9.6-47.5	9.07	\$113,837
Gram Pos (GP)2016 – uncomplicated sepsis	26	13.36	6-19.7	7.92	\$90,504
Gram Neg (GN) 2014 – uncomplicated sepsis	53	43.04	21.8-92.6	7.62	\$84,172
Gram Neg (GN) 2016 – Uncomplicated sepsis	39	9.07	3.7-16.9	5.18	\$52,675
Yeast 2014	8	40.12	19.4-69.9	n/a	n/a
Yeast 2016	7	13.18	6-23.6	n/a	n/a
Contaminants 2014	97	29.04	9.13-8.62	n/a	n/a
Contaminants 2016	50	15.33	6-40	n/a	n/a

The average PBID time decreased by 45.6% for GP, 77.6% for GN, 40.5% GP for uncomplicated sepsis, 78.9% for GN uncomplicated sepsis, 47.2% for contaminants, and 67.1% for yeast. The “ALL” population patients included all diagnoses/procedures, many not associated with sepsis. The LOS and charges were dependent on the severity of the diagnosis and procedures. We investigated if the shorter PBID times provided better LOS outcomes for the subset of “uncomplicated” sepsis patients (no septic shock, severe sepsis or bacteremia). For this subset, the LOS for gram positive sepsis patients dropped from 9.07 days to 7.92 days and associated charges dropped 15.89% (unadjusted for inflation). The LOS for gram negative sepsis patients dropped from 7.62 days to 5.18 and charges fell 36.25% (unadjusted).

**Conclusion:** The implementation of MALDI TOF for identification of positive blood cultures markedly decreased the time to identification for all positive blood cultures. It appears from this study that the shorter time to ID, led to shorter LOS and lower charges for all patients and more significant decreases were noted with uncomplicated sepsis.

**A-417****Improved Liquid Chromatography Mass Spectrometric Determination of Vitamin D Metabolites in Human Plasma/Serum by Phospholipid Removal with Zirconia Sorbents**

X. Lu, D. S. Bell. MilliporeSigma, Bellefonte, PA

**Background (Objective)**

Liquid chromatography with mass spectrometry (LC/MS) has been gaining more and more popularity in the clinical analysis due to the unparalleled speed, selectivity and sensitivity. However, the application of LC/MS to the determination of vitamin D metabolites in plasma/serum has been compromised by the considerable matrix effects. The present study is attempt to the development of a simple and quick method

to remove the highly abundant phospholipids as well as proteins in plasma/serum and thus reduce the matrix effects.

**Method**

Zirconia sorbents packed into a 96-well plate were exploited for the selective removal of phospholipids from the matrices. Proteins in the matrices were firstly precipitated by the addition of organic solvent such as acetonitrile and methanol, into the plasma/serum samples. This can be done either inside or outside the 96-well plate. The protein-precipitated samples were then passed through the 96-well plate where the phospholipids were retained by the zirconia sorbents but vitamin D metabolites went through and were collected. The resulting samples were directly injected for LC/MS/MS analysis with a pentafluorophenyl (F5) phase HPLC column and triple quadrupole mass spectrometer.

**Results**

Four vitamin D metabolites including 25-OH vitamin D3 (D3), 3-epi-25-OH-vitamin D3 (epi-D3), 25-OH vitamin D2 (D2), 3-epi-25-OH vitamin D2 (epi-D2) spiked in human plasma, are separated and determined by the LC/MS/MS. Without phospholipid removal, the signals of the vitamin D metabolites in the plasma are about half of those of vitamin D neat standards in solutions. This indicates considerable matrix effects. Further study reveals multiple phospholipid species of high intensity, 1,000,000 to 7,000,000 cps, co-elute with the vitamin D metabolites. The zirconia sorbent-packed 96-well plate were exploited and found consistently removing >99.5% of the phospholipids from the plasma and the intensity of the phospholipids drops to <5,000 cps. Additionally, the signals of vitamin D metabolites in plasma are fully restored to that of the neat standards. The reproducibility of the recovery of the vitamin D metabolites ranges from 4% to 12%.

**Conclusion**

A method has been developed for the LC/MS/MS determination of vitamin D metabolites in human plasma with minimum matrix effects. The method utilizes zirconia sorbent-packed 96-well plate for quick and efficient phospholipid removal. The signals and recovery of vitamin D metabolites are significantly improved when phospholipids are removed from the plasma.

**A-418****Comparison of blood sirolimus level measured by LC-MS/MS and immunoassay method**

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**Background:** Sirolimus is a one of the widely used immunosuppressant for preventing organ rejection after kidney transplantation. Therapeutic drug monitoring of this agent is important because of its narrow window of therapeutic efficacy. In this study, we performed method comparison of analytical accuracy between liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and antibody-conjugated magnetic immunoassay (ACMIA).

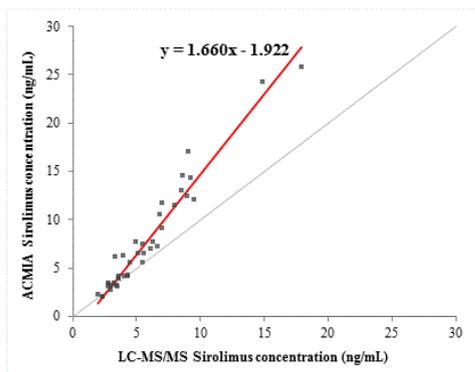
**Methods:** We used LC-MS/MS and Dimension RXL System (Siemens Healthcare Diagnostics) for measuring sirolimus concentrations. A total of 42 samples from patients treated with sirolimus were analyzed using these two methods. We also measured blood samples spiked with 6 different levels of sirolimus using these methods. We performed correlation and Passing-Bablok regression analyses between levels measured by two different methods with 95% confidence interval.

**Results:** For the real patient samples, the results measured by ACMIA showed significant (about 66%) deviation in sirolimus concentrations from those of LC-MS/MS. We obtained the following relationship:  $y = 1.660x - 1.922$  (correlation- $r = 0.975$ ), where  $y$  is the value obtained with LC-MS/MS and  $x$  is that obtained with ACMIA. However, for artificially spiked samples, these two methods did not show significant deviation:  $y = 1.099x - 1.969$  (correlation- $r = 0.996$ ) (Fig. 1).

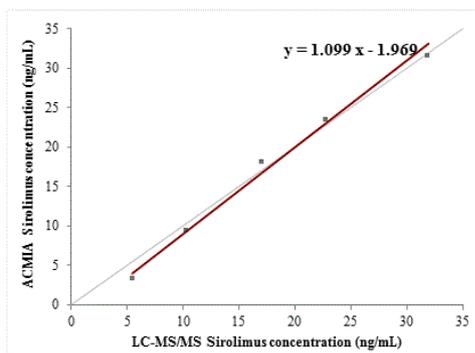
**Conclusion:** Sirolimus concentrations measured by ACMIA showed good agreement with those measured by LC-MS/MS for artificially spiked samples, but not for real patient samples. Compared to LC-MS/MS, 66% positive deviation in ACMIA was observed. The difference in agreement between sample types might be attributed to cross-reactivity of ACMIA with sirolimus metabolites (such as 12-hydroxy sirolimus, 39-O-demethyl sirolimus, and 27-39-O-didemethyl sirolimus). So, we recommend the use of LC-MS/MS for measuring sirolimus concentrations to prevent cross-reactivity by its metabolites.

**Fig. 1.** Method comparison of analytical accuracy between LC-MS/MS and ACMIA. These are the results using the real patient samples (A) and artificially spiked samples (B) measured by these two methods.

(A)



(B)

**A-419****Development of an LC-MS/MS Method for Measurement of a Steroid Panel in Serum for Clinical Research**X. Xie. *Thermo Fisher Scientific, San Jose, CA***Background**

Over the past few years there has been a growing interest to use liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure steroids in serum for clinical research. LC-MS/MS offers the potential for more reliable measurement and capability of measuring multiple steroids simultaneously compared to other detection systems, such as immunoassays. However, the challenges are time-consuming sample pre-treatment, matrix interference and isobars separation. We developed a simple and fast solid phase extraction (SPE) sample preparation method for an 11-steroid panel (11-deoxycortisol, 17-OH progesterone, aldosterone, androstenedione, corticosterone, cortisol, cortisone, estradiol, estrone, progesterone and testosterone) and evaluated the analytical performance on an LC-triple quadrupole MS/MS system. This analytical method is to demonstrate analytical capabilities of instruments and workflow, and it has not been validated and it is not intended to be used for reporting anything else other than analytical performance.

**Methods**

Extraction was performed on a micro 96-well SPE plate. Calibrators/QCs were spiked into neat solution or charcoal stripped serum and mixed with water and methanol. The mixture was loaded directly onto the SPE plate; no preconditioning was required. After washing the plate with 30% methanol, the elution was performed with two volumes of 25  $\mu$ L of methanol each. Eluates were diluted with 50  $\mu$ L water. 50  $\mu$ L of the diluted eluate was injected for LC-MS/MS analysis. Extracted compounds were separated on a reverse phase column chromatographically followed by analysis on a triple quadrupole mass spectrometer with heated electrospray ionization. The total LC run time is 7 min. Data were acquired in selected-reaction monitoring (SRM) mode. Two SRM transitions for each analyte/IS were measured with polarity switching. Ion ratios were calculated for confirmation.

**Results**

The whole SPE process takes less than 20 minutes and no pre-conditioning, evaporation or reconstitution is required. Comparing to conventional SPE method which involves those steps, our method is simpler and faster. We optimized the washing step for

SPE and determined that 30% methanol yielded the best overall recovery rate. The recovery rate ranged from 42% (aldosterone) to 95% (testosterone). Lower limit of quantitation (LOQ) of androstenedione is 1 pg/mL. LOQ of testosterone is 2 pg/mL. LOQ of 11-deoxycortisol, 17-OH progesterone, cortisone, estradiol, estrone, and progesterone is 5 pg/mL. LOQ of aldosterone, corticosterone, and cortisol is 10 pg/mL.

**Conclusion**

We demonstrated a simple and fast SPE method for sample pre-treatment of an 11-steroid panel with acceptable recovery rate. The LOQs of 1-10 pg/mL indicate LC-MS/MS is a sensitive and selective analytical method for simultaneous measurement of multiple steroids in serum for clinical research.

**A-420****A Collaborative Approach for Mass Spectrometry Education for Medical Laboratory Scientists**

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**Background:** Laboratory scientists, until recently, have had limited exposure to the principles, practice and methodologies of mass spectrometry. Since the 80's, gas chromatography/Mass spectrometry (MS) was the method of choice for drug confirmation for Workplace Drug Testing, Clinical and Forensic Toxicology. Recent technological advances have rapidly expanded clinical and translational applications of mass spectrometry and LC-MS/MS. These included the use of MALDI-TOF for microbiology, selected areas of clinical chemistry such as endocrine applications, and Therapeutic Drug Monitoring such as immunosuppressants, signaling the emerging Next Generation MS. Thus, the clinical laboratory is challenged to implement new tests using this methodology. Selected laboratory staff have gained mass spec proficiency by on the job training, but it is estimated that more personnel will be needed. In order to increase training and education of MS, professional societies have offered workshops and lectures to meeting attendees. Hands-on workshops have also been offered by some institutions. Because of our own needs of expanding LC-MS/MS capabilities, we developed the current collaborative and focused approach for training Medical Laboratory Scientists (MLS) in MS, which evolved as an extension of the overall educational missions of both Wake Forest Baptist Health (WFBH) and Winston Salem State University (WSSU).

**Methods:** WFBH and WSSU have recently modified the general clinical pathology rotation for their MLS students to offer both greater exposure and more in-depth hands-on training opportunities for students. The educational training contents were developed in a complementary collaborative planning process. The basic principles and introduction to MS were incorporated as part of the third and fourth year curriculum. Students were introduced to chromatography, MALDI-TOF and triple quadrupole MS instruments by didactic lectures at WSSU. This was complemented by the general clinical laboratory rotations at WFBH which provided limited "shadowing" opportunities. From this initial exposure and with feedbacks from the faculty and staff, a select number of students was identified to continue focused training. On-site clinical presentations of selected area of clinical applications of MS for microbiology and clinical chemistry/toxicology were offered.

**Results:** Based on approximately 1.5 years of collaboration, we have two students doing the general clinical laboratory rotation. From this group, one student was selected for additional training in Mass Spec. The student was able to participate in the validation of a newly developed assay for clinical use and also participate in research projects related to MS. At the end of the rotation, the student was competent in the basic areas of Mass Spec. The student presented her experiences in MS training at WFBH to her fellow students and increased their interest for this additional training. We now have new students interested in a summer internship in MS.

**Conclusion:** In developing a collaborative and complementary program, medical laboratory scientists are able to gain introductory knowledge and skill sets. For selected individuals, the exposure might evolve into more advanced training, enhancing clinical MS applications and the availability of trained laboratory personnel.

## A-421

## Steroid detection in small volume blood by LC-MS/MS

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**Background:** Liquid chromatography- tandem mass spectrometry (LC-MS/MS) has become a successful immunoassay substitute for steroid detection in clinical laboratories. The LC-MS/MS platform provides superior specificity and the possibility to detect a vast number of hormones in a small amount of blood in just one acquisition. However, the sensitivity of the LC-MS/MS assay is still equal or even lower than that of immunoassays. The aim of the present study is to demonstrate the ability of LC-MS/MS to reach a higher level of sensitivity in patient samples using a small volume collection device for blood.

**Method:** An LC-MS/MS quantitative method was set up on a Shimadzu 8060 triple quadrupole mass spectrometer for 16 steroids (Cerilliant, Round Rock). Steroids were spiked in charcoal strip FBS serum (Fisher Scientific), which was tested negative for all hormones, present in study. Sample preparation was done by solid-phase extraction using SPE trace T-20 column (Tecan SP) and Biotage positive pressure manifold. After elution, samples were dried under nitrogen steam and reconstituted in a water/methanol solution. 10 ul of reconstitute was injected directly into the LC-MS system, which was operating in both positive and negative electrospray ionization mode. Compounds (including isobaric) were separated on a Restek Biphenyl column, with a total run time of 6 minutes (including column reequilibration). Concentration of steroids from vein and capillary blood across 40 individuals was compared using set-up LC-MS/MS assay.

**Results:** Method was clinically validated, and demonstrated acceptable accuracy (>90%) and precision (CV>80%). Linearity range for all compounds in this study covered published normal reference ranges, and LOQ's that were compatible with immunoassay, available for the Abbott i1000 chemistry analyzer. Method performance was confirmed by cross-validation with CAP proficiency testing samples and with 20 patient results from Abbott i1000 analyzer. Hormone concentration from vein and capillary blood demonstrates significant correlation ( $R^2 > 0.98$ ).

**Conclusion:** The current sensitivity level for steroid detection, which was demonstrated by our LC-MS/MS assay allows for a successful substitute to the classical immunochemistry assay in clinic. The hormones concentration in capillary blood (finger stick) near identical to conventional serum venipuncture level. Therefore finger-stick could be an excellent source of blood for hormone testing due to convenience, cost, and timing of collection.

## A-424

## Identification of FLT3 Internal Tandem Duplications by Liquid Chromatography/Tandem Mass Spectrometry

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**Background:** Using sophisticated molecular methods, several mutations of prognostic significance have been identified in cytogenetically normal cases of acute myeloid leukemia (AML). The genetic loci most commonly affected, in order of relative frequency, include the coding regions for FMS-related tyrosine kinase 3 (*FLT3*), nucleophosmin family member 1 (*NPM1*), and CCAAT/enhancer-binding protein alpha (*CEBPA*). *FLT3* mutations are heterogeneous and can involve either the juxtamembrane domain (JM) or the tyrosine kinase domain (TKD) of the protein product. Current methods of identifying *FLT3* mutations are based on polymerase chain reaction (PCR) amplification and subsequent analysis using a variety of techniques (capillary electrophoresis, etc.) which all impose significant cost on the clinical laboratory. With an ever increasing amount of information being stored in nucleic acid and protein databases, more rapid identification and accurate quantification of molecular species is now possible. Mass spectrometry (MS) is one technique that allows for the timely characterization of such entities while avoiding the tedious benchwork historically required for molecular identification.

**Methods:** Purified *FLT3* protein (OriGene Technologies, Rockville, MD) was resuspended according to the manufacturer's instructions for subsequent LC-MS/MS analysis. Paraffin-embedded bone marrow samples from 56 AML patients were sectioned, stained with hematoxylin, and placed on standard glass slides. Separate areas of the bone marrow clot sections with the highest blast fractions were identified and laser capture microdissection (LCM) performed using a XT-TI system (Arcturus Engineering Inc., Sunnyvale, CA). Following LCM, protein extraction was carried out using the Liquid Tissue® MS Protein Prep Kit (Expression Pathology, Rockville, MD) for each specimen. Samples were analyzed on a Q-Exactive HF mass

spectrometer (Thermo Scientific, Rockford, IL) coupled to a Dionex Ultimate-3000 nano-UPLC system (Thermo Scientific, Rockford, IL).

**Results:** A small oligopeptide EYEYDLK (480.2 > 375.2) was identified using data dependent acquisition and the top 20 most abundant peptide fragments. This small peptide is centered around arginine 595, the most frequently involved amino acid in *FLT3* internal tandem duplications (ITDs), and demonstrates linear concentration-dependent changes in signal intensity.

**Conclusions:** Mass spectrometry is a promising modality for proteomic studies spanning a wide range of disease states. Acute myeloid leukemia is one area that may benefit from this technology as *FLT3* mutational status becomes rapidly available and therapeutic regimens personalized according to the results of such molecular analysis.

## A-425

## Evaluation of Clinical, Genetical, and Steroid Profile Features of Cases with 3Beta-Hydroxysteroid Dehydrogenase Type 2 Deficiency

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**Background:** 3-beta hydroxysteroid dehydrogenase 2 ( $\beta$ -HSD2) is the enzyme which catalyzes the conversion of D4 steroids (pregnenolone; Preg, 17-hydroxypregnenolone; 17OHPreg, dehydroepiandrosterone; DHEA) to D5 steroids (progesterone; Prog, 17-hydroxyprogesterone; 17OHProg, androstenedione; AS) and is coded by the *HSD3B2* gene.  $\beta$ -HSD2 deficiency is the rarest cause of congenital adrenal hyperplasia and results in deficiency of glucocorticoid, mineralocorticoid, and sex steroids. Deficiency of adrenal sex steroids and increase of their precursors may cause sexual maturation disorders in both genders and premature adrenarche and hyperandrogenism in females. The impact of this disease on steroid profile is not known. We aimed to investigate the clinical, genetic and steroid profile properties of  $\beta$ -HSD2 deficiency. **Methods:** Patients with  $\beta$ -HSD2 deficiency (as confirmed by genetic testing or clinical and laboratory findings) were included in this multicentric study. The treatments of patients using glucocorticoids/mineralocorticoids, were discontinued for 48 h. Blood samples obtained between 08-09 A.M., were analyzed with liquid chromatography - tandem mass spectrometry (LC-MS/MS).  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratio was used for the diagnosis of  $\beta$ -HSD2 deficiency, and the ratio was compared to the healthy control ratios. *HSD3B2* gene sequence was analyzed in patients with elevated  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratios.  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratios were calculated and compared in patients with and without *HSD3B2* mutation, in  $\beta$ -HSD2 heterozygote individuals (parents of patients with *HSD3B2* mutation), and in patients with genetically confirmed 21-hydroxylase deficiency patients for the diagnosis and differential diagnosis of  $\beta$ -HSD2 deficiency.

**Results:** Of the 29 patients suspected to have  $\beta$ -HSD2 deficiency, 12 had *HSD3B2* mutation. Four of the six mutations found, were new mutations.  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratios could be calculated in 7 patients with *HSD3B2* mutation and was significantly elevated compared to other groups (median; 4.3,  $p < 0.0001$ ). Genetic testing was not done in ten patients with normal  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratios. *HSD3B2* mutation was not found in 7 patients with elevated  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratios (n=7, median; 0.16, IQR; 0.09-0.23) compared to healthy controls (n=43, median; 0.03, IQR; 0.02-0.08) ( $p=0.0002$ ). **Conclusion:** LC-MS/MS is the gold standard in the measurement of steroid hormone analysis.  $(\text{Prog}+17\text{OHPreg}+\text{DHEA})/(\text{Prog}+17\text{OHPreg}+\text{AS}+\text{cortisol})$  ratio is very useful in the diagnosis of  $\beta$ -HSD2 deficiency. In patients with a similar steroid profile to that of  $\beta$ -HSD2 deficiency, further genetic analyses are required to elucidate the etiology of diseases such as hyperandrogenism and polycystic ovary syndrome and the effects of  $\beta$ -HSD2 action.

## A-426

**Development of a novel, high-sensitivity LC-MS/MS serotonin assay for assessing platelet function using a minimal amount of whole blood**

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**Background:** Light transmission aggregometry (LTA) is presently the gold standard employed in patients with suspected bleeding disorders for *in vitro* investigation of an underlying platelet function abnormality. LTA instruments equipped with a second luminescence channel (“lumi-aggregometer”) additionally can simultaneously measure released ATP that occurs upon secretion of platelet dense granules. The dense granules contain ATP, ADP, additional phosphates, serotonin (5-HT), and calcium. The released ADP is thought to be most critical, since it activates additional platelets that then contribute to formation of the hemostatically essential platelet aggregate. Performance of lumi-aggregation employing a battery of platelet stimuli typically requires up to 50 mL of patient blood, with a platelet count of at least 150,000/ $\mu$ L. The development of a novel LC-MS/MS approach was undertaken in an effort to extend the opportunity for the study of platelet dense granule secretion to newborns, as well as to adult patients with decreased platelet counts.

**Methods:** 200  $\mu$ L of citrated whole blood was incubated with exogenous deuterated serotonin ( $D_5$ -HT). Upon  $D_5$ -HT uptake, blood was aliquoted, and separately incubated with a variety of classic platelet stimuli, closely paralleling the approach used in LTA. Following stimulation, blood was centrifuged and the releasate obtained. A platelet lysate was used to assess total  $D_5$ -HT uptake. For serotonin sample quantitation, calibrators and quality controls (QC) were prepared by spiking  $D_5$ -HT into modified Tyrode’s buffer (MTB). Internal standard (IS) methylated-5HT (10 ng/mL) was added into samples followed by precipitation using ascorbic acid (50 mg/mL) and perchloric acid (70%), followed by centrifugation. The supernatant fraction was subjected to solid phase extraction. The eluent was concentrated using evaporation technique followed by reconstitution with 5% methanol/95%  $H_2O$  with 0.1% formic acid (FA) prior to injection. Samples were separated under a gradient elution on a Triart C18 Column (50 x 0.5 mm, 3 $\mu$ m, YMC America, Inc. PA) with a flow rate of 25  $\mu$ L/min and a total run time of 4 min using the Eksigent microLC 200 (Sciex, MA). Analytes and IS were detected by the Sciex QTrap 6500 mass spectrometer (Sciex, MA) with an ESI source.

**Results:** This assay showed excellent analytical measuring range from 50-2000 pg/mL ( $r^2 > 0.99$ ). QC samples prepared at the lower limit of quantitation, as well as low, mid and high QC levels yielded an inter-assay precision showing a CV of 9.6%, 4.8%, and 5.5%, respectively. Intra-assay precision showed 6.7%, 7.6%, and 3.6% respectively. Accuracy study demonstrated 75% extraction recovery, while showing 28% ion suppression.

**Conclusion:** This novel methodology permits extensive study of platelet dense granule secretion using extremely small blood volumes. The use of exogenous, non-radioactive serotonin has allowed us to construct a highly sensitive, and robust serotonin release assay to assess platelet function in patients being evaluated for a bleeding disorder. This approach offers a means of overcoming serious limitations of current platelet function testing by expanding the availability to pediatric patients and to adult or pediatric with decreased platelet counts.

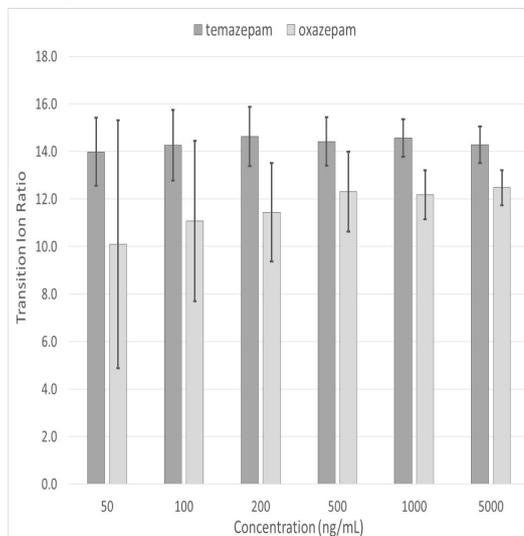
## A-427

**Variation of transition ion ratios for urine benzodiazepine analysis by liquid chromatography-tandem mass spectrometry**

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**Background:** Transition ion ratio (TIR) is the ratio of fragment-1 over fragment-2 from the same precursor and is frequently monitored for liquid chromatography tandem mass spectrometry (LC-MS/MS) quantification. If used correctly, TIR monitoring should improve the specificity of an LC-MS/MS assay. The Clinical and Laboratory Standards Institute (CLSI) C50-A guidelines give a static percent allowable TIR deviation based on the TIR level. For example, a TIR of 10-20% has a static allowable deviation (SAD) of 30%. Anecdotally, we have observed failures of these rules for some of our LC-MS/MS assays. **Objective:** To investigate whether TIRs always follow the SADs defined by the CLSI guidelines and whether TIRs may be concentration dependent for certain analytes and instruments. **Method:** Multiple spiked human urine lots with known levels (calibrators and quality controls) of 7-aminoclonazepam (Precursor ion: 286m/z  $\rightarrow$  Product ion 1: 121m/z; Product ion 2: 222m/z), lorazepam (321  $\rightarrow$  229;194), nordiazepam (271  $\rightarrow$  140;208),  $\alpha$ -hydroxyalprazolam (325  $\rightarrow$  279;243),  $\alpha$ -hydroxytriazolam (359  $\rightarrow$  176;242),

oxazepam (287  $\rightarrow$  241;104), and temazepam (301  $\rightarrow$  177;255) were analyzed by an LC-MS/MS method along with patient samples. TIRs for the calibrators and QCs on a Thermo TSQ™ Quantum Ultra from July 2016 to February 2017 were monitored. The TIRs were extracted and compiled using a Perl script with statistics and graphs produced using Microsoft Office Excel. **Results:** The mean TIR was 24.4%, 33.2%, 16.3%, 95.5%, 11.7%, and 14.4% for 7-aminoclonazepam, lorazepam, nordiazepam,  $\alpha$ -hydroxyalprazolam,  $\alpha$ -hydroxytriazolam, oxazepam, and temazepam, respectively. One of 7 displayed concentration dependent TIR, while four of seven showed concentration dependent CV for TIR. Figure 1 demonstrates an analyte (temazepam) of which TIR met the SAD requirement and another analyte (oxazepam) of which TIR was concentration dependent and failed the specified criteria by CLSI guidelines at the lowest concentration. **Conclusion:** TIR may be concentration dependent for certain analytes and instruments in LC-MS/MS analysis. TIR acceptance criteria should be assessed during method development for each individual analyte.



## A-430

**Simultaneous Measurement of ThioTEPA and its Metabolite TEPA in Serum and CSF by Turbulent Flow LC-MS/MS**

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**Background:** *N,N',N''*-Triethylenephosphoramidate (thioTEPA) is an alkylating agent that has been used in the treatment of multiple solid malignancies for over 20 years. Recently, interest in thioTEPA has been renewed as part of conditioning regimens prior to hematopoietic cell transplantation for hematologic malignancies owing to its potent myelosuppressive effects. Once administered thioTEPA is rapidly metabolized to *N,N',N''*-triethylenephosphoramidate (TEPA). ThioTEPA and TEPA have similar alkylating activity therefore it is necessary to monitor both compounds. Interestingly, thioTEPA and TEPA exhibit excellent central nervous system (CNS) penetration resulting in cerebrospinal fluid (CSF) concentrations comparable to simultaneous plasma concentrations making it an effective conditioning agent for use in CNS lymphoma.

**Objective:** The objective was to develop a highly sensitive and specific turbulent flow LC-MS/MS (TFLC-MS/MS) method that is also suitable for application in a high volume clinical laboratory to simultaneously monitor thioTEPA and TEPA in plasma and CSF.

**Methods:** Plasma and CSF samples were prepared by protein precipitation using methanol containing deuterated thioTEPA and TEPA as internal standards. TFLC-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 (50 X 0.5 mm) and an Accucore®C18 (50 X 3 mm i.d.) analytical column. The HPLC gradient elution was 20-80% of 10 mM ammonium formate + 0.1% formic acid in methanol over 0.25 minutes which was then held for 1.5 minutes. Calibrators (6) were prepared in blank human plasma and CSF.

**Results:** The analytical measurement range for thioTEPA and TEPA was established based on relevance to current practice at 19.5-2500 ng/mL with calibration curves

linear over the AMR ( $R^2 \geq 0.995$ ). The limit of quantitation of thioTEPA and TEPA was 5 ng/mL (CV <20%). Imprecision studies were conducted over a twenty-day period using concentrations of both drugs that spanned the AMR. Between-day CVs for both compounds in plasma and CSF did not exceed 15% over the entirety of the study. Similarly within-day (n=10) CVs did not exceed 10%. Given the lack of availability of a comparison method, the accuracy of the assay was assessed via recovery studies in patient plasma and CSF. Both thioTEPA and TEPA recoveries at three different concentrations were between 99 and 112% in plasma and CSF. Interferences from hemolysis, icterus and lipemia were evaluated in plasma and cross reactivity from other drugs was evaluated in both plasma and CSF.

**Conclusion:** We have developed a fast, accurate and sensitive assay to measure thioTEPA and TEPA levels in plasma and CSF by TFLC-MS/MS. To our knowledge this is the most rapid method described to date for monitoring thioTEPA and TEPA in plasma with an analytical run time of less than five minutes and the only LC-MS/MS method developed to monitor thioTEPA and TEPA in CSF. The ability to rapidly and accurately monitor these drugs allows for timely dose adaptations in hopes of maintaining treatment effectiveness while mitigating excessive regimen-related toxicity.

### A-431

#### A Comparison of Blood Collection Tube Types for Analysis of Testosterone by LC-MS/MS

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**Background:** Testosterone is the major androgenic hormone and is present in both males and females. In males, the hormone is secreted by the testicular Leydig cells and is responsible for physical characteristics including external genitalia and secondary sexual characteristics. In females, it produced in the ovaries and is a precursor of estrogen. Testing for testosterone levels can be used to detect several medical conditions; however, because females, males on testosterone suppressing therapy, and children have very low levels compared to normal males, a more sensitive method of testing such as LC-MS/MS is required. Blood collection tube type is critical as it has been reported in literature that certain tube types can cause the value of testosterone to be falsely elevated due to the anticoagulants and/or serum separator gels. Pathology Associates Medical Laboratories (PAML) recently switched to a new LC-MS/MS method and evaluated different types of collection tubes.

**Methods:** A group of 10 volunteers consisting of both adult males and females were utilized. Five blood collection tubes were drawn from each participant; A serum tube (red top), serum separator tube (SST), lithium heparin, lithium heparin plasma separator tube (PST), and a K2 EDTA tube. Manufacturer instructions were followed for collection. Once the blood samples were centrifuged, the plasma and serum were aliquoted off except for the SST and PST, which only had an aliquot of 300  $\mu$ L used. The serum and plasma were allowed to stay in contact with the gel in the SST and PST and aliquots of 300  $\mu$ L were taken from them at 0 hours, 2 hours, 8 hours, and 24 hours after collection. The samples were then analyzed on an LC-MS/MS instrument. **Results:** We evaluated testosterone results from all blood collection tube types compared to the red top tube as our predicate device, since red top tubes do not contain any anticoagulant or gel, and we also evaluated the effects of serum/plasma left sitting on the gel for up to 24 hours. The data was analyzed using EP Evaluator 11. The SST correlation had an R value of 0.9990, lithium heparin had an R value of 0.9998, PST Lithium Heparin had an R value of 0.9998, and K2 EDTA had an R value of 0.9997. To evaluate if the exposure to the SST or PST gel caused the value of testosterone to change over time, the aliquots at 0 hour, 2 hours, 8 hours, and 24 hours, were compared to the value at the time of collection, and the CV was less than 4.5% over a 24 hour period.

**Conclusion:** We have shown that different blood collection tubes can be used and that SST and PST gels up to 24 hours do not interfere with the assay if a robust LC-MS/MS method is used. Every lab should evaluate the blood collection tube types that they will be using with their testosterone method to ensure that no interferences are noted before testing patient samples.

### A-432

#### Quantification of Infliximab in Human Serum by LC-MS/MS Using A Full-Length Stable Isotope Labeled Internal Standard

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##### Background:

Infliximab, a chimeric monoclonal antibody used to treat rheumatoid arthritis, psoriatic arthritis and many autoimmune diseases by binding to tumor necrosis factor-alpha (TNF $\alpha$ ) to reduce the inflammatory response. Clinical responses are different among patients due to inadequate amount of drug in blood and the formation of autoantibodies, which can also interfere with ELISA assays. Therefore, there is a growing demand for reliable LC-MS/MS assays to support quantification of serum Infliximab. The accurate quantification of Infliximab is enabled by early introduction of an internal standard that behaves identically to the native target protein throughout the analytical workflow. We have developed a full-length stable isotope labeled Infliximab monoclonal antibody internal standard, which allows significant improvements in accuracy and reproducibility in routine quantification of serum Infliximab using LC-MRM assay. We demonstrate a lower limit of quantitation, without immunoenrichment, of 500 ng/mL with less than 15% CV and  $\pm$ 15% accuracy.

**Methods:** SIL-Infliximab was expressed in CHO cells which were grown in serum-free media enriched with <sup>13</sup>C<sub>6</sub>15N<sub>4</sub> Arginine and <sup>13</sup>C<sub>6</sub>15N<sub>2</sub> Lysine. The SIL-Infliximab was analyzed at the intact protein level and after trypsin digestion. Intact mass analysis (SEC-MS) was used to confirm the amino acid composition of the protein and level of glycosylation. The sequence and isotope incorporation were determined at the peptide level after trypsin digestion. For quantification, samples were prepared by spiking 20  $\mu$ g/mL of SIL-Infliximab as an internal standard into human serum containing 0.5 - 250  $\mu$ g/mL of an Infliximab target antibody. Samples were precipitated by adding saturated ammonium sulfate, reconstituted with 50 mM ammonium bicarbonate, and digested using trypsin. Tryptic peptides were separated on a Supelco Biosoil A160 Peptide C18, 2.7  $\mu$ m fused core particle column; 10 cm x 500  $\mu$ m. Detection was performed in MRM mode on Sciex QTRAP 5500 system. Transitions of four unique Infliximab peptides, GLEWVAEIR, SINSATHYAESVK, YASESMSGIPSR, and DILLTQSPAILSVSPGER, were monitored.

**Results:** Stable isotope labeled (SIL) full-length Infliximab monoclonal antibody has been produced with high purity and isotopic incorporation > 99%. The sequence was confirmed at peptide level using peptide mapping and at protein level by intact mass analysis. SIL-Infliximab was used as a full length internal standard for quantification of Infliximab in human serum using pellet digestion followed by microflow LC-MRM analysis. SIL-Infliximab was spiked into human serum containing 0.5 - 250  $\mu$ g/mL of an Infliximab target antibody. Each standard was precipitated using saturated ammonium sulfate and digested using trypsin. The calibration curve was created using the ratio of area under the peaks of target antibody and internal standard. The linear regression coefficient was greater than 0.99 for four unique

Infliximab peptides. The lower limit of quantitation was determined to be 500 ng/mL with less than 15% CV and  $\pm$ 15% accuracy for triplicate calibration standards.

**Conclusions:** We demonstrate that the use of a full length SIL-Infliximab internal standard allows sensitive, accurate, and reproducible quantification of Infliximab in human serum.

### A-433

#### A clinical research LC-MS/MS method for busulfan pharmacokinetics and pharmacodynamics studies in plasma

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**Background:** Busulfan is a bifunctional alkylating agent whose bioavailability varies greatly between individuals due to factors such as age, underlying diseases and drug-drug interactions. An accurate, sensitive and specific analytical method may play a role in assessing the pharmacokinetic and pharmacodynamic effects of busulfan administration in clinical research.

**Methods:** Samples (50  $\mu$ L) were deproteinised with busulfan-<sup>2</sup>H<sub>8</sub> internal standard in methanol. Elution was achieved within three minutes using a Waters HSS-T3 C18 UPLC column (2.1x50mm, 1.8  $\mu$ m) on the Waters ACQUITY UPLC<sup>®</sup> I-Class with a water/methanol/ammonium acetate/formic acid gradient. Ammonium adducts of busulfan were analysed using electrospray ionization in positive mode with multiple reaction monitoring using the Waters XEVO<sup>®</sup> TQD mass spectrometer.

Matrix-matched calibrators (0.025-5  $\mu$ g/mL) and quality control samples (0.05, 0.75, 1.5 and 3.5  $\mu$ g/mL) were prepared by gravimetric weighings of independent stocks

of busulfan. Due to known instability of busulfan in plasma, aliquots of in-house calibrators and quality control samples were stored frozen and thawed prior to use each time the method was used.

**Results:** Analytical sensitivity was calculated to be 0.020 µg/mL (n=10 extractions, over five occasions, ≤16.0% CV). Linearity was demonstrated over the concentration range 0.0175-6.51 µg/mL and system carryover was negligible in samples ≤10 µg/mL.

Precision studies (n=5, over five occasions) demonstrated repeatability and total precision ≤7.3%. A comparison was made by analysing anonymized plasma samples (n=40, range 0.20–2.28 µg/mL) against an independent UPLC-MS/MS method. Good agreement was evident from an ordinary linear fit comparison of  $r=0.998$ , a mean bias of 5.3% from Altman-Bland analysis and a Deming equation of  $y=1.01x+0.04$ .

The mean recovery for busulfan pooled plasma samples (n=3 at 0.05 and 3.5 µg/mL) was between 85.1-106.1% in the presence of high concentrations of endogenous compounds albumin, bilirubin, cholesterol, triglycerides and uric acid and exogenous Intralipid®. The mean recovery was between 93.2-103.0% in the presence of acetaminophen, fluconazole, ketoconazole, itraconazole, phenytoin, posaconazole and voriconazole.

Negligible matrix effects were observed at low (0.05 µg/mL) and high (3.5 µg/mL) concentrations as indicated by respective mean internal standard adjusted matrix factors of 0.99 (range 0.97–1.02) and 0.98 (range 0.95–1.00).

**Conclusions:** A method for the quantification of busulfan in plasma for clinical research has been developed. The method demonstrates good linearity, analytical sensitivity and precision with negligible matrix effects.

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#### A-435

##### Liquid chromatography-tandem mass spectrometry (LC-MSMS) quantitation of buprenorphine, norbuprenorphine and naloxone using the AC Extraction Plate-TM(ACP) - simple but sensitive

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**Background:** Buprenorphine (BP) for treatment of opioid dependency has some advantages versus methadone. Urine testing to monitor compliance, parenteral administration or adulteration is a standard recommendation. Desirable features for LC-MSMS BP methods include ease of use, quantification limits <1 µg/L and detection of BP, norbuprenorphine (NBP) and naloxone (NX). Dilution methods are simple but less sensitive and solid phase extraction is sensitive but complex. We developed and validated a simple automated LC-MSMS method for BP, NBP and NX using the ACP with 0.5, 0.5 and 5 µg/L lower limits of quantification(LLoQ) respectively.

**Methods:** We investigated the acetonitrile:H<sub>2</sub>O ratio(5:95-95:5) and pH(2-11) of extraction, wash and elution/injection reagents to optimize extraction recovery and chromatography. The LC-MSMS was a Waters Acuity LC-XEVO TQS in positive ESI mode with a Waters BEH C18, 2.1x50mm, 1.7 µm column. Mobile phases A and B (MP-A/B) were 2 mmol/L ammonium acetate/0.1% formic acid and acetonitrile/0.1% formic acid. The LC gradient was 95:5 to 40:60 MP-A:MP-B, flow rate 0.5mL/min, run time 3.02min. MRMs were BP-468/396, 468/414, BP\_D4-472/101, NB-414/101, 414/187, NB\_D3-417/83, NX-328/212, 328/268, NX\_D5-333/258. Injection volume was 7 µL. Six calibrators (BP, NB, NX) and two QC (BP-, NB- and NX-glucuronides) were prepared in drug free urine(DFU). The master-mix of beta-glucuronidase, Rapid Hydrolysis buffer (IMCSzyme®, IMCS-LLC, Irmo, SC) and internal standard(IS) was 20,000 U/mL glucuronidase and 80/80/2,400 µg/L of BP\_D4/NBP\_D3/NX\_D5 respectively in 95:5 buffer:acetonitrile. Reagents were: Extraction(Na bicarbonate buffer, 1 mol/L, pH 9.2), Wash(1% NH<sub>4</sub>OH) and Elution/Injection(80:20 H<sub>2</sub>O:acetonitrile/1% formic acid). A Freedom EVO automated liquid handler with orbital shaker performed all extraction steps. The extraction protocol was addition of 200 µL urine and 50 µL of master-mix to an AC plate, then 2 min of mixing. Glucuronide hydrolysis was performed at 65 °C for 30 min. Addition of extraction reagent (post-hydrolysis) was followed by 10 min of mixing to partition the analytes into the non-polar coating of the ACP. The extraction mixture was discarded, followed by a wash step. The wash residue was discarded and elution reagent was added. After 5 min of orbital shaking the 100 µL eluate was transferred to an injection plate.

**Results:** Hydrolysis recovery was >95%. No interference was observed from 54 other drugs/metabolites at 3,000-50,000 µg/L. Within- and between run precision studies of the 6 calibrators (n=16), 2 QC and LLoQ patient pool (n=20) over 5 days yielded coefficients of variation <10%, bias <10% at means ranging between

0.5/0.5/5 to 98/102/1,029 µg/L for BP/NBP/NX respectively. Mean %recovery was 42/33/6 and mean %matrix effect was 52/65/92 for BP-BP\_D4/NBP-NBP\_D3/NX-NX\_D5 respectively. Post-column infusion studies showed matching patterns of ion suppression between analytes and the corresponding IS. No carryover was found at 100/100/1,000 µg/L for BP/NBP/NX. The assay was linear from 0.5-100/0.5-100/5-1,000 µg/L(BP/NBP/NX). Patient samples were tested on 1:9 dilutions with DFU or undiluted. Deming regression statistics for patient sample testing (n=42) versus two other LC-MSMS methods were (BP/NBP) slope 1.01/1.05, intercept -1.6/10.9, Sy/x 6.1/32.9, % bias -0.1/1.1, ranges 7/8-605/982 µg/L).

**Conclusion:** This automated method has good precision and accuracy, low quantitation limits and expands the options available for BP urine monitoring.

#### A-436

##### Comparison of LC-MS based alpha 1 antitrypsin evaluation algorithm with isoelectric focusing based algorithm.

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**Background:** Alpha 1 antitrypsin (A1AT) deficiency affects 1 in 1500 to 3500 individuals of European ancestry. The disease develops due to levels of A1AT that are the result of mutations in the SERPINA1 gene. Multiple mutations of the gene have been described with the vast majority (>95%) of the disease caused by mutations resulting in the Z and S phenotypes. The current gold standard algorithm to diagnose A1AT deficiency is accomplished by documenting both low serum A1AT concentration and a disease associated phenotype by isoelectric focusing (IEF). Recently, as an effort to increase efficiency and cost effectiveness, our lab has implemented a LC mass spectrometry (MS) method to screen for S and Z phenotypes to decrease the number of samples to needing IEF. The material costs of a single MS based test is about 10 times less than cost of IEF test. Here we compare our experience with the LC mass spectrometry based testing algorithm (A1AT proteotyping) to the traditional IEF method during the same 1.5 year period.

**Methods:** The A1AT proteotyping algorithm utilizes both A1AT level and LC-MS S and Z detection as a screen prior to IEF. If a patient has a no detectable S or Z mutation and the protein level is greater than 100 mg/dL, no further testing performed as the patient is by definition not A1AT deficient. If the patient screen positive for an SS or ZZ phenotype, no further testing is done as the diagnosis has been established. However, patients with A1AT levels less than 100 mg/dL who are screen negative for S and Z mutations or those with levels below 70 mg/dL who are screen positive for heterozygous for S and Z phenotypes are reflexed to IEF to detect less common deficiency phenotypes.

**Results:** During a period spanning 7/2015 to 11/2016, our lab performed 5500 A1AT proteotypes. Of the 5500 tests, 96.7% of the samples were resulted from the LC-MS screen. Of the 113 samples reflexed to IEF for being less than 100 mg/dL and screen negative for the S or Z mutation (most likely MM phenotype), 88% had no other phenotype found by IEF while 3 IM, 7 PM and 2 F phenotypes were reported. For the screen positive S and Z heterozygous phenotypes reflexed for being below 70 mg/dL, 90% were confirmed MS and MZ while 2 FZ, 1IZ and 1 PZ phenotypes were reported.

During the same time period 16,324 tests were performed using the traditional IFE approach. In an effort to determine the number of deficient phenotypes that may have been missed by the proteotype algorithm, we retrospectively applied the algorithm to these specimens. In total, 4 potential risk phenotypes would have been missed (1 I, 2 IS and 1 IZ), which represents 0.02% of total tests.

**Conclusion:** In conclusion, the proteotype algorithm appears to be an efficient and cost effective method to detect A1AT deficiency.

#### A-437

##### Measurement of 17-hydroxyprogesterone and cortisol in dried blood spots by liquid chromatography-tandem mass spectrometry as a candidate confirmatory test for congenital adrenal hyperplasia

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**Background:** We developed and validated a liquid chromatography-tandem mass spectrometry (LC-MC/MC) method for measuring 17-hydroxyprogesterone (17-OHP) and cortisol in dried blood spots (DBS) as a confirmatory test for congenital adrenal hyperplasia (CAH). The most common cause of CAH is 21-hydroxylase deficiency leading to accumulation of its substrate, 17-OHP, and reduction of

cortisol. Newborn screening (NBS) plays an important role in reducing morbidity and mortality, as early diagnosis and timely initiation of therapy can prevent adrenal crisis caused by CAH. Most NBS programs use immunoassays to measure the concentration of 17-OHP in DBS. Although immunoassays are generally sensitive, the specificity is low leading to a low positive predictive value. A reliable second tier confirmatory test that will reduce the false positive rate while minimizing the chance of missing clinically significant cases would be beneficial.

**Methods:** Cortisol and 17-OHP of DBS (4 x 3 mm) were suspended in 0.5 mL water, extracted with 1.4 mL tert-butyl-methyl-ether, and separated on a C18 column (50x2.1 mm, 2.6  $\mu$ m). The analysis was carried out using electrospray ionization tandem mass spectrometry in negative-ion mode for 17-OHP by monitoring m/z 329 $\rightarrow$ 285 transition for quantification, m/z 329 $\rightarrow$ 123 for qualification, and [2,3,4-<sup>13</sup>C]<sub>3</sub>17-OHP internal standard by monitoring the m/z 332 $\rightarrow$ 126 transition. Cortisol was detected in positive-ion mode by monitoring m/z 363 $\rightarrow$ 121 for quantification, m/z 363 $\rightarrow$ 91 for qualification, and [2,3,4-<sup>13</sup>C]<sub>3</sub>cortisol internal standard by monitoring m/z 366 $\rightarrow$ 124 transition. Concentrations of 17-OHP and cortisol in QC and patient DBS samples were determined using the LC-MS/MS method and compared with the 17-OHP concentrations measured using the AUTODELFIA Neonatal 17 $\alpha$ -OH-progesterone immunoassay kit (Perkin Elmer).

**Results:** The LC-MS/MS method had a limit of detection, for 17-OHP and cortisol, of 5 and 2.5 ng/mL, and limit of quantification of 10 and 5 ng/mL, respectively. Both inter- and intra-assay imprecisions were < 12% at 20, 80, and 120 ng/ml for 17-OHP, and 30, 80, 120 ng/ml for cortisol. Recoveries were 91-109% for 17-OHP at 40, 80, 160 ng/ml and cortisol at 50, 100, 200 ng/ml, respectively. Linear response was obtained for 17-OHP ( $R^2 = 0.9938$ , range 10 - 160 ng/mL), and for cortisol ( $R^2 = 0.9963$ , range 5 - 200 ng/mL). No interference was observed in DBS samples spiked with 10 steroids at concentrations >10 times the upper limit of their corresponding reference ranges. Correlation analysis of results by LC-MS/MS versus AUTODELFIA showed  $R^2 = 0.9787$  (AUTODELFIA = 0.9215\*LC-MS/MS, n=43) for positive and negative QC materials, and  $R^2 = 0.3649$  (AUTODELFIA = 1.5305\*LC-MS/MS, n=44) for patient samples. When using 56 ng/mL as the positive cut-off value for 17-OHP, a sensitivity of 82.4% and specificity of 96.3% was achieved. Combining this cut-off value with a cut-off value of 1.0 for the ratio of 17-OHP to cortisol, the sensitivity increased to 94.4% with a specificity of 100%.

**Conclusions:** The LC-MS/MS method for measuring 17-OHP and cortisol produced more reliable 17-OHP results than the immunoassay, possibly by specifically detecting the analytes of interest and being not subject to interfering materials existed in patient samples. The method can be used for confirming CAH.

#### A-438

##### A highly reproducible automated proteomics sample preparation workflow

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**BACKGROUND:** Sample preparation for protein quantification by mass spectrometry requires multiple processing steps including denaturation, reduction, alkylation, protease cleavage, and peptide cleanup. Scaling these procedures for the analysis of numerous complex biological samples can be tedious and time-consuming, and there are many liquid transfer steps and timed reactions where technical variations can be introduced and propagated.

**METHODS:** An automated sample preparation workflow was established on a Biomek NX<sup>3</sup> Span-8 Laboratory Automation Workstation. The total processing time for 96 samples is 5 hours, including denaturation, reduction, alkylation and a 2-hour incubation with trypsin. Peptide cleanup is accomplished by online diversion during the LC-MS analysis.

**RESULTS:** For albumin, complement C3, alpha-1-antitrypsin, alpha-1-acid glycoprotein 1, hemopexin, and apolipoprotein C-III and spiked  $\beta$ -galactosidase mean intra-day CVs for 5 samples ranged from 5.5%-8.9% for serum and 3.9%-6.2% for plasma, and mean inter-day CVs over 5 days ranged from 5.8%-10.6% for serum and 3.9%-6.0% for plasma. In an SRM assay targeting >70 proteins, 90% of the transitions from 6 plasma samples repeated on 3 separate days had total CVs below 20%. Similar results, with at least 93% of peptides having CVs below 20%, were obtained when the workflow was transferred to a second site. In an analysis of  $\beta$ -galactosidase-spiked plasma samples from 48 individuals, the average CVs for 3  $\beta$ -galactosidase peptides were less than 10%.

**CONCLUSIONS:** An automated trypsin digestion workflow yields uniformly-processed samples in less than 5 hours. Reproducible quantitation of hundreds of peptides from >70 proteins was seen across replicates, days, instruments, and laboratory sites, demonstrating the broad applicability of this approach.

#### A-439

##### Characterization of Standard Reference Material 2924 C-reactive Protein Solution

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**Background:** The National Institute of Standards and Technology (NIST) has developed a standard reference material (SRM) - SRM 2924, C-Reactive Protein Solution. This material is a recombinant C-reactive protein (CRP) intended to serve as a "pure substance" calibrant providing traceability to SI units in the analysis of future serum-based reference materials containing CRP. The material was analyzed using a variety of techniques to determine concentration, molar mass, structure, stability and density using the highest order methods possible. This material will complement the existing reference material CRM 6201b produced by the National Metrology Institute of Japan.

**Methods:** The concentration of SRM 2924 was determined using amino acid analysis (AAA) using double isotope-dilution tandem mass spectrometry. Samples were selected for analysis using stratified random sampling from the sample boxes and were subjected to hydrochloric acid hydrolysis following spiking with isotopically labeled free amino acids (isoleucine, leucine, phenylalanine, proline and valine) and calibrated by similarly processed unlabeled amino acids. NMIJ CRM 6201-b, C-reactive Protein Solution, was included as a quality control material. The pentameric structure of CRP was analyzed using size exclusion chromatography (SEC) with detection by monitoring fluorescent detection (295 nm excitation, 350 nm emission). Retention times were calibrated using protein size standards. Molar mass of the material was assessed using electrospray ionization mass spectrometry with deconvolution. Density determinations were made using the Lang Levy pipet method. Optimal storage conditions were determined using AAA and SEC comparing frozen and refrigerated groups. Short term stability of the material was analyzed by incubating the material at a variety of times and temperatures and assessed by retention of pentameric structure.

**Results:** The concentration of SRM2924 was 20.6  $\mu$ mol/L with an expanded uncertainty of 1.2  $\mu$ mol/L ( $k=2.15$ ) with good linearity of the calibrating curves ( $r^2 > 0.999$ ). The material concentration was determined to be homogeneous although slight differences were observed dependent upon from which box the material was selected. These differences were added into the overall uncertainty. Values for the QC material were within 2.8% of the certified value. Relative molar mass values determined to be 23028.0 (dimensionless) with an expanded uncertainty of 0.3 ( $k=2.20$ ). This agrees with the theoretical value of 23027.8 based on the sequence and known post-translational modifications. Pentameric structure was assessed comparing the retention time of SRM2924 to calibrating proteins of known size. The principle peak was 99.6% of the total signal observed between the calibrating masses of 158,000 Dalton and 44,000 Dalton verifying the pentameric structure. Additional peaks were observed eluting at later times but were determined to be either preservative (sodium azide) or small levels of nucleic acids based on ultraviolet absorbance characteristics. Long term storage of the material will be at a temperature of -80  $^{\circ}$ C. Stability studies showed no effects on structure for short term temperature excursions.

**Conclusion:** The evaluation of SRM2924, C-reactive Protein solution was performed using the highest order methods available for a variety of characteristics and has been found to be acceptable as a secondary reference material with anticipated release in the second quarter of 2017.

#### A-441

##### Enhanced Lipids Removal from Biological Matrices to Prepare Samples for LC/MS/MS Analysis

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**Background:** Matrix components like proteins and lipids can significantly impact bioanalysis quality by liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS). Proteins can be removed effectively by protein precipitation, while phospholipids and other matrix components can take more effort and cost for efficient removal. The unremoved phospholipids and matrix interferences can cause ion suppression resulting in lower detection limits and poor method reliability. Using the Agilent EMR-Lipid method, protein precipitation can be performed in-well

followed by pass-through cleanup for highly selective and efficient matrix removal, significantly improving the reliability of bioanalysis by LC/MS/MS.

**Methods:** In order to demonstrate the EMR-Lipid method on biological matrix cleanup, eight generic drug compounds were tested for the quantitation in human serum, including 5-Fluorouracil, Gemcitabine, Amphetamine, Metoprolol, Hydrocortisone, Warfarin, Androstenedione, Atorvastatin, and Diclofenac. These compounds were selected to cover different compound properties, from hydrophilic to hydrophobic, and acidic to neutral to basic. In addition, different biological matrices were tested for phospholipid removal, including human plasma, human serum, and animal plasma. The quantitative method was investigated for calibration curve linearity, inter- and intra-day accuracy and precision, method selectivity and recovery.

**Results:** Calibration range were 0.5 - 200 ng/mL including duplicated calibration curves, and the  $R^2 > 0.99$  for all of compounds with  $>90\%$  of calibration points within 20% of expected concentration at LOQ level and 15% at all higher levels. The accuracy and precision data were collected based on five levels of QCs, with average accuracy within 15% of expected concentration. Precision is demonstrated with  $<15\%$  RSD for both intra-day ( $n=6$  at each level) and inter-day ( $n=18$  at each level) reproducibility. The absolute compounds recoveries were between 80-120% for all of compounds at level of 1 ng/mL. Among all of the tested biological matrices,  $>99\%$  removal of phospholipids was obtained using the EMR-Lipid cleanup with protein precipitation. The post-column infusion test demonstrated significantly reduced ion suppression in samples using the EMR-Lipid cleanup compared to samples without cleanup or other cleanup methods.

**Conclusion:** The results demonstrate that the Agilent EMR-Lipid cleanup method provides easy sample treatment workflow, highly selective and efficient lipid/matrix removal and reduces matrix ion suppression, and thus support the accurate and precise quantitation of multiple drug compounds in biological matrices. For Research Use Only. Not for use in diagnostic procedures.

#### A-442

##### A Validated Turboflow LC-MS/MS Method for Plazomicin - Its Role as a Reference Method in Diagnostics Development

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**Background:** LC-MS/MS is a practical reference method to demonstrate immunoassay performance in complementary or companion diagnostics programs, where the drug is new and the immunoassay to be commercialized might, during the course of drug development, be found to be beneficial or even essential for therapeutic drug management. Here, we present the development and validation of a novel TurboFlow™ LC-MS/MS method for the detection and quantitation of plazomicin in human serum/plasma. This LC-MS/MS method will serve as a reference method for development of a QMS® Plazomicin Immunoassay on the Beckman Coulter® AU 680. Plazomicin is a next generation aminoglycoside antibiotic being developed to treat serious bacterial infections due to multi-drug resistant (MDR) Enterobacteriaceae, including carbapenem-resistant Enterobacteriaceae (CRE).

**Methods:** The method involves a simple single step extraction/protein precipitation from 50  $\mu$ L serum/plasma using trichloroacetic acid (TCA), followed by centrifugation. The extract is then injected into the TLX-HESI-MS/MS in the positive ion mode using optimized TurboFlow and LC-MS/MS conditions. Cyclone P 0.5x50 mm TurboFlow and Hypersil Gold 50x2.1mm, 3  $\mu$ m analytical columns are used. The TurboFlow clean-up and analytical separation were achieved within 7-minute run time. The method was validated as per the FDA guidelines on bioanalytical method validation. The validation studies used 9 calibrator and 4 control levels.

**Results:** A TurboFlow LC-MS/MS method for the sensitive detection and accurate quantitation of plazomicin in human serum/plasma was developed. A nine-point linear calibration curve ranging from 0-100  $\mu$ g/mL with a correlation coefficient R of 0.999 was established. The lower limit of quantification (LLOQ) was established at 0.08  $\mu$ g/ml. Minimal carryover was observed. The method demonstrated intra-day precision from 0.56% to 5.87% CV and inter-day precision from 1.96% to 4.91% CV, with recovery bias less than  $\pm 10\%$ . The method validated that plazomicin analyte in human serum/plasma can be subjected to three freeze/thaw cycles. The auto sampler reinjection stability achieved 24 hours at 4°C. For dilution integrity a recovery bias less than 7.24% and coefficient of determination ( $R^2$ ) at 0.9986 were obtained. This indicated that the samples could be accurately quantitated after diluting 10-fold. No interferences were observed when tested with other aminoglycosides or potential interfering substances. Matrix effects were minimal. Method comparison studies performed using plazomicin clinical samples between the in-house TurboFlow LC-MS/MS method and a separate LC-MS/MS method, developed and validated at

Alturas Analytic, Inc., typically used in pharmacokinetic studies, yielded a Passing-Bablok's regression equation of  $y = 1.043x - 0.4103$  with an R of 0.9657 ( $n=62$ ).

**Conclusions:** The developed TurboFlow LC-MS/MS method for the quantitation of plazomicin in human serum/plasma is highly sensitive, specific, robust and accurate. TurboFlow clean-up saves a significant amount of time in sample preparation. Due to its excellent performance characteristics, the method can be successfully used as a reference method for the development of the QMS® Plazomicin Immunoassay on the Beckman Coulter® AU 680.

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#### A-444

##### Making the Right Call: Distinguishing Residual M-proteins from Monoclonal Antibody Therapies Used in the Treatment of Multiple Myeloma

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**Background:** Diagnosis and management of Multiple Myeloma (MM) is largely supported by the identification and quantification of the disease-associated M-protein. The most commonly encountered M-protein is an intact monoclonal immunoglobulin (Ig) of the IgG $\kappa$  isotype accounting for up to 40% of M-proteins. Recently, the breakthrough therapeutic monoclonal antibodies (t-mAbs), daratumumab and elotuzumab, were FDA-approved for treatment of refractory MM. Furthermore, additional t-mAbs (isatuximab) have entered phase III clinical trials for treatment of MM. These t-mAbs are of the IgG $\kappa$  isotype and are given at doses where a majority of patients will achieve steady-state concentrations that exceed the detection limit of immunofixation electrophoresis (IFE) and in a subset of patients will also be detectable by protein electrophoresis (PEL). Each of these therapies migrates within the  $\gamma$ -region making it challenging to distinguish between residual IgG $\kappa$  M-proteins and t-mAbs using conventional methods with certainty. One solution is to identify t-mAbs through their accurate molecular mass, which is distinct for each t-mAb, using mass spectrometry (MS). The objective of this study was to develop and assess the analytical performance of different MS methods to differentiate residual M-proteins from t-mAbs used in the treatment of MM.

**Methods:** Serum was immunoenriched for IgG antibodies using a camelid-derived single domain antibody fragment coupled to agarose beads that recognize all IgG subclasses independent of the light chain subclass. Bound antibodies were washed with PBS/water and eluted with 50 mM TCEP in 5% acetic acid to dissociate bound Igs into heavy and light chain components. Dissociated Igs were spotted onto a MALDI plate and analyzed using a Bruker Microflex LT MALDI-TOF instrument and were also analyzed by a high resolution, accurate mass (HRAM) LC-MS method using microflow liquid chromatography coupled with an ESI-QTOF mass spectrometer. Patient samples with IgG $\kappa$  M-proteins that co-migrated with daratumumab, elotuzumab or isatuximab were used for spike-in studies. Each M-protein was diluted to a range of M-protein concentrations (0.1g/dL - 1g/dL) with pooled healthy donor serum and then spiked with a range of concentrations (range: 0 g/dL - 0.1 g/dL) of co-migrating t-mAbs ( $N=173$ ). In addition, residual serum samples were collected from patients with a history of IgG $\kappa$  MM who had received daratumumab ( $N=17$ ), elotuzumab ( $N=2$ ) or isatuximab ( $N=2$ ) therapy. All samples in this study were evaluated by IFE, MALDI-TOF and HRAM-MS.

**Results:** T-mAbs could be resolved from endogenous M-proteins using HRAM LC-MS in 100% of spike-in samples (173/173). MALDI-TOF MS could resolve 83% of samples (143/173). In samples collected from patients receiving a t-mAb, the presence of an interference was ruled out correctly in 100% of cases (21/21) by both MS methods. In contrast, blinded reviewers were uncertain in nearly all samples evaluated by IFE.

**Conclusion:** HRAM LC-MS could differentiate t-mAbs from residual M-proteins in all samples evaluated in this study. In contrast, both MALDI-TOF MS and traditional IFE were unable to resolve all cases with a high degree of certainty. HRAM LC-MS provides a reliable method to differentiate residual M-proteins and exogenous t-mAbs.

**A-446**

**Parallel Reaction Monitoring using Q-Exactive Exhibit Improved Sensitivity and Selectivity in Sub-amole Testosterone Quantification**

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**Background:** Precise and accurate measurement of low level testosterone in highly complex clinical samples remains challenging. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the technology of choice for this analyte due to its high sensitivity and specificity. However, wide range of interferences present in a complex biological specimen impair reliable quantification by LC-MS/MS, especially at the sub pg/mL level for free testosterone in circulation. Parallel Reaction Monitoring (PRM) using high-resolution mass spectrometry (HRMS) not only has target quantitative analysis capabilities, but also performs MS/MS full scan at the same time, offering additional confidence of analyte identification.

**Methods:** Testosterone in serum was extracted, dried, and derivatized with Amplifex™ Keto reagent (AB SCIEX). The resulting solution was injected onto an EASY-Spray™ PepMap™ RSLC (C18 15 cm x 75 μm ID column) and eluted with a 40-90% acetonitrile gradient in 0.1% formic acid over 12 min at a flow rate of 300 nL/min. The analytical system was a Thermo Scientific™ EASY-nLC 1000™ HPLC system with EASYSpray™ source and coupled to a Q-Exactive mass spectrometer (Thermo Scientific). The Q-Exactive was set for time scheduled parallel reaction monitoring.

**Results:** A blank Seracon serum was spiked with 1.0 pg/mL testosterone. After derivatization, 10 μL resulting solution was analyzed using the nLC-MS method. Ion chromatograms were constructed using a mass tolerance window of 500 mmu (A) and 2 mmu (B), respectively. As shown in Figure, the chromatogram for 500 mmu mass tolerance window had unresolved peaks with significant interference, while the 2 mmu mass tolerance provided two nice isoform peaks with much less interference.

**Conclusion:** HRMS was demonstrated to reduce interference, resulting in reliable quantification of low abundance testosterone in a complex biological matrix.

