Wednesday, August 1, 2018

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

Mass Spectrometry Applications

B-211

Determination of Methylmalonic acid in urine by LC-MS/MS.

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Methylmalonic acid (MMA) is a specific diagnostic marker for vitamin B12 deficiency and for the methylmalonic acidemia. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electrospray ionization is a rapid, robust and selective technique. So the objective of this work was to develop and validate a simple method for the determination of methylmalonic acid in urine by LCMS/MS for application in clinical diagnostic. Chromatographic separation was obtained on Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm x 1.7 µm) held at 30°C using isocratic mobile phase constituted by 45% of water, 45% of acetonitrile, 10% of methanol and 0.1% of formic acid at a flow rate of 0.400 mL min^{-1}.The chromatographic run time was 4.10 min. The experiments were performed on an Agilent 6460C (Santa Clara, CA) triple quadrupole LC-MS/MS system operated in a positive mode, with an Agilent 1290 Infinity LC system. For the sample preparation, 100 µl of sample was spiked with 20 µl of deuterated internal standard and 30 µl of Hydrochloric acid 0.86 $molL^{-1}$ solution. The mixture was stirring for 5 seconds, 500 µL of ethyl acetate was added and it was vortexed for more 90 s. This mixture was centrifuged and 350 µL of the supernatant was evaporated to dryness with a vacuum concentrator. The extract was derivatized with 100 µl of n-butanol in hydrochloric acid 3.0 $mol.L^{-1}$. Linearity was achieved from 0.2 to 98.2 $µmol.L^{-1}$. The average of recovery was 94.1-110.1%. The intra and inter day imprecision was lower than 10.3%. In conclusion, the method has been developed and validated successfully for the quantitative analysis of MMA in urine and can be applied in clinical diagnosis.

B-212

Selective, simple and fast determination of Pyridoxal 5-Phosphate in plasma samples using a C8 column and LC-MS/MS analysis.

B. F. P. Paulo, E. Cueva Mateo, A. C. S. Ferreira. *Hermes Pardini Institute (Research & Development Division), Vespasiano, Brazil*

Vitamin B6 is important for normal brain development and for keeping the nervous system, immune system and skin healthy. Vitamin B6 has several derivatives, where pyridoxal 5-phosphate (PLP) has been determined to be the biologically active form due to its role as a cofactor in a number of enzymatic reactions. Patients with kidney disease or deficiency in absorption of nutrients are able to be vitamin B6 deficient. PLP is an acid and very polar compound (logP -2.09) and their chromatographic separation is difficult due to its poor retention in C18 reversed-phase (RP) columns. Although the MS/MS is considered a technique with excellent selectivity, we was observed that there an interfering peak close to the PLP retention time when a C18 RP column are used (Zorbax Eclipse Plus C18 RRHD 100 mm X 2.1 mm X 1.8 µm - Agilent Technologies). This interfering peak compromises the exact quantification of PLP and are present in 2-3% of samples analyzed in our laboratory. To solve this problem, was developed and validated a selective and simple analytical method for determination PLP in plasma by LCMS/MS using a C8 RP column. C8 columns are more polar than C18 columns and is capable to retain better the PLP to provide adequate chromatographic selectivity. The chromatographic separation was performed on Agilent Poroshell 120 HPH C8 column (50 mm X 3.0 mm X 2.7 µm) held at 25 °C using a gradient separation constituted of mobile phase A - 0.1% of acid formic solution and B - methanol with 0.1% of acid formic, at a flow rate of 0.400 mL min^{1}. The chromatographic run time was 3.2 min. All experiments were performed on Agilent 6460C (Santa Clara, CA) triple quadrupole LC-MS/MS system, with an Agilent 1290 Infinity LC system. The source was operated in positive mode. Sample preparation was performed adding 40.0 µL of plasma sample, calibrator or quality control and 130.0 µL of 5% trichloroacetic acid solution with 258.0 ng mL^{-1} of internal standard (PLPD3) to conical bottom PCR plate. The plate was sealed and vigorously agitated for 60 seconds and then, centrifuged at 4.500 rpm for 10 minutes. The supernatant was collected and injected on a LC-MS/MS system. The method was successfully validated achieving a LoD of 0.5 ng mL^{-1}, linearity of 3.0 to 120.0 ng mL^{-1} and imprecision was less than 5.3%. The recovery was between 95.5 and 85.3%. The selectivity was tested by analysis of 427 real samples. No interfering peaks was detected near the PLP retention time. In conclusion, the method has been developed and validated successfully for the quantitative analysis of PLP in plasma samples.

B-213

A rapid LC-ESI-MS/MS method for quantification of plasma oxysterols with dimethylglycine derivatization

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Background: Oxysterols are derivatives of cholesterol which are formed by oxidation through numerous chemical reactions and play an important role in many physiological processes and in various degenerative and metabolic diseases, lipid metabolism disorders. In this study, analytical validation of liquid chromatography-tandem mass spectrometric (LC-MS / MS) method was evaluated by using various extraction and derivation steps in the measurement of C-triol and 7-KC which are important oxysterols. **Methods:** Optimization studies were performed in LC-MS / MS with positive electrospray ionization (ESI) in multiple-reaction monitoring (MRM) mode. The analytical validation of C-Triol and 7-KC multiplex measurements with dimethylglycine (DMG) derivatization was evaluated in plasma from 25 healthy individuals. For this purpose; linearity, accuracy, repeatability, detection and quantitation limits (LLOD and LLOQ), recovery and carry-over analysis were studied and the results were evaluated statistically. **Results:** The time of the analysis was 10 minutes (min) for both parameters (C-Triol: 3.2 min, 7-KC: 7.4 min). The values of r-squared (r^2) were found for C-triol as 0,999, for 7-KC as 0,994 in generated calibration curves. Concentrations of C-triol and 7-KC were within ± 20% in quality control samples prepared at least two levels for accuracy analysis. Plasma C-triol levels were determined in a control group of 25 individuals (min: 15.7, max: 38.61; mean±SD: 25.61±9.2 ng/mL). The standard deviations were found high for 7-KC measurements, it was considered that the plasma matrix interfered or the 7-KC stability in the plasma deteriorated. **Conclusion:** In this study, oxysterol measurement has been developed and ready for use in our laboratory with a sensitive and specific method. 7-KC was considered to be a useful parameter to reflect the stability of the analyte in the plasma.

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Rapid preparation for analysis of steroid hormones by LC-MS/MS with Microelution at positive pressure.

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Background: Steroid hormones are of great importance in clinical endocrinology. However, the immunoassay method analyzes have methodological interferences that, in the majority, result in false high results. Liquid chromatography tandem mass spectrometry (LC/MS/MS) is replacing classical methods for steroid hormone analysis. It offers analytical specificity superior to that of immunoassays or conventional high performance/pressure liquid chromatography (HPLC) for low molecular weight analytes. However, the preparation time of the samples in the LC-MSMS may be a limiting factor for the analysis of a large number of samples. **Objective:** This study evaluates the reduction of preparation time of the samples through the process of positive pressure extraction with solid phase extraction cartridge and subsequent analysis for LC-MS/MS composed of an ACQUITY UPLC system combined with a XEVO tandem quadrupole mass spectrometer both from Waters. It was controlled by the MassLynx software **Methods:** We analyzed Testosterone, Androstenedione, 17α-hydroxyprogesterone, 11-deoxycortisol, Progesterone, Corticosterone, DHEA and cortisol, serum samples were prepared in the same extraction method and in only a single chromatographic run. All with deuterated steroid standards and solvents were purchased from SIGMA, Cabridge Isotopes Laboratories (CIL) and Merck. Samples were extracted using the positive pressure-96 Waters and the Oasis Prime HLB microelution cartridge. The LCMS system used was Waters ACQUITY UPLC, Xevo TQ-S positive ionization mode (ESI +) with ACQUITY UPLC BEH C18 2.1x100mm, 1.7µm column. The flow rate was 0.5mL / min. with gradient of solvents A and B, consisting of water and methanol with 0.1% formic acid respectively. Running time is 3.5 minutes. **Results:** To validate the method, we performed intra-day and inter-day comparative studies, for a total of 40 samples for each analyte. The method exhibited linear response in the range of 0.2 ng / mL to 250 ng / mL and CVs within the expected range. **Conclusion:** The use of micro-elution cartridge in positive pressure equipment has reduced the number of steps, eliminating evaporation and reconstitution processes, and optimizing sample preparation compared to traditional cartridges.

B-215

Development and validation of Nicotinamide in human serum by liquid chromatography electrospray ionization tandem mass spectrometry.

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Nicotinamide (NA) is the active form of vitamin B3 and is an essential component of several coenzymes. A rapid LC-MS/MS method was developed for quantitative determination of nicotinamide in human serum for clinical practice. Detection was obtained on a 6460 MS system (Agilent Technologies) and it was conducted by monitoring the fragmentation ions of protonated molecules of *m/z* 122.9→80.0 for NA quantifier, 122.9→78.0 for NA qualifier and 128.9→85.0 for internal standard NA-$^{13}C_6$. Chromatographic separation was performed on a Poroshell 120 PFP column (100 mm x 2.1 mm, 2.7 µm) held at 30°C and isocratic mobile phase containing water:methanol (95:5, v/v) with 0.1% de formic acid at a flow rate of 0.2 mL.min^{-1}. The chromatographic run time obtained was 2.8 minutes. The extraction procedure is a simple protein precipitation with 400 µL of trichloroacetic acid 10%, using only 100 µL of sample and 25 µL of internal standard (NA-$^{13}C_6$). The linear range was achieved from 5.0 to 2000.0 µg.L^{-1}. The medium range of recovery was between 91 and 102%. Total imprecision ranged from 1.2 to 5.1%. The tests of quantification limits, linearity, precision and recovery were adequate for clinical evaluation. In conclusion, a simple and rapid method has been developed successfully for the quantitative analysis of vitamin B3 in human serum for clinical diagnosis.

B-216

LC-MS/MS assay for detection of elevated biotin in plasma

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BACKGROUND: Elevated biotin due to self-administration or prescription is a potential interferent for many immunoassays that utilize biotin-streptavadin interaction in their design. A recent FDA Safety Alert (November 2017) has brought widespread current attention to this issue. Laboratories will ideally be prepared to assess interference in individual cases by pre-treatment of samples with streptavadin. A biotin assay can assist in validation of such procedures, as well as provide a means for direct verification of biotin elevation in suspected cases. To this end, we developed a simple LC-MS/MS assay designed specifically to measure elevated biotin in plasma (biotin >1 ng/mL). **METHODS**: Stock solution (1000 ng/uL) of biotin (Sigma-Aldrich) was used to prepare elevated biotin standards (0, 1, 20, 100, 500 ng/mL) by addition to 7% bovine serum albumin (BSA). Desthiobiotin (Santa Cruz Biotechnology) was used as internal standard (IS). Samples were prepared as follows: 200 ul of MeOH containing IS was added to 200 uL of sample; after vortexing and centrifugation, 100 uL of supernatant was diluted with 50 uL of mobile phase A (H_2O, 0.1% formic acid). 20 uL of the final extract was injected for LC-MS/MS. LC (Shimadzu UFL Prominence) was performed using a Phenomemex Synergy Hydro RP column (50 × 4.6 mm) at 50 oC, with binary mobile phase (B = MeOH, 0.1% formic acid) at fixed flow rate of 0.6 mL/min as follows (interval (min), %B): -1-0, 30%; 0-3.5, 30%-80%; 3.5-4.5, 80%; 4.5-5.0, 30%. MS/MS was performed using positive APCI on a Sciex 3200MD and monitoring for m/z 245.1>227.1 (biotin) and m/z 215.2>179.1 (IS). Retention times were 2.4 min (biotin) and 3.1 min (IS), with injection-to-injection time of 6.0 min. Quality control samples were prepared in 7% BSA from biotin purchased from Santa Cruz Biotechnology. **RESULTS**: The assay was linear over the range of standards (r2>0.99, 0-500 ng/mL). Accuracy was verified by 1:1 ± 4% correspondence with biotin standards from an independent source (Vector Laboratories). Process recovery was 51% at 100 ng/mL biotin. The lower limit of quantification (LOQ) in plasma was 2 ng/mL (CV=8.4%), which is greater than the upper limit of the reference range for biotin (nominally 1 ng/mL). Intra- and inter-assay coefficients of variation were <3.9% and <4.3% at 10 and 100 ng/mL, respectively. No interferences were found in measurements made using biotin-spiked high QC materials (Biorad) for a wide range of immunoassay analytes and drugs of abuse, or for samples with elevated hemoglobin, hyperbilirubinemia or lipemia. Matrix effects, if any, were stable and equal across standards, controls and patient samples as assessed by metric plots for IS. Pooled plasma demonstrated biotin below LOQ. A survey of 20 individual patient plasma samples showed no results above LOQ. **CONCLUSIONS**: The LC-MS/MS assay showed acceptable performance characteristics for quantitation of plasma biotin elevated beyond the upper limit of the reference range. Availability of such an assay will be useful in validation of biotin-stripping procedures used to remove immunoassay interference, as well as for direct evaluation for elevated biotin in suspected interference case samples.

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LC-MS/MS method for detecting paclitaxel in human plasma

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Background: Paclitaxel is a natural product commonly used to treat a wide range of cancers including ovarian, breast, lung, bladder, prostate, melanoma, esophageal, and Kaposi's sarcoma. Recently, exposure-guided paclitaxel dosing (i.e. therapeutic drug monitoring - TDM) was demonstrated to be less toxic and similarly effective to standard dosing when combined with platinum drugs in patients with lung cancer. As efforts continue to develop new drugs to treat the sequelae of chemotherapy or to enhance the efficacy of paclitaxel, it will be important to confirm that its pharmacokinetics are unaltered and anti-neoplastic efficacy is maintained. Therefore, TDM of paclitaxel and/or monitoring the possible interaction of paclitaxel with other drugs is worth further investigation in additional tumor types and chemotherapy regimens. To this end, we developed a rapid and sensitive LC-MS/MS method for measuring paclitaxel in human plasma. **Methods:** Human plasma samples were treated with stable isotope labeled paclitaxel-d5 solution in methanol to precipitate proteins and analyzed by ABSciex 5500 Triple Quadrapole mass spectrometer with electrospray ionization in a positive ion mode. A Shimadzu Nexeral LC equipped with a Phenomenex C18 column (50 x 3.0 mm, 2.6 µm) and a Phenomenex C18 guard cartridge (4.0 x 2.0 mm) was 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 0.75 min, and reverted to 20% B at 4.6 min. For sample preparation, 100 µL of matrix blank, calibrators, controls, and samples were mixed with 300 µL of internal standard solution and centrifuged at 16,500 g for 5 min. Then 250 µL of each supernatant was mixed well with 250 µL water. 25 µL of each prepared sample was analyzed by LC-MS/MS. The results were quantified using the internal standard method of quantitation. **Results:** The developed method was specific for paclitaxel with no interference of peak at the retention time of paclitaxel. Linearity (r^2 >0.99) was established for the range of 10-1000 ng/mL. Coefficients of variation for low, middle, and high quality controls were less than 4.6% for intra-day assays and less than 7.2% for inter-day assay. Accuracies for all quality controls were greater than 94.5% for both intra- and inter-day assays. Further validation studies did not show apparent matrix effects (2.3%) and carry-over (-2.2%). **Conclusions:** The developed LC-MS/MS method can be used to quantify blood paclitaxel concentrations in cancer patients during chemotherapy with minimal sample preparation, rapid turn-around, and acceptable precision and accuracy.

B-218

Validation of a LC-MSMS method for the quantification of plasma curcumin levels in clinical analyses

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Background: Curcumin is a polyphenol with a long history of being used as a dietary spice with a broad spectrum of effects described in the medical literature as antioxidant, anti-inflammatory, antimicrobial and anticancer. The objective of this study was to develop and validate a method with sensitivity and specificity enough to be used in clinical studies with curcumin. **Methods:** Standards for Curcumin and Curcumin-D6 were acquired from Sigma-Aldrich and Toronto Research Chemicals, respectively. A Waters Corporation Acquity TQD was used in positive ionization mode with an electrospray source. Samples were collected in plasma, and the calibrators were made by adding the standards in blank plasma. Samples and calibrators were extracted by addition of zinc sulfate 0.1M and acetronitrile followed by centrifugation and separation of the supernatant. The triple quadrupole mass detections with multiple reaction monitoring mode was used to monitor the ion transitions (m/z) 369.1>177 and 369.1>285 for curcumin, and 375>180 for the internal standard curcumin-D6. The method was validated following the CLSI C62-A document. After validation the pharmacokinetics was tested in a healthy volunteer by drinking a curcumin mixture with 200 mg of curcumin. **Results:** The lower limit of detection was 0.13 ng/mL, the lower limit of quantification was 2.3 ng/mL, and the linearity was 1021.0 ng/mL. The intra-day and inter-day precision was calculated on two levels. The intra-day CV was 11.6% for the low level and

6.8% for the high level. The inter-day precision was 6.5% for the low level and 4.9% for the high level. Recovery was determined on three levels and the results were 99.0 to 104.6%. The matrix effect was calculated in three levels by addition of curcumin standards in blank plasma compared to the standard in mobile phase. The results were 0.60, 1.2, and 2.3%. The method was tested for carry-over and none was noticed at the highest tested concentration (1200 ng/mL). The sample was shown to be stable for 15 days in a -30°C freezer. For the pharmacokinetics the maximum concentration (C_{max}) was 236.6 ng/mL and the time to achieve maximum concentration (T_{max}) was 90 minutes. **Conclusion:** The method was shown to be simple, fast, and reproductible. This provides an analytical tool to study the pharmacokinetics of curcumin and on clinical studies with curcumin

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Measurement of Thiopurine Metabolites in Erythrocytes to Optimize Thiopurine Therapy

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Background: Azathioprine (AZA), 6-Thioguanine (6-TG) and 6-mercaptopurine (6-MP) are thiopurine-based molecules requiring enzymatic conversion into thiopurine nucleotides to exert cytotoxicity for the treatment of acute lymphoblastic and myeloblastic leukemia. The metabolism of AZA and 6-MP by a series of enzymes including Thiopurine Methyltransferase (TPMT) yields 6-thioguanine nucleotides (6-TGN) whose levels correlate with therapeutic efficacy and 6-methylmercaptopurine (6-MMP) nucleotides which are associated with hepatotoxicity and myelotoxicity. TPMT activity is affected by genetic polymorphisms with significantly different activity profiles. Hence, the accurate quantification of thiopurine metabolites is clinically informative and essential in the mitigation of toxicity. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with multiple reaction monitoring (MRM) was developed by the Clinical Pharmacokinetics Laboratory at St. Jude Children's Research Hospital to assay for 6-TGN and 6-methylmercaptopurine (6-MMP) nucleotides in erythrocytes. The aim of the current study was to transfer the assay to an LC-MS instrument platform at the Advanced Clinical Chemistry Diagnostics Laboratory at Johns Hopkins, harmonize the assays between platforms from different LC-MS vendors, and to validate the assay towards a goal of offering the assay for clinical testing purposes. **Methods:** Remnant, de-identified whole blood specimens collected in K_2-EDTA tubes were used to prepare 6-TG and 6-MMP calibrators and QC samples in lysed erythrocytes. 6-TG-$^{13}C_2$,^{15}N and 6-MMP-d_3 (Toronto Chemicals) were used as internal standards (IS). Red blood cells (RBC) were counted using a Sysmex XP cell counter. 6-TGN and 6-MMP nucleotides extracted from erythrocytes were converted to their respective bases by acid hydrolysis. Reversed phase HPLC was conducted with a Vanquish system (Thermo Scientific). A 2.8 min binary gradient (6 min total run time) from 2 - 80% mobile phase B (0.1% formic acid in acetonitrile) was delivered to a 2.1 x 50mm high strength silica column (Waters). The column was interfaced with an Endura triple quadrupole mass spectrometer (Thermo Scientific). A constant dwell time of 10 ms was used and two transitions were monitored for each analyte. Results were reported in units of pmol/8x10^8 RBCs. Validation experiments included linearity, LLOQ, precision/repeatability, accuracy, specificity and robustness. The clinical performance of the assay was evaluated by measuring 6-TGN and 6-MMP nucleotide levels in acute lymphoblastic leukemia patients who received thiopurine therapy at St. Jude Children's Research Hospital. **Results:** The thiopurine metabolites assay was successfully transferred and adapted to an LC-MS platform distinct from the instrumentation that was used to develop the original method. Calibrators from St. Jude Children's Research Hospital were used to enable instrument method optimization towards a goal of inter-laboratory harmonization. Additional studies are ongoing to explore the feasibility of coupling this LC-MS based test with a TPMT genotype test to enable the prediction of patient response by assessing TPMT activity while providing clinicians with clinically actionable data to facilitate the optimization of drug concentrations by measuring thiopurine drug metabolites. **Conclusion:** An LC-MS thiopurine metabolites assay was validated and harmonized across distinct liquid chromatography and mass spectrometry instrument platforms to permit the accurate determination of thiopurine metabolite concentrations to optimize drug dosing and prevent toxicity in leukemia patients.

B-220

Rapid determination of 25-hydroxyvitamin D by supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS)

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Background: Determination of 25-hydroxyvitamin D has become a critical test for evaluation of calcium metabolism and homeostasis. At present, liquid chromatography-mass spectrometry (LC-MS) is the most reliable method. However, 25-hydroxyvitamin D could not produce a strong signal in LC-MS due to its lack of positive charge. To ensure the detection of 25-hydroxyvitamin D in low concentration, the system noise has to be strictly controled by using expensive ultra pure solvent as mobile phase. In this study, a supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) method was developed for determination of 25-hydroxyvitamin D. Inert, non-toxic, non-flammable, and low cost supercritical carbon dioxide was applied as eluent with modification of methanol in small amount. 25-hydroxyvitamin D was analyzed within less than four minutes. **Methods:** The separation was performed using a SFC system coupled to a triple quadrupole mass system. The backpressure was set by the automatic back pressure regulator. Human plasma samples were extracted using a liquid-liquid extraction method. Calibration curves, limit of detections (LODs), intra-assay precision, and accuracy were calculated for method validation. **Results:** The 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 were baseline separated. The calibration curves were linear from 1 to 200 ng/mL with regression coefficients (R^2) > 0.99. The limits of detection (LOD) were found to range between 0.5 and 5 ng/mL for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. The intraassay relative standard deviation (RSD) was lower than 10%, and the accuracy of QC sample was between 92% to 110%. **Conclusion:** A new SFC-MS method was developed for determination of 25-hydroxyvitamin D within four minutes. A baseline separation of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 with satisfied precision and accuracy was achieved by optimizing the outlet pressure, the column temperature, the flow rate and the methanol gradient program.

B-221

Vitamin D and its metabolites- Which LC-MS methodology do I choose?

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Background: Liquid chromatography triple quadrupole (QQQ) mass spectrometry (LC-MS/MS) is suited for rapid analysis of multiple analytes. Various LC-MS/MS analytical methodologies were developed for the quantitation of the Vitamin D metabolites such as 25-Hydroxy-Vitamin D, 1,25-Dihydroxy-Vitamin D, 24,25-Dihydroxy-Vitamin D and others to ascertain under which LC and MS conditions and sample preparation options resulted in the most consistent and appropriate results. A simple sample preparation technique that involved a simple protein crash and liquid-liquid extraction were utilized along with a one (1D) dimensional liquid chromatographic configuration with and without the PTAD derivatization. The described method achieves the required sensitivity and is capable of determining the various vitamin d metabolites over their dynamic range. Therefore, simple and accurate quantitative analytical methods were developed for the quantitatively measurement of vitamin D metabolites in serum. Method: A Thermo Scientific™ Quantis™ tandem mass spectrometer in positive Electrospray and Atmospheric Pressure Chemical Ionization modes and a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system were initially utilized for this analysis. 200 µl of serum were used for the analysis of the various vitamin D metabolites in serum. Various columns were evaluated and a Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 1.5 µm with a water:methanol mixture containing and 0.1% Formic Acid along with methylamine achieved baseline chromatographic separation for all the vitamins D metabolites in serum 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 mode and the precision and accuracy of the method was verified using pooled quality control materials and serum samples. Result: Good linearity and reproducibility were obtained with the concentration range of 5 pg/ml to 1000 ng/ml for the respective vitamin D metabolites in serum 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 5 pg/ml to 50 pg/ml and excellent reproducibility was observed for all compounds (CV < 10%). Conclusion: Sensitive, simple, specific and accurate liquid chromatography tandem mass spectrometry methods were developed and verified for the determination of the

vitamins D metabolites in serum. The sample preparation techniques were kept quick for easy application and the various ionization modes were evaluated that resulted in no major differences while the addition of the methylamine gave increased responses. The use of the derivative PTAD boosted the response and sensitivity of the compounds that were all baseline separated particularly for the dihydroxyvitamin D metabolites. Thus the various vitamin D metabolites can be evaluated together albeit at different concentrations without any major issues due to the design of the methodologies.

B-222

Impact of Seminal Plasma Copper to Retinol Ratio in Infertile Subjects

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Introduction: Infertility is a complex problem that influenced by multifactorial causes. One of known cause is micronutrient and also trace element. Retinol plays an important role in sperm quality through its antioxidant activity to protect spermatozoa against free radical. Copper also suggested have higher potency as a sensitive indicator on sperm quality. Quantification method also play critical role in accuracy and sensitivity. There is no available data that combine these two potential infertility marker as a ratio and its correlation with sperm quality parameters quantified using mass spectrometry. **Objective:** This study objectives were for investigating impact of retinol, copper, and copper to retinol ratio in infertility from seminal plasma quantified by mass spectrometry. **Method:** Seminal plasma was obtained by consecutive sampling of sperm analysis in Prodia Clinical Laboratory consist of 33 normal zoopermia subjects and 20 abnormal zoospermia subjects with mean age 33 years old. Retinol quantified by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and copper quantified by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Data analysis was using SPSS 24. **Result:** There was no significant age difference between normal and abnormal zoospermia subjects (33 ± 4 vs 35 ± 9, p = 0.713). There was significant negative correlation between retinol with number and concentration of spermatozoa (r = -0.472, p = 0.001 and r = -0.522, p = 0.000) and also significant positive correlation between copper with number and concentration of spermatozoa (r = 0.454, p = 0.009 and r = 0.302, p = 0.028). Copper to retinol ratio shown significant positive correlation with number and concentration of spermatozoa (R = 0.517, p = 0.000 and R = 0.470, p = 0.000) which was also significantly higher ratio in normal zoopermia subject compared to abnormal zoospermia (7.6 ± 3.2 vs 2.8 ± 2.7, p = 0.000). Cut off value of ratio suggested that subject have abnormal zoospermia was below 5.2 (AUC = 0.880 95%CI: 0.78 - 0.98). Copper to retinol ratio shown higher AUC compared to copper and retinol alone in infertility (0.880 vs 0.808 vs 0.850). **Conclusion:** Result suggest that retinol and copper have individual potential marker in infertility status based on abnormal zoospermia. Combination of these markers as a ratio give potential impact to distinguish infertility status based on micronutrient and trace element. **Keywords:** Copper, Retinol, Seminal Plasma, Infertility

B-223

Investigation of calibration matrix materials to achieve optimal sensitivity of total testosterone by LC-MS/MS

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Background:
Superior specificity and sensitivity are two benchmarks, which support the analysis of testosterone in serum by LC-MS/MS as the gold standard methodology, especially for females, children and hypogonadal males. According to CLSI C-62A guidelines, “background peaks should be absent or <20% of the peak area for the analyte at the LLMI.” In addition, a minimal difference between the internal standard responses of the calibration material and patient samples is essential for accurate concentration calculation. The ideal calibrator matrix would provide the lowest background (Blank) response combined with the highest correlation of internal standard (ISTD) response between patients and blank matrix. The objective is to identify the matrix material most suitable for use as blanks and calibration standards in an assay for the quantitative analysis of serum total testosterone by LC-MS/MS.
Methods:
Eight different types of matrix materials were investigated: charcoal stripped human serum (MSG-4000, Golden West Biologicals), synthetic human serum (SigMatrix, Sigma Aldrich and SMx Serum, UTAK Laboratories), commercially available calibration standard kits which contained a lyophilized serum blank (ChromSystems and Cerrilliant), 5% human serum albumin (Sigma Aldrich), normal saline and 50% methanol. The LLMI target was 2 ng/dL for testosterone. A simple LLE extraction was performed on 200 µl of the calibrator matrix and patient serum, using ethyl acetate:hexane (90:10), followed by flash freezing the aqueous layer and then pouring off and drying the organic layer. The dried extract was reconstituted in mobile phase and 25 µl was injected. Double blank, blank and a calibration standard near the anticipated LLMI were analyzed for each matrix type. An Agilent 1260 series HPLC and 6460 QQQ were used to separate and detect the presence or absence of testosterone in the calibrator matrix and serum samples. A 50 mm x 2.1 µm x 2.7 µm Poroshell 120 EC-18 column was used to separate testosterone and similar structured moieties. Generally, a 3.0 minute HPLC gradient was run from 53% to 56% using a mobile phase of 5 mM ammonium formate in methanol and 5mM ammonium formate in water. Detection in the mass spectrometer was accomplished using positive mode ESI and monitoring two ion transitions, 289.2>97.1 and 289.2 > 109.1 m/z, for testosterone and one for the ^{13}C internal standard, 292.1>112.1 m/z.
Results:
Saline, 50% methanol, SigMatrix and SMx Serum produced the least percentage background peak and most consistency ranging from 2%, to 5%.. The commercial calibrator set, 5% human serum albumin and stripped serum had the largest percent background peaks at 11% to 16%.. Unexpectedly, the human serum albumin had a greater than expected lot variation of 3% and 14% between the two lots. Internal standard response difference between calibrators and patient samples ranged from 12% to 24%, with SMx Serum at 12%.
Conclusions:
The SMx Serum matrix from UTAK Laboratories enabled the optimal assay LLMI by providing the lowest background peak, an acceptable correlation between the testosterone internal standard responses of the calibration standards compared to the patient specimens, the greatest consistency between batches and was most cost effective.

B-224

Evaluation of a High Sensitivity Estrone and Estradiol Assay by LC-MS/MS

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Background: High sensitivity measurement of estrone/estradiol is important as part of the diagnostic workup of delayed puberty in females, disorders of sex steroid metabolism, antiestrogen therapies of breast cancer, and bone health of postmenopausal women. LC-MS/MS based assays are superior to conventional immunoassays with improved sensitivity and specificity. We aim to develop a high sensitivity assay for estrone/estradiol by LC-MS/MS for clinical diagnostic use. We evaluated performance of the assay focusing on sensitivity using several approaches recommended by guidelines. **Methods:** We evaluated options of sample volume and preparation, column chromatography and instrumentation, and constructed a LC-MS/MS method without derivatization. Sample preparation is by SLE+ cartridges using 200 µL serum of adult or pediatric patients. Column chromatography is by 2D reverse phase separation using mobile phases containing ammonium fluoride. A QTRAP LC-MS/MS instrument and ESI in negative mode were used to detect and quantify estrone and estradiol. The assay performance including linearity, sensitivity, specificity, and accuracy was validated for clinical diagnostic use. Sensitivity was further evaluated using CLSI, FDA, ISO, CAP, and EU guidelines. **Results:** The assay sensitivity for estrone/estradiol was in range of 2-10 pg/mL when evaluated by the precision profiles, signal-to-noise ratios, and maintenance of ion transition ratios. The assay was linear up to 1,000 pg/mL for estrone and 2,000 pg/mL for estradiol. Correlation data were y=0.94x-6.8 [LC-MS/MS, Ref Lab 1], n=20, r^2=0.98 for estrone and y=1.0x+1.1 [LC-MS/MS, Ref Lab 2] n=75, r^2=0.98 for estradiol. **Conclusion:** The LC-MS/MS method is highly sensitive and with easy to follow steps in sample preparation. It has satisfactory clinical performance for estrone and estradiol measurement in both adult and pediatric patients and it may have broad clinical application due to its extensive measuring range.

B-225

BIUXX

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Background: The abundance of mass spectrometry data can be beneficial yet overwhelming. Automated platforms many times only provide a numeric value but mass spectrometry platforms provide countless pieces of data as to why that numeric value is analytically valid. Many laboratories recognize the power of these data elements and manually review them to enhance the validity of the fi-

nal value. But this review takes an enormous amount of technologist time. Solutions surrounding auto-data review for mass spectrometry based assays exist but use "static" rules that define acceptable peak characteristics. Unfortunately, static rules may eventually fail entire batches, not because the quality of data has suffered but because the entire assay has shifted in an acceptable way. Many laboratories realized that mass spectrometry assays are dynamic processes which ebb and flow as the method/mass spec/LC/columns age. Because calibrators are generally run with each patient batch, what if the calibrators themselves could calibrate more than just the final sample value? What if these calibrators could also provide "dynamic" information regarding retention time? Relative retention time? IS peak area? If these data elements could be analyzed, then the dynamic rules developed can move with the changes in the assay. This greatly reduces the amount of IT/mass spec department maintenance of specific mass spec assays over time. **Methods:** The data described in the presentation was obtained using a Sciex 5500 Mass Spectrometer coupled with a Shimadzu LC20 Liquid Chromatograph at Providence Regional Laboratories in Portland Oregon. Multiquant was used as the quantitation software. The driver was designed by Data Innovations in S. Burlington, Vermont. This is a beta driver designed to average data elements from any samples designated as "calibrators". These averages are then populated under each patient sample which can then be compared to the individual patient data. Rules are generated from this comparison using specific criteria defined and designed by Providence Regional Laboratories. The rules and scenarios were tested using test patients with artificially integrated mass spectrometry data to mimic potential problems with samples. **Results:** After validation of our in-house opiates LCMS assay using the new driver, we observed a significant decrease in review time. Analytes which required manual review were reduced to less than 15% of the total number of analytes reviewed. Error rates for auto-data review were significantly lower than with manual review of data by technologists. The overall time to completely review each patient batch was reduced by over 50%. **Conclusion:** Dynamic auto-data review is a concept that is not entirely new, but requires individual laboratories to produce highly customized, in-house solutions. There needs to be an easier way for laboratories to access these solutions, preferably using technologies and strategies many labs have already acquired. This new data innovations driver provides a "jump start" to middleware rule writing that can be semi-customized based on the individual laboratory's needs. By elevating and incorporating clinical mass spectrometry data into middleware, we bridge the gap between mass spectrometry and routine automated instrumentation platforms.

B-226

New Card for Blood Collection Anytime and Anywhere

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Background: Non-traditional blood collection is growing rapidly for drug compliance, nutritional and wellness testing. The leading method for blood collection outside a clinical facility is the dried blood spot (DBS) card, but DBS results can be difficult to correlate to plasma levels which are needed for clinical significance. Plasma cards are a new alternative to DBS cards that produce dried plasma spots from a drop or two of whole blood. A new plasma card will be evaluated for use in drug and wellness testing. **Methods:** Human blood was collected in accordance with our IRB protocol. Vitamin D reference standards SRM 968 L1/L2 and SRM 968d L1 were obtained from NIST. Isotope labeled Vitamin D and indomethacin were obtained from Medical Isotopes (Pelham, NH). Vitamin D was analyzed as described in Anal. Chem., **2013**, *85* (23), pp 11501-11508. The Vitamin D reagents were obtained from Novilytic. Vitamin D samples were analyzed on a Sciex 4000 mass spectrometer. Indomethacin was analyzed by Alturas Analytics according to their method presented at the AAPS 2016 Conference in Denver, CO. Samples were analyzed on a Sciex 5500 mass spectrometer. One and two disc plasma cards were obtained from Novilytic. **Results:** Combining two plasma collection discs resulted in a total plasma volume of 10 µL. The lower limit of detection (LOD) for vitamin D2 was just under 2 ng/mL using two plasma collection discs. The LOD for vitamin D3 was also under 2 ng/mL. The CV at the limit of detection for both vitamin D2 and D3 was less than 12%. A single plasma collection disc agreed with the vitamin D3 reference standard at 12 ng/mL to within 7% and two plasma discs combined agreed within 5%. Indomethacin was detectable down to 9 ng/mL with a simple methanol extraction using two plasma collection discs. The bias and the CV for two plasma discs were less than 14% at the limit of the detection, 9 ng/mL. The quality control at 1,200 ng/mL showed excellent results with the bias and CV less than 7% for either a single plasma collection disc or two combined discs. **Conclusion:** A new plasma card enables remote blood collection and fast plasma preparation within minutes. The resulting plasma samples are sufficient to measure vitamin and drug levels at the low ng/mL levels using LCMS.

B-227

Development and validation of a quantitative method for plasma markers of transmethylation and transsulfuration

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Background: Metabolism of methionine (Met), homocysteine (Hcy) and cysteine (Cys) is critical for folate-dependent transmethylation and transsulfuration. Abnormal metabolism of these sulfur-rich amino acids may be associated with genetic or epigenetic factors. The primary markers of defects in remethylation and transsulfuration include not only Met, Hcy, and Cys, but also cystathionine (CSH), S-adenosylhomocysteine (SAH), and S-adenosylmethionine (SAM). Our goal was to develop and validate a comprehensive method for the measurement of these markers in plasma using liquid chromatography with tandem mass spectrometry (LC-MS/MS). **Methods:** Plasma samples prepared in 1M acetic acid were mixed with stable isotope internal standards for each of the measured components. For the sulfur-rich amino acids, a strong reductant, dithiothreitol, was added to yield free forms of homocysteine and cysteine from their disulfides, with subsequent derivatization with ethylchloroformate to prevent oxidation and facilitate efficient liquid-liquid extraction with ethyl acetate. For SAH and SAM sample preparation, phenylboronic acid solid phase extraction and nonafluoropentanoic acid, an ion paring reagent were employed to facilitate separation and ionization. Certified standards were acquired to derive a 5-point calibration curve for each analyte. Calibrators and plasma extracts were analyzed on an Agilent 6460 LC-MS/MS with chromatographic separation achieved on a C18 analytical column, permitting identification and quantitation of the compounds of interest. Precision, accuracy, linearity, recovery, and stability were evaluated. **Results:** The intra-assay and total imprecision coefficients of variation (CV_W, CV_T) (n=24) in plasma samples was determined for Met: 0.8%, 8.7% at 1.7 µmol/dL, and 1.2%, 7.5% at 3.4 µmol/dL; Hcy: 1.2%, 8.6% at 5.0 µmol/dL, and 1.2%, 4.8% at 10.2 µmol/dL; Cys: 1.0%, 10.8% at 16.2 µmol/dL, and 0.4%, 8.9% and at 36.0 µmol/dL; CSH: 5.6%, 19.2% at 0.08 µmol/dL, and 2.7%, 5.5% at 0.30 µmol/dL; SAH:1.0%, 1.8% at 61.0 nmol/L, and 0.9%, 1.2% at 113.2 nmol/L; and SAM: 2.4%, 6.6% at 40.4 nmol/L, and 2.9%, 3.3% at 141.7 nmol/L. Linearity range (n=11) and percent recovery were confirmed in spiked plasma samples for Met: 0.4-4.0 µmol/dL, 95.9%-105.6%; Hcy: 2.0-20.0 µmol/dL, 96.3%-101.5%; Cys: 4.0-40.0 µmol/dL, 96.9%-102.9%; CSH: 0.04-0.40 µmol/dL, 94.0%-104.5%; SAH: 5.0-120 nmol/L, 98.1%-102.9%; SAM: 11.0-300.0 nmol/L, 96.7%-102.3%. Least-squares regression analysis comparing split plasma extracts on the Agilent 6460 LC-MS/MS to a previously validated LC-MS/MS method (n=76) yielded correlation coefficients for Met: 0.892, y = 1.107x + 0.363, range 0.6-3.9 µmol/dL; Hcy: 0.986, y = 1.027x + 0.107, range 3.6-16.9 µmol/dL; Cys: 0.972, y = 1.049x + 0.431, range 8.0-35.3 µmol/dL; CSH: 0.994, y = 1.086x - 0.004, range 0.04-0.31 µmol/dL; SAH: 0.995, y = 1.071x - 2.772, range 10.5-58.7 nmol/L; and SAM: 0.925, y = 0.925x - 4.684, range 48.6-219.8 nmol/L. Adequate stability of all analytes in plasma acidified with 0.1% acetic acid (v/v%) was demonstrated for 10-days stored at 2-8°C, and 30-days stored at -20°C. **Conclusion:** This LC-MS/MS method was validated to provide sensitive, precise and accurate evaluation of plasma Met, Hcy, Cys, CSH, SAH, and SAM to guide clinical intervention to improve or normalize methionine metabolism and ameliorate or prevent the potential adverse consequences associated with inadequate methylation and transsulfuration capacity.

B-228

Development and Validation of a LC/MSMS Method for Simultaneous Quantification of Five Vitamin D Metabolites

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Background: There has been a growing interest in Vitamin D and its clinical associations in the last decade. Since it generally correlates well with vitamin D stores of the body and numerous automated immunoassays are readily available, plasma concentrations of 25(OH)D is the most measured metabolite to investigate vitamin D metabolism. However, simultaneous measurement of various forms and isomers of vitamin D metabolites can reveal how vitamin D metabolism is affected in different clinical conditions. Therefore, we aimed to design a method that can analyze the frequently investigated vitamin D metabolites, namely 25(OH)D3, 1,25(OH)2D3, 24R,25(OH)2D3, 25(OH)D2, and 3-epi-25(OH)D3 simultaneously. **Methods:** We developed and validated a high-performance liquid chromatography tandem mass spectrometry (LC/MSMS)-based method by using standards and internal standard solutions for vitamin D metabolites to quantitate each of these in plasma samples. 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and methylamine were

used for derivatization after liquid/liquid extraction, and vitamin D metabolites' derivatives were chromatographically separated using pentafluorophenyl (PFP) and C18 columns. We evaluated performance parameters e.g., matrix effect, carryover, measuring range, limit of quantitation, linearity, imprecision, interferences and accuracy of the method according to the Clinical & Laboratory Standards Institute: Liquid Chromatography-Mass Spectrometry Methods, Approved Guideline (C62-A). **Results:** The method met the linearity and imprecision criteria within the measuring ranges [3.1-100.0 ng/mL for 25(OH)D_3, 15.6-500.0 pg/mL for 1,25$(OH)_2D_3$, 0.6-20.0 ng/mL for 24R,25$(OH)_2D_3$, 1.3-40.0 ng/mL for 25(OH)D_2 and 0,6-20,0 ng/mL for 3-epi-25(OH)D_3], chosen in accordance with the clinical decision-making levels of vitamin D metabolites. The validation process was completed with imprecision and accuracy evaluation; coefficient of variations of all metabolites at various concentrations did not exceed 9.3% at all CV classifications (within-run, between-run, within-day, between-day and total) and the highest bias% obtained from the measurement of 4 levels of the certified reference materials (SRM972a) provided by NIST for 25(OH)D_3, 24R,25$(OH)_2D_3$, 25(OH)D_2 and 3-epi-25(OH)D_3 were (+1,3%), (-3,8%), (-8%), and (-8%), respectively. Reduction of isomeric and/or isobaric interferences on 1,25(OH)2D3 measurement and separation of 25(OH)D3 and 3-epi-25(OH)D3 peaks were established by using two analytical columns together. **Conclusion:** LC/MSMS-based methods have high sensitivity and specificity and can be used to monitor changes on the concentration of vitamin D metabolites simultaneously in various clinical conditions. In the future, they will reach much better performances with improved extraction, derivatization, chromatographic separation and more advanced instruments. Although they are rapidly gaining ground in clinical chemistry laboratories, precise and accurate results can only be obtained with carefully and accurately validated methods due to the complexity of the LC/MSMS systems.

B-229

High Sensitivity Determination of Underivatized Estradiol and Testosterone in Human Serum by LC-MS/MS

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Background: Accurate measurement of estradiol (E_2) at low concentrations is required in postmenopausal women, men, pediatric patients, and to assess the efficacy of anti-estrogen therapies. The same is true for testosterone (T) in women, children, hypo-gonadal men and the need to control anti-androgen therapies. This study aims to develop and validate high-sensitive methods for analysis of underivatized E_2 and T in low volume of human serum. **Methods**: E_2 and d_3-E_2 (internal standard) were extracted from 200 µL of human serum with 1-chlorobutane, and T and d_3-T (internal standard) were extracted from 250 µL of human serum with ethylacetate. Chromatographic separation was performed on a core shell C18 analytical column under specific gradient elution for each analyte, with mobile phases consisting of methanol (phase A) and 0.2 mM ammonium fluoride (phase B). Negative electrospray ionization was used for E_2 to follow the predominant transitions: collision energy (CE) -64, m/z 271→143 (qualifier); CE-50, m/z 271→145 (quantifier), CE -50, m/z 274→145 for d_3-E_2; positive electrospray ionization was used for T to follow the predominant transitions: collision energy +25, m/z 289→97 (qualifier), m/z 289→109 (quantifier) и m/z 292→109 for d_3-T. Raw data of mass chromatograms were collected and processed by specialized software, and weighted (1/X) linear regressions were performed to determine the concentration of the analytes. Validation strategy was adhered to current industrial and clinical guidance. **Results**: Selectivity was assessed with 20 individual native matrices of human serum applying the technique of standard additions: 5 from children under the age of 6 years, 5 from postmenopausal women, 5 from men, and 5 from premenopausal women, at each point, in the range 2 - 1000 ng/L for E_2, and 0.01 - 20 µg/L for T. Normalized matrix effect averaged 89-112% (percent matrix bias: -11 ÷ 12%), imprecision being within 15%. Inaccuracy ranged from -12.0 to 8.9 % within runs and from -14.9 to 12.2 % between runs. Imprecision was up to 12.7% within-runs, and up to 14.8% between-runs. Linearity was assured in the range 1.0 ÷ 1000 ng/L, R^2>0.996 for E_2, and 0.005 ÷ 20 µg/L, R^2>0.995 for T. Freeze-thaw stability was determined for three cycles each lasting 24 h, post-preparative stability was documented for 24 h at 4°C, short-term stability at room temperature was proven for 6 h at daylight and 4 h in the dark; stock solution stability and long term stability in serum were documented for 96 days at -20°C. With run time of 6 min, a throughput of over 100 samples per working day could be achieved for each analyte. **Conclusion**: The two methods were validated according to current industrial and clinical requirements and allow the accurate and precise determination of E_2 and T in human serum at very low concentrations.

B-230

Analysis of Serum Androgens and Corticosteroids for Clinical Research by LC-MS/MS

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Background: Here we evaluate an offline automated method for the measurement of serum androgens; testosterone, androstenedione and dehydroepiandrosterone sulfate (DHEAS), and serum corticosteroids: 17-hydroxyprogesterone (17-OHP), cortisol, 11-deoxycortisol and 21-deoxycortisol, enabling steroid profiling for the investigation of metabolic dysfunction biomarkers for clinical research. An LC-MS/MS method was developed using a novel Solid Phase Extraction (SPE) sorbent in 96-well plate format, reducing sample preparation time and removing more matrix interference in comparison to other sample preparation techniques. **Methods:** Certified testosterone, androstenedione, DHEAS, cortisol, 11-deoxycortisol and 21-deoxycortisol reference material purchased from Cerilliant (Round Rock, TX) were used to create calibrators and QC materials in stripped serum purchased from Golden West Biologicals (Temecula, CA). Serum samples purchased from UK NEQAS (Birmingham, UK) for testosterone, androstenedione, DHEAS, 17-OHP and cortisol were analyzed and concentrations were compared to the EQA MS mean for each steroid hormone. 100 µL serum samples were pre-treated with internal standard, methanol and water. SPE was carried out with a Waters® Oasis® PRiME HLB µElution 96-well plate, providing phospholipid removal and allowing direct injection of the SPE eluate. Offline automated extraction was performed using a Tecan® Freedom Evo 100. Using an ACQUITY UPLC® I-Class system, samples were injected onto a 2.1 x 50 mm Waters ACQUITY UPLC HSS T3 column with a pre-column T3 VanGuard™ using a water/methanol/ammonium acetate/formic acid gradient and quantified with a Waters Xevo® TQ-S micro mass spectrometer. **Results:** The developed method was shown to be linear for the serum androgens and corticosteroids. No significant carryover was observed from high concentrations serum samples into serum blanks. A 1:5 dilution was successfully performed on over-range samples for the serum steroids with recoveries ranging from 97-107% with CVs < 7%. Total precision and repeatability on five separate days for low, mid and high QC samples were all ≤ 7.6% CV (n = 30) for all analytes. Analytical sensitivity investigations performed over three occasions demonstrate a CV < 20% at 0.03 ng/mL for testosterone, 0.025 ng/mL for androstenedione, 0.063 ng/mL for 17-OHP, 4 ng/mL for DHEAS, 0.25 ng/mL for cortisol, 11-deoxycortisol and 21-deoxycortisol. S/N (PtP) calculations at each of these concentrations were > 10:1. Matrix Factor experiments demonstrate the internal standard compensates for ion suppression observed in the method, with matrix factor range of 93 - 100% and CVs < 6.7% for the steroid hormones. The method has shown to be analytically selective through separation of isobaric steroid species and matrix specific interferences such as albumin, triglycerides and bilirubin that could affect accuracy and imprecision. Excellent agreement between this analytical method and the EQA LC-MS mean values have been demonstrated with mean method bias of -0.1%, -5.1%, 5.2%, -5.8% and -1.0% for testosterone, androstenedione, 17-OHP, DHEAS and cortisol, respectively. **Conclusions:** We have successfully quantified serum androgens and corticosteroids using SPE with LC-MS/MS for clinical research purposes. This offline automated method demonstrates excellent linearity, analytical sensitivity, selectivity, precision and accuracy, while providing high sample throughput capabilities. For Research Use Only, Not for use in diagnostic procedures.

B-231

Development of an LC-MS/MS method for measurement of human glycated serum albumin.

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Background: Monitoring glycemic control in patients with Diabetes Mellitus is important to avoid many long term complications associated with the disease. In most patients, glycated hemoglobin (HbA1c) testing is used for long-term monitoring. However, in some situations, e.g. those that affect erythrocyte lifespan, HbA1c results may not be accurate. Glycated Albumin (GA) is a good alternative measurement of glycemic control in these situations. The main goal of this study was to develop a mass spectrometry assay for GA quantitation. **Methods:** A QTRAP 6500+ (Sciex) coupled with Shimadzu HPLC system were used. The assay was initially developed using in-vitro glycated human serum albumin samples. The samples were reduced and alkylated with DTT/IAA, then digested with Glu-C enzyme. The information dependent acquisition was used to identify

all glycated albumin sites. The MRM transitions of two peptides RQIKKQTALVE (521-531) and FKPLVEEPQNLIKQNCE (377-393) and their glycated forms (Lys-525 and Lys-378 accordingly) were chosen for quantitation. The developed protocol was further used for serum samples from subjects with and without diabetes. **Results:** There was a linear correlation between our LC-MS/MS and the commercial Asahi Kasei (Tokyo, Japan) Lucica method for human serum samples, r^2 values were 0.91 and 0.86 for Lys-525 and Lys-378 sites respectively. The CV for human serum samples was 5.2%. **Conclusion:** In summary, we have developed and validated a novel method for glycated albumin quantitation in human serum samples.

B-232

Evaluation of the first FDA-cleared LC-MS/MS assay for quantification of 25-hydroxyvitamin D2/D3 and C3-epimers on the Sciex Topaz system.

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INTRODUCTION: Measurement of 25-hydroxyvitamin D (25(OH)D), i.e., the sum of 25(OH)D2 and 25(OH)D3, has long been recognized as most sensitive and specific when measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) versus other methods such as immunoassay. However, LC-MS/MS for 25(OH)D testing is not widely implemented in routine clinical laboratories due to the need for specialized personnel to operate what are generally considered intricate, open-source platforms. We present one of the first published evaluations of the FDA-cleared Vitamin D 200M assay on the Topaz™ LC-MS/MS system with regard to ease-of-use and prevalence of measurable 25(OH)D2 and C3-epimers in a randomly selected patient population at Cedars-Sinai Medical Center. **METHODS:** Two staff members received one-day of training on the Topaz system and Vitamin D 200M assay. 100µL aliquots of 27 adult serum remnants, 6 calibrators, and 3 quality controls were analyzed with the Vitamin D 200M assay protocol and kit. LC-MS/MS results on these samples were compared to Abbott Architect immunoassay results, which measures total 25(OH)D. Accuracy was assessed by evaluating linearity and reportable range with replicate calibrator extractions. Intraday precision was evaluated with 5 replicate injections of 3 extractions of each of 3 QC levels. The Sciex locked version of Vitamin D 200M for 25(OH)D3 and D2 was also used to evaluate patient C3-epimer concentrations. **RESULTS:** Expected linearity and reportable range were demonstrated for 25-OH Vitamin D3 (R=0.996, 4.37-135.0 ng/mL) and 25(OH)D2 (R=0.999, 2.5 -123.2 ng/mL. %CVs for intraday precision (n=15) of controls were as follows: For 25(OH)D3 Q1=2.1%, Q2=2.7%, and Q3=2.7%, which all fell within expected %CVs; and for 25(OH)D2 Q1=4.0%, Q2=4.1, and Q3=3.2%, which were again within expected limits. The average %bias of the Sciex LC-MS/MS method vs. immunoassay was -7.8%. Amongst 27 patient samples, 10 (32%) had quantifiable 25(OH)D2, and 4% had D2 concentrations >20ng/mL. All samples had measurable concentrations of C3-epimer-25(OH)D3 (range: 2.3-7.7 ng/mL); only one had a quantifiable C3-epimer-25(OH)D2 concentration of 2.8 ng/mL, related to their high 25(OH)D2 concentration (45 ng/mL). However, it has been previously demonstrated that the C3-epimer is primarily related to the 25(OH)D3 level; in our patient population we would estimate that this ~8.6% of the 25(OH)D3 concentration as the linear regression equation of the correlation to immunoassay is: y=0.0855x+0.9812. Additionally, 4 samples had C3-epimer-25(OH)D3 >5 ng/mL (i.e., 15% of the study population). **CONCLUSION:** Although other parameters can be used to assess ease-of-use, after one day of training on the Topaz™ LC-MS/MS system, staff could quantify 25(OH)D2/D3 with linearity, reportable range, and precision as expected based on cleared specifications. Correlation to immunoassay was biased by -7.8%, which is to be expected when both 25(OH)D2 and C3-epimers are present at appreciable concentrations. Lastly, the observation of measurable 25(OH)D2 and the C3-epimers in 32% and 100% of patient samples tested, respectively, emphasizes two points: 1) The importance of the specificity offered by the Topaz™ LC-MS/MS system and vitamin D assay in routine hospital populations. And 2) Recognition that C3-epimer is not inconsequential in the adult population and should likely be quantitated, yet many lab-developed 25(OH)D LC-MS/MS assays do not.

B-233

Determination of cadmium, lead, mercury, and nickel in blood for assessment of environmental exposure by inductively coupled plasma mass spectrometry: a comparison with atomic absorption spectrometry and stability test of samples under refrigerated condition

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Background: The purpose of this study is to establish Cadmium, Lead, Mercury, and Nickel analytical methods by Inductively coupled plasma-mass spectrometry (ICP-MS) for investigating environmental exposure to the trace metals in Korean general population and to ensure the continuity with previous national biomonitoring data by atomic absorption spectrometry (AAS) through comparing ICP-MS with AAS. In order to confirm the change with the time delay from sampling to the measurement, the stability of samples under the refrigerated condition was also evaluated. **Methods:** We established and validated the dilution methods for Cd, Pb, Hg, and Ni in whole blood sample using ICP-MS (7900X ICP-MS, Agilent Technologies, Japan). We compared our routine external calibration method and the matrix-matched calibration method with G-EQUAS (German External Quality Assessment Scheme) materials with assigned value. Whole Blood samples from 100 healthy Korean population were collected in trace element EDTA tubes (BD, USA). Cd, Pb, and Hg levels were determined using both ICP-MS and AAS (Cd; AAnalyst 800, Perkin Elmer, Singapore, Pb; 240Z AA, Agilent Technologies, Australia, and Hg; DMA 80, Milestone, Italy). The stability test under the refrigerated condition was carried out for Cd, Pb, Hg, and Ni with 10 patient samples on the 1st, 3rd, 7th and 21st days after blood sampling. **Results:** Limit of quantifications were 0.31 µg/, 2.11 µg/dL, 1.06 µg/L, and 0.99 µg/L for Cd, Pb, Hg, and Ni, respectively. The average within-batch and total coefficients of variation were below 10 % in all analytes. The percent bias against the assigned value of two levels of G-EQUAS material were 2.51, 4.90 for Cd, -1.00, 0.04 for Pb, and -1.28, -3.36 for Hg with external calibration and 5.26, 4.70 for Cd, -2.24, -0.07 for Pb, and 0.22, -0.80 for Hg with standard addition method. The differences between ICP-MS and AAS method for Cd, Pb, and Hg were statistically significant ($P < 0.001$). The regression equation for the comparison between ICP-MS (y) and the AAS method (x) was $y = 1.061x + 0.018$ ($r^2 = 0.964$) for Cd, $y = 0.910x - 0.242$ ($r^2 = 0.855$) for Pb, and $y = 0.978x - 0.048$ ($r^2 = 0.985$) for Hg. The average of differences of ICP-MS against AAS were +11.6 % for Cd, -21.8 % for Pb, and -3.4 % for Hg. In regression analysis for the stability test, the probability of F of all items was> 0.05. **Conclusion:** Our ICP-MS method for the determination of Cd, Ob, Hg, and Ni showed a good performance, and the ICP-MS results could be converted to be equivalent to the previous AAS results, so it can be used for biomonitoring in general population. There was no significant change in the stability according to the shelf life, therefore, there is no concern about the quality of specimens during refrigerated storage and transportation.

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Development of an LC-MS/MS method for quantifying DNA methylation in whole blood for assessment of hematologic malignancies.

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Background: DNA methylation is a dynamic physiologic mechanism for silencing genes. In cancer, these epigenetic modifications can be dysregulated to be advantageous for tumorigenesis. Numerous cancers, including some hematologic malignancies, are well-described in the literature as having hypermethylator phenotypes. Clinically, DNA methylation status has been of recent interest in acute myeloid leukemia (AML) as a new paradigm of prognostic stratification, often showing increased survival. Despite this, a clinically validated method for assessing total genomic methylation of these cancers in clinical samples has yet to arise. Our research provides a mass spectrometry-based method for quantifying methylated DNA extracted from whole blood samples for assessment of hypermethylator phenotypes in hematologic malignancies. **Materials and Methods:** Analysis of remnant clinical samples was approved by the UCSF Institutional Review Board. Genomic DNA was extracted from 200uL of whole blood, concentrated, and enzymatically hydrolyzed into nucleosides. Chromatographic separation of analytes was performed with a Kinetix C18 column (50x3mm, 2.6um; Phenomenex). Data was acquired on a QTRAP 4500 (SCIEX) using positive-ion mode multiple reaction monitoring. Quantifier and qualifier fragment ion transitions for 2'-deoxycytidine (C), 5-methyl-2'-deoxycytidine (5mC), and deuterated internal standards (Toronto Research Chemicals) were used to measure peak area. A calibration curve was constructed for concentrations 10 to 2000 ng/mL. Appropriate DNA hydrolysis

was confirmed each run with native and methylated DNA oligomer controls (ZYMO Research). Percentage of DNA methylation was calculated from the concentrations of 5mC divided by the sum of 5mC and C measured in each sample. Validation of this method was performed with 5 separate extractions from analyte-spiked whole blood for a 10-point calibration curve with 3 quality controls spanning the range of the curve. **Results:** Inter-assay calibration curves (n=5) were linear and reproducible over 10-2000 ng/mL (r^2>0.99). Accuracy, as measured by percent error from nominal value between inter-assay calibrator and QC concentration mean values ranged from 0.74-11.55%, with an average percent error of 5.95%. Inter-assay coefficient of variance (CV) for each calibrator and QC ranged from 4.14-8.10%, with an average CV of 6.08%. Calculation of DNA methylation in healthy control whole blood showed percent cytidine methylation of 3.08%. **Conclusion:** This method is novel in that it provides a clinical diagnostic tool for assessing total genomic methylation from whole blood, which can potentially be used to assess methylation status of hematologic malignancies. Further analytical and clinical studies using this method will be performed to confirm and explore prognostic correlations that have been preliminarily reported for hypermethylator phenotype hematologic malignancies, specifically AML.

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Measurement of Serum Iohexol by LC-MS/MS to Assess Glomerular Filtration Rate in Kidney Transplant

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Background: We have developed a new liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to measure iohexol, a filtration marker, for accurate glomerular filtration rate (GFR) determination as part of living kidney donor evaluation. In the recently published guidelines by Kidney Disease Improving Global Outcomes (KDIGO), measured GFR (mGFR) is recommended as confirmatory test for estimated GFR (eGFR) to better assess the long-term end-stage renal disease (ESRD) risk for donor candidates. The current mGFR method implemented in our hospital involves the use of a radioactive marker (^{125}I-iothalamate), which poses potential safety risk and often suffered from supply shortage. The purpose of the current study is to develop a simple yet sensitive method to allow the use of the non-radioactive iohexol as an alternative. **Methods:** After a single bolus of iohexol (5mL Omnipaque 300™), three blood samples were collected at 120, 180 and 240 minutes' interval after injection of iohexol. Upon separation of serum from blood, iohexol concentrations were then measured by LC-MS/MS. From serum iohexol levels and the administration dose, GFR would be calculated using the one-compartment open model system corrected according to the Brochner-Mortensen formula. Standard GFR would also be calculated by correcting GFR to body surface area (BSA). To measure serum iohexol, a quick sample extraction method was developed using strong acid protein precipitation. The LC-MS/MS method was developed on Thermo Vanquish UHPLC system coupled with TSQ Endura triple quadruple mass spectrometer. Iohexol and d5-iohexol, the internal standard, were simultaneously eluted by a Kinetex EVO C18 column (5µm, 50*3.0 mm), using a 2.5-minute gradient of 0.1 % formic acid in water and methanol. Two multiple reaction monitoring (MRM) transitions were set for iohexol: 822.0◊804.0 (for quantitation) and 822.0◊603.0 (for confirmation); and one MRM transition is set for d5-iohexol: 827.0◊809.0. Simultaneous peak integration and quantitation for iohexol were achieved automatically using the pre-installed TraceFinder™ Software. **Results:** Validation study shown great linearity ($R^2 \geq 0.99$) across the analytical measurement range (AMR) from 5 to 1000 µg/mL. Assay within-day and between-day precisions were assessed at three different QC levels, with coefficient of variation (CV) less than 15% achieved for each level. To assess the assay accuracy, results from 20 iohexol-spiked serum with concentrations across the AMR were compared to that obtained from a reference laboratory. The regression analysis shown a slope of 0.995 (0.968 to 1.023, 95% CI), an intercept of 11.092 (-1.447 to 23.632, 95%CI), and the standard error estimate of 14.817. The correlation efficiency is 0.998 with percent bias of 2.4%. Further validation study, including ion suppression and specimen stability, is currently underway. **Conclusion:** In summary, we have developed a new LC-MS/MS assay to accurately measure iohexol, providing a safer GFR assessment method that is easy to implement, from which both our patients and clinicians can benefit.

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Determination of urinary metabolites of gasoline using LC-MS; method validation

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Abstract

Background: Biological monitoring (BM) is an evaluation of occupational exposure to volatile organic compounds in gasoline and an important measure for the prevention and protection of occupational intoxication. Objective: We aimed to develop a method to simultaneously assess 4 metabolites of gasoline in urine samples of exposed workers; trans, trans-muconic acid (MUC), mandelic acid (MAN), hippuric acid (HIP) and orthomethylhippuric acid (MHP) which are metabolites of benzene, ethylbenzene, xylene & toluene (4 main constituents of gasoline) using Liquid Chromatography-Mass Spectrometery (LC-MS). Method: The developed method was suitable for quantitative analysis of the targeted metabolites in the urine of gas stations workers. FDA regulations for analysis in biological fluids were followed for validation. Procedure: Standards of HIP, MHP, MAN and MUC were provided from Sigma-Aldrich, St. Louis, Missouri, USA. Urine samples & standard solutions were used. Shim-pack® XR – ODS II column was used as stationary phase with a gradient eluting solvent of 1% formic acid and acetonitrile. The metabolites were resolved with retention times of 3.1, 5.8, 6.2 and 11.1 min for MUC, MAN, HIP and MHP, respectively. The MS detector was APCI (atmospheric pressure chemical ionization) adjusted to negative SIM (selected ion monitoring) mode to eliminate the interference and enhance sensitivity. The selected m/z values were 141, 151, 178, and 192 for MUC, MAN, HIP and MHP, respectively. Results: Accuracy: recoveries ranged from 92.87-105.01 % at three levels of concentration, and from 82.5 – 88.9 % at the limit of quantitation. Precision: The within-run coefficient of variation (CV) ranged from 0.580% to 8.138% for all analytes. The repeatability CV ranged from 1.052% to 6.316%. Measuring range & linearity: The limits of detection were 0.117, 0.251, 0.139, and 0.109 µg/mL, and the limit of quantification values were 0.355, 0.759, 0.422, and 0.329 µg/mL for MUC, MAN, HIP and MHP, respectively. The method was linear up to 20 µg/mL for all analytes. No extraction procedure was required for analysis in urine and being a simple non-invasive method, it could be applied for routine check ups of workers in gas stations. Matrix ion suppression was avoided using many strategies including sample dilution, APCI ionization. The method was sensitive and selective to simultaneously analyze the four metabolites in presence of possible urine interferences as albuminuria & hemoglobinuria. Stability parameters were also tested at room temperature, refrigeration, 3 freeze-thaw cycles & long term storage at -80°C Conclusion: The developed method was suitable for quantitative analysis of the targeted metabolites in the urine of gas stations workers. It will be further used in a study to assess vestibular system dysfunction among workers exposed to gasoline. Metabolites level will be assessed in urine samples pre and post shift. Urinary metabolites will be normalized analyte concentration-to-urine creatinine concentration to compensate for fluctuations in absolute concentration related to physiologic variation as urine dilution or concentration.

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Candidate reference method of serum thyroxine using Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry

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Background: There are some reference methods to determine serum total thyroxine by isotope dilution gas chromatography mass spectrometry and liquid chromatography mass spectrometry which are time consuming and complicated. A need exists for a simple reference method that can be easily adopted to verify the accuracy of serum thyroxine measurements. So candidate reference methods involving isotope dilution liquid chromatography tandem mass spectrometry (ID-LC/MS/MS) for total thyroxine were established. **Methods:** Serum samples were sampled by weight and the $^{13}C_6$-thyroxine internal standards were added volumetrically using automated dilutors,followed by equilibration,protein precipitation,and cation exchang solid-phase extractions(SPE). After SPE,the eluates were evaporated to dryness under nitrogen and then the evaporated residues were reconstituted to prepare samples for liquid chroma-

tography-mass spectrometry electrospray ionization (LC/MS-ESI) analysis using electrospray for ionization (ESI). For separation, a Zorbax Eclipse XDB C18 column was used with a mobile phase consisting of 0.05% formate in water-methanol (30:70 by volume) for positive ions. The quantitative ion transitions of $[M+H-HCOOH]^+$ at *m/z* 777.7→731.6 and *m/z* 783.7→737.6 were monitored for thyroxine and $[^{13}C_3]$-thyroxine, respectively. The qualitative ion transitions of $[M+H-HCOOH-I]^+$ were at *m/z* 777.7→633.8 and *m/z* 783.7→639.8, respectively. **Results:** The within-run,between-run and total coefficients of variation were:0.60%(0.35%~0.82 %),0.54%(0.27 %~1.23 %) and 0.84%(0.57%~1.37 %),respectively. The analytical recoveries ranged from 99.6% to 100.7%. The limit of detection was 0.12 nmol/L (S/N ratio 3:1) and the limit of quantification was 0.41nmol/L (S/N ratio 10:1) for thyroxine in human serum. The results of analyzing the certified reference material of German Societies for Clinical Chemistry (DGKC) CRM21201 and CRM21202 showed biases of -0.30% (ranged from -0.13% to 0.73%). **Conclusion:** Isotope dilution LC/MS/MS method for serum thyroxine has been developed. The method is less sample volume demand and less time-consuming and may be used as a candidate reference method. This method was been used in international laboratory comparison including RELA (IFCC) and domestic Reference measurement comparison of EQA. Results showed that this ID-LC/MS/MS method was well-characterized for serum thyroxine with a theoretically sound approach, demonstrated good accuracy and precision, and low susceptibility to interferences qualifies as a candidate reference method. Use of this reference method as an accuracy base may reduce the apparent biases in routine methods along with the high interlaboratory imprecision.

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Clinical Validation and Implementation of a High Throughput Method for Measuring Whole Blood Lead Levels Using an Alkaline Digestion and ICP-MS

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Background: Lead is a cumulative toxicant from environmental exposures with effects on virtually all organ systems including the CNS, liver, gastrointestinal, renal, and hematological systems. Of great clinical concern are the deleterious effects of lead exposure on neurocognitive function in pediatric populations. Low blood lead levels (<5ug/dL) have been shown to affect cognitive function and these effects appear to be irreversible. Atomic Absorption Spectroscopy (AAS) is the most prevalent method for lead testing. Though AAS is adequate for detecting levels >5ug/dL, it is suboptimal for detecting low blood lead levels, uses highly corrosive solvents, and is not conducive to a high throughput environment. We validated a method for whole blood lead testing using Induction Coupled Plasma Mass Spectrometry (ICP-MS) that is sensitive, precise, accurate, and allows for high throughput testing in a CLIA environment. **Methods:** Commercially available lead standards were used to spike into donor blood as well as a matrix matched sample medium (7.5g/L sodium chloride) devoid of lead. Results were verified against commercially available whole blood quality control material (UTAK), CAP proficiency testing material, and clinical specimens that were analyzed in our laboratory using a method that has full approval for patient care (AAS). Whole blood (100uL) was diluted 1:40 in a solution containing 4% butanol, 1% tetramethylammonium hydroxide, 0.01% EDTA, and 0.01% Triton X-100. The diluent was designed to reduce matrix concentration, increase pH, solubilize lead, and minimize the memory effects that can be seen in some ICP-MS methods. The sample is automatically introduced into a 1mL sample loop using a high-speed sample introduction system (ISIS-3; Agilent Technologies) that was optimized for throughput and low carryover, atomized, and subsequently quantified by ICP-MS (Agilent 7900) using the sum of the 3 naturally occurring lead isotopes (206, 207, and 208). An internal standard mixture containing bismuth (209), lutetium (175), and terbium (159) are continuously infused at a 1:10 ratio relative to the primary flow. **Results:** A 1:40 dilution was sufficient to reduce the matrix concentration for robust analysis of a large batch of samples (up to 150 per batch) and still meet the desired limits of detection (0.1ug/dL). The method is linear over 4 orders of magnitude between 0.1 to 100ug/dL with low carryover (<0.25%). The intra- and inter-run imprecision estimates are 2.6% and 3.1% for the lower end of the analytical measurement range (1.8ug/dL) and 1.3% and 3.6% for the upper end of the analytical measurement range (59.7ug/dL) respectively. Accuracy is 84% at 0.1ug/dL and >98% at concentrations greater than 0.3ug/dL. The 3 internal standards are all linear between 1ug/dL and 1000ug/dL and all were accurate for normalization of ionization variability. The method correlated well to AAS ($R^2=0.98$) as well as CAP proficiency results with no fixed or variable bias. **Conclusion:** We successfully validated and implemented a method in a large university hospital for measuring blood lead by ICP-MS with sensitivity down to 0.1ug/dL using alkaline conditions that can be used for high throughput heavy metal testing.

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Determination of bottled mass of Angiotensin I in candidate Standard Reference Material 998a by amino acid analysis

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Background: The National Institute of Standards and Technology (NIST) has offered a Standard Reference Material (SRM) 998 Angiotensin I (AT-I) for use in the calibration and standardization of renin functional assays. Each vial of this material contained 0.5 mg of AT-I and was reconstituted by adding a desired quantity of solvent. A replacement lot has been procured having an expected mass of 5 mg in each bottle. The purity of the bulk material was assessed prior to packaging and was determined to be 789 mg/g with expanded uncertainty of 58 mg/g (k=2) using amino acid analysis. The current study seeks to determine the average mass and variation of AT-1 as vialed. The true value is critical to determining the reconstitution protocol for this material, whether to add a known amount of solvent or to weigh out milligram quantities for solubilization.**Methods:** Six bottles of the material were selected and approximately 1 mL of 0.01 M hydrochloric acid was added and the mass recorded. The samples were diluted approximately 160-fold and two replicates were taken of each diluted sample. The concentration of AT-1 was determined via amino acid analysis (AAA) using double isotope-dilution tandem mass spectrometry after gas phase hydrochloric acid hydrolysis. Samples were spiked with isotopically labeled free amino acids (isoleucine, leucine, phenylalanine, and valine) either before (n=3) or after (n=3) hydrolysis. Calibration was accomplished using unlabeled amino acids also spiked with labeled amino acids, both without acid hydrolysis. The mass in each bottle was calculated using the determined concentration and known solvent addition and then corrected for purity. **Results:** Values were assigned using linear regression of an external ratiometric calibration curve for each individual amino acid. The regression coefficient was found to be > 0.99 in all cases with slope and y-intercept values close to unity and zero, respectively. The average mass determined for AT-1 was 5.46 mg (5.0 %cv) and 6.00 mg (4.1 %cv) for the groups for which the internal standard was added either pre-hydrolysis or post-hydrolysis, respectively. The means of the two groups were not shown to be different (t-test, $p > 0.05$) and the results were combined to yield an average of 5.7 mg (6.6 %cv). **Conclusion:** The evaluation of the mass contained within each bottle of candidate SRM998a, Angiotensin-I was performed using amino acid analysis and the variation was determined. The bottle to bottle variation shown may be acceptable for use as a calibrant prepared by fixed volume addition in functional assays but will require investigation into each specific case. The larger mass contained in SRM998a will offer the additional ability to measure out specific quantities of material to avoid the bottle-to-bottle variability.

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Fully automated high-throughput urinary creatinine method by liquid chromatography tandem mass spectrometry

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Background: Creatinine is a by-product of muscle metabolism that is excreted in urine at a constant rate through glomerular filtration, and its concentration in urine is proportional to muscle mass. Reliable creatinine measurements are important to evaluate kidney function and for normalizing urinary analyte concentrations. There is a need for specific and sensitive measurements of creatinine to ensure results are accurate and reliable. To address this need, we developed a high-throughput, routine mass spectrometry-based method that requires small sample volumes and provides high accuracy. **Methods:** A high-throughput, fast and accurate liquid chromatography tandem mass spectrometry method for the quantitation of human urinary creatinine was developed and validated. Sample preparation was fully automated including decapping of cryovials, sample ID scanning, and sample aliquoting and processing. Sample processing was performed using a liquid handling system and 96-well plates. This allows for processing of over 600 samples in 8 hours. Quantitation was performed using a stable isotope-labeled internal standard. Multiplexed chromatographic separation of creatinine was achieved within an one-minute run on two Waters Aquity UPLC HSS C18 SB 1.8µm, 2.1x50 mm columns and followed by tandem mass spectrometry on a Thermo Electron triple quadrupole Quantum mass spectrometer in positive electrospray ionization mode. The precursor and product ions of creatinine and D3-creatinine were monitored in selected reaction monitoring mode using the following transitions: m/z 114→44 (quantitation ion (QI) for creatinine), m/z 117→47 (QI for D3-creatinine), m/z 114→72 (confirmation ion(CI) for creatinine) and m/z 117→75 (CI for D3-creatinine). **Results:** Method validation results showed great reproducibility with a within-run precision of 3.59%, 3.49% and 2.84% and among-run precision of 4.01%, 3.28% and 3.57% for low, medium and high quality control materials, respectively. The calibration curve

was linear from 7.5 to 300 mg/dL (R^2=0.9999). Matrix effects were studied in four different matrices and found to have minimal impact on the method with a 95.15 % mean matrix effect observed. Analytical specificity was achieved by chromatographically separating creatinine from potentially interfering creatine within a one-minute run and monitoring the QI/CI ratios in samples. The method showed excellent accuracy with a bias of -2%, -0.8% and -1.1% for three levels of NIST certified reference material. **Conclusion:** A simple, accurate and high-throughput method was successfully developed for measuring urinary creatinine in human urine samples.

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Consumption of Movantik (Naloxegol), an opioid antagonist, results in detection of naloxone in confirmatory urine drug testing

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Background: Many patients that receive chronic opioid therapy suffer from constipation, one of the most common side effect of opioid. Movantik (naloxegol) is an opioid antagonist that is recently introduced in the market to treat opioids-induced constipation and contains naloxegol, as the active ingredient. Naloxegol is a pegylated (polyethylene glycol-modified) derivative of α-naloxol. Confirmatory methods of urine drug testing are known to have high specificity and producing minimum false positive results compared to the screening methods. In pain management clinics appearance of naloxone in the confirmatory urine drug testing report of patients that are prescribed movantik may mislead the clinicians. This study was conducted to investigate the presence of naloxone in the urine of patients that consume movantik in pain management clinics. **Methods:** In a retrospective study the presence of naloxone in the urine of 36 patients that had consumed movantik in pain management clinics of Houston, Texas was investigated. The presence of naloxone was tested using a dilute and shoot liquid chromatography mass spectrometry (LC-MS) method. In concurrence the urinary concentration of naloxone was evaluated in four volunteers that took one pill of movantik. The presence of naloxone in movantik pills was assessed using liquid chromatography mass spectrometry. **Results:** Naloxone was detected in the urine of 34 individuals that were prescribed movantik. All patients observed were also prescribed opioids. Urinary concentration of naloxone showed a bimodal distribution with a mean of 28 ± 20 ng/ml for 26 patients and 133± 49 ng/ml for 8 patients. Consumption of one pill of 25 mg movantik resulted in the detection of naloxone in the urine of four volunteers one hour after taking the pill. Naloxone was not detected 24 hours after the pill consumption. The peak of urinary concentration of naloxone in the volunteers` urine was almost 20 ng/ml. Analysis of movantik pill by liquid chromatography mass spectrometry demonstrated very low concentration of naloxone. **Conclusion:**This study demonstrated that consumption of movantik leads to appearance of naloxone in the urine of patients undergoing opioid therapy in pain management clinics.

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Evaluation of Substance Abuse and Mental Health Services Administration (SAMHSA) pH criteria for definition of adulterated and invalid result specimens in confirmatory urine drug testing.

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Background: Federally-regulated toxicology laboratories that mainly perform workplace urine drug testing utilize certain criteria defined by Substance Abuse and Mental Health Services Administration (SAMHSA) to interpret urine validity tests and report the specimen as valid, dilute, adulterated, substituted and invalid result. If urine specimen pH is ≤ 4 or ≥ 11 the specimen is labeled as adulterated and when the pH is between 4-4.5 or 9-11 the specimen is called invalid result. In contrast to screening immunoassay methods, liquid chromatography mass spectrometry are not prone to many interferences. We tested this hypothesis whether SAMHSA pH criteria for definition of adulterated and invalid result are applicable for both screening and confirmatory urine drug testing. **Methods:** Drug free urine specimens were spiked with common drugs that are tested in clinical toxicology laboratories and the specimen pH altered to a range from 1.6 to 14. The urine specimens were tested with both screening (immunoassay) and confirmatory (liquid chromatography mass spectrometry) using Beckman coulter AU680 Siemens reagents and Sciex 4500 dilute and shoot method, respectively. The confirmatory method measured the concentration of 87 drugs, while the screening method assayed the presence of 9 drugs (amphetamines, barbiturates, benzodiazepine, THC, benzoylecgonine, Methadone, opiates, oxycodone and phencyclidine). Urine specimens were directly used for immunoassay methods. However, the specimens for the confirmatory method were first prepared in a process that included hydrolysis using beta-glucuronidases and dilution and then injected to the machine. The concentration of drugs in urine specimens with a pH of 6.8 was used as the base line and other specimens were compared to these specimens. Alterations greater or lesser than 20% was defined as significant changes in drugs concentration. The drugs concentrations were selected at the cutoff concentrations. **Results:** Concentration of drugs measured by screening immunoassay method did not significantly change when specimen's pH adjusted between 4.5 or 9, reference range for urine pH. In addition, no changes in the concentrations of drugs were detected when specimen's pH were adjusted in the SAMHSA defined pH for invalid result (4-4.5 or 9-11). In specimens with pH ≤ 2, concentrations of benzodiazepines, opiates and phencyclidine were significantly reduced using immunoassay method. The specimens that had a pH ≥12 demonstrated alteration in concentration of benzoylecgonine, THC, methadone, and phencyclidine, when measured by immunoassay method. Amphetamine, barbiturate and oxycodone concentrations did not change in any of tested pH in the immunoassay method. No significant changes in the concentrations of the 88 drugs in the tested pH were detected in the confirmatory liquid chromatography mass spectrometry method except for cocaine, 6 monodactyl morphine, flunitrazepam, methylphenidate and buspirone, which showed a reduction in pH ≥12. **Conclusion:** In contrast to screening immunoassay methods of urine drug testing, the urinary concentration of drugs tested with confirmatory liquid chromatography mass spectrometry method are not altered by changes in pH except for cocaine, 6 monodactyl morphine, flunitrazepam, methylphenidate and buspirone.

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Quantitative analysis of organic acids in plasma using acidified methanol extraction and detection by gas chromatography-mass spectrometry

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Background and objectives: Plasma contains a variety of compounds, and represents a challenging matrix for organic acid analysis. We developed and validated a method based on liquid-liquid extraction using acidified methanol and detection by gas chromatography mass spectrometry. This method allows for the analysis of ten organic acids: lactic, pyruvic, succinic, 3-hydroxybutyric, acetoacetic, 2-keto-3-methylvaleric, 2-ketoisocaproic, 2-ketoisovaleric, glutaric, and citric. **Methodology:** Organic acids are extracted by deproteinizing with acidified methanol, oximated to preserve otherwise unstable short chain ketoacids, and converted to volatile trimethylsilyl (TMS) derivatives before analysis by gas chromatography-mass spectrometry (GC-MS). Compound identification is obtained by retention time and characteristic fragmentation spectra using Agilent MassHunter software. Organic acids are quantified using a six-point calibration curve with 2-ketocaproic acid as internal standard. **Results:** Analytes are linear within the following ranges: lactic acid = 400-6000 uM, pyruvic acid = 10-1000 uM, succinic acid = 10-500 uM, 3-hydroxybutyric acid = 50-1500 uM, acetoacetic acid = 50-1000 uM, 2-keto-3-methylvaleric acid = 5-500 uM, 2-ketoisocaproic acid = 5-500 uM, 2-ketoisovaleric acid = 10-500 uM, glutaric acid = 5-300 uM, and citric acid = 30-800 uM. Intra-assay variability (n=6 in 2 experiments) was <15% for most analytes, and inter-assay variability was <30% (n=3). Recovery in matrix was assessed by testing plasma of 10 healthy donors before and after spiking with a mixture of standards. Recovery of spiked analyte was calculated after accounting for donor endogenous analytes. Recovery was 77% or greater for most analytes. Measurement of lactic, pyruvic and 3-hydroxybutyric acids by GC-MS were comparable to alternative, single-analyte methods (n=10, slopes = 0.913-1.062, R^2 = ≥0.9446). **Conclusion:** We have developed a robust analysis of ten organic acids in plasma. Although this test is not recommended as a routine test for inherited disorders of metabolism, it is utilized in addition to other biochemical genetics testing in some patients.

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Investigating the interferences of lidocaine and its primary metabolites for cocaine metabolites using liquid chromatography mass spectrometry

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Background: Cocaine is a commonly abused drug in the United States that can be also prescribed as a local anesthetic to block nerve impulses. Lidocaine is a locally applied anesthetic in the treatment of arrhythmias. There is a growing concern in the scientific community about the impediment caused by lidocaine in detec-

tion of cocaine in patient populations. The common method for detection of drugs and their metabolites in urine samples are urine drug screen (USD) immunoassays. USD immunoassays are quick and inexpensive; however, they are prone to false-positive results that could affect individual's life, employment and legal citations. In this study, we aim to determine if lidocaine exposure during routine medical procedures can interfere with cocaine or its metabolites, particularly benzoylecgonine. **Methods:** We developed a LC-MS assay to measure lidocaine primary metabolite, two metabolites of cocaine and two metabolites of a common cocaine adulterant. We applied this method to analyze urine from 300 volunteer patients prescribed for lidocaine. The LC-MS assay was developed on a Waters TQD UPLC and detector using a CN column. The mobile phase's compositions were 2mM Ammonium acetate and 0.1% formic acid in either water or methanol. Data was analyzed using Waters MassLynx software. **Results:** Initial results of the first 25 samples show no cross-reaction between lidocaine and any of cocaine metabolites. The assay was linear from 10-2000 ng/mL for all the compounds tested. The total assay precision was less than 15% for all the analytes. The LoD, LoQ and LoB concentrations were 2, 5 and 0.5ng/mL, respectively. The assay is in progress for the rest of our patient samples. **Conclusion:** We developed this assay to help to support or refute the claim that lidocaine and its primary metabolites (nor-lidocaine) cause false-positive reactions with cocaine and its metabolites. This assay should be useful in evaluating immunoassay drug screens for false positivity due to lidocaine metabolites.

B-245

Quantitative Analysis of Iohexol and Iothalamate in Urine and Plasma using LCMS for clinical research use

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

Background: Iothalamate and Iohexol are triiodinated derivatives of benzoic acid that are used to assess renal function particularly the kidneys glomerular filtration aspect. The compounds are cleared from the body very rapidly with a clearance rate of 8-11 hours. A simple, sensitive and specific LC/MS/MS analytical method was developed for the quantitation of Iothalamate and Iohexol which are light sensitive using protein crash in plasma and a dilute and shot in urine sample preparation techniques. This easy method achieved good analyte recovery, post-extraction cleanliness and is capable of the sensitivities to quantitate the iohexol and iothalamate in urine and plasma over their dynamic range in bot matrices despite some challenging issues. Method: A Thermo Fisher TSQ Quantis tandem mass spectrometer in positive Electrospray mode and a Thermo Fisher Vanquish Horizon HPLC system were utilized. 200 ul of urine and plasma were used for the analysis of these compounds. Various columns were evaluated and a Thermo Fisher Accucore C18 50 x 2.1 mm, 2.6 um with a water:acetonitrile mixture containing 0.1% formic acid achieved baseline chromatographic separation in less than 6 minute run time for all compounds. Quantitative analysis was performed using scheduled reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive mode and accuracy of the method was verified using quality control materials and serum samples. Results: Good linearity and reproducibility were obtained with the concentration range from 0.01 to 500 ug/ml for the iothalamate and iohexol with a coefficient of determination >0.995 for both sample preparation. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range initially from 0.01 and 0.05 ug/ml. Excellent reproducibility was observed for both compounds (CV < 10%) and all techniques and configurations. Conclusion: A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry method was developed and verified for the simultaneous measurement of iothalamate and iohexol in urine and plasma using ioversol as an internal standard. The sample preparation techniques are quick and easily applied for high throughput analysis and included protein precipitation in plasma and urine dilution but improvements are being investigated and the method demonstrates that it is appropriate for GFR determinations and can be used to measure GFR in renal transplant populations.

B-246

Development and Implementation of One-Step, Broad-Spectrum, High-Sensitivity Drug Screening by Tandem Mass Spectrometry in a Pediatric Population

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Background: Urine drug screening by immunoassay is common in pediatric clinical settings. Cross-reactivity, limited scope, and high limits of detection lead to high rates of false positive and negative results. False-positive results are discoverable using higher level analytic techniques such as mass spectrometry. False-negative results last forever. Errors in drug screening, particularly in newborns, have far-reaching medico-legal implications. To circumvent these problems, our laboratory has developed and implemented an LC-MS/MS technique designed to replace immunoassays in urine drug screening. Our objective was to describe the impact of this technique at a large, urban, academic, pediatric teaching hospital and compare it retrospectively to results obtained using our previous immunoassay first-confirmatory approach. **Methods:** Immunoassay screens were performed using a Cobas 6000 system and confirmed by mass spectrometry at a reference laboratory. LC-MS/MS profiles were performed using a Waters Acquity UPLC system equipped with TQ Detector. Urine specimens were diluted with acetonitrile containing 5 internal deuterated drug standards prior to injection. Drug identification was based on detection of precursor ion at an appropriate retention time and the yield of two fragment ions in a ratio characteristic of standard drug. We conducted a retrospective analysis of 4258 pediatric drug screens performed a year before and after the implementation of LC-MS/MS. We reviewed the medical record from the patients of all 1139 samples that tested positive during the two-year surveillance period. We extracted presumptive positive immunoassay results and subsequent confirmatory data and tabulated the identities of all compounds identified by screen-confirm algorithm or the one-step LC-MS/MS approach. **Results:** Prior to LC-MS/MS, 1272 drug screens were performed by the immunoassay in the general pediatric population. Twenty-one percent of these urine specimens were presumptively positive. Of these, 86% compounds were confirmed by MS making the false positive rate 14%. The 3 most prevalent drugs confirmed were THC (44.3%), morphine (18.2%) and amphetamine (18.2%). After implementation of the one step LC-MS/MS approach, 2322 drug screens were performed. 676 (29%) were positive and 28 different compounds were detected. The 5 most prevalent were THC (14.4%), amphetamine (11.5%), fentanyl (7.6 %), benzoylecgonine (7.5%), and morphine (7.4%). In the nursery population, 394 drug screens were performed by immunoassay and 144 (37%) were presumptively positive. Of these presumptive results only 40% were confirmed by MS. The false positive rate of 60% was largely due to high rates of false positive THC detection. Morphine (32.9%), methadone (26.3%) and benzoylecgonine (10.5%) were the most commonly confirmed compounds in this patient cohort. After implementation of LC-MS/MS only testing, 270 drug screens were performed and 48 (18%) were positive. The five most prevalent compounds detected were benzoylecgonine (17.0%), morphine (14.8%), methadone (13.6 %), EDDP (7.5%), and oxycodone (9.1%). **Conclusion:** Primary drug screening using LC-MS/MS increased detection of a broader spectrum of compounds in our pediatric population. This approach has proven to be a reliable substitute for immunoassay-based drug screening as it offers superior specificity and sensitivity while obviating undesired confirmation cost and delay. Moreover, the LC-MS/MS provides a dynamic platform adaptable to changes in local patterns of drug supply and abuse.

B-247

Development of an LC-MS/MS method for creatinine measurement in icteric subjects

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Background
Routine serum creatinine measurement is performed using automated chemistry analyzers that utilize either the Jaffé alkaline picrate or an enzymatic creatininase mechanism. Both of these methodologies are sensitive to bilirubin interference. In this study, we developed a sensitive and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantitative determination of creatinine in human serum samples that is insensitive to bilirubin interference.
Methods
Creatinine and its internal standard were analyzed using a Waters UPLC system (Milford, MA) coupled to an AB Sciex QTRAP 5500 mass spectrometer (Washington,

D.C.) in multiple reaction monitoring (MRM) mode. Chromatographic separation was performed using a Phenomenex normal phase column (Torrance, CA). The mobile phases consisted of 10 mM ammonium formate and 1.0% formic acid in water (phase A), and 1.0% formic acid in acetonitrile (phase B). Mobile phase B (90%) was ramped up to achieve 50% B over 2.0 minutes where it was maintained, followed by 1.30 minutes of re-equilibration in mobile phase A. Creatinine and its internal standard were detected by positive electrospray ionization with the following transitions: creatinine m/z 114→44 and internal standard m/z 117→47. Calibrators were created by spiking phosphate buffered saline with 1% bovine serum albumin (PBS w/ 1% BSA) with creatinine concentrations ranging from 0.05 to 5.0 mg/dL. Human serum samples were combined with labeled internal standard (creatinine-d3), and extracted using protein precipitation and dilution in a 96-well plate format. Method comparison studies were conducted on the Roche cobas 8000 (Indianapolis, IN) using the Roche Creatinine Plus Ver. 2 and Jaffé Gen. 2 reagent systems. Quality control (QC) samples were assayed on each day prior to testing. Bilirubin was evaluated indirectly by icterus measurement on the cobas 8000. Data reduction was performed using the Alternate Method Comparison Module on Data Innovations EP Evaluator® Version 9.4.0 software (South Burlington, VT).
Results
The LC-MS/MS method for creatinine measurement was linear from 0.05 to 5.0 mg/dL. Intra-day and inter-day precision was <3.3% and <10.8%, respectively. Accuracy by spike-and-recovery yielded recoveries from 88-100% for sixteen samples spanning the analytical measurement range. Specimen dilution was verified up to eight-fold. The reference interval was verified at 0.60-1.35 mg/dL for males, and 0.50-1.10 mg/dL for females. Stability was established for up to 7 days at ambient (20-25° C and refrigerated temperatures (2-8° C). Freeze-thaw stability was established for 5 cycles at both -70°C and -20°C. Specimen stability was verified up to 10 months at both -70°C and -20°C. No interference was observed for bilirubin concentrations exceeding 100 mg/dL. Creatinine measurement by LC-MS/MS demonstrated acceptable correlation to the automated enzymatic methodology (positive 1.2% average bias), but unacceptable correlation to the Jaffé methodology (positive 7.0% average bias). Both the Jaffé and enzymatic methodologies were more sensitive to bilirubin interference compared to LC-MS/MS.
Conclusions
LC-MS/MS provides an excellent alternative for the measurement of serum creatinine compared to routine automated methodologies that is insensitive to interference exceeding 100 mg/dL bilirubin. This methodology provides accurate creatinine determination in the context of hepatitis, HIV, and liver failure care patients.

B-248

Validation of Prostate Cancer Biomarkers and Inflammation: A Proteomics Study

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Background: In this study serum protein profiles were analyzed in order to investigate possible confounding parameters in the discrimination between prostate cancer (PCa) and benign prostatic hyperplasia (BPH).
Methods: Patients with clinical suspect of PCa and candidates for trans-rectal ultrasound guided prostate biopsy (TRUS) were enrolled. Histological specimens were examined in order to identify PCa, BPH and detect inflammation. Surface Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (SELDI-ToF-MS) and two-dimensional gel electrophoresis (2-DE) coupled with Liquid Chromatography-MS/MS (LC-MS/MS) were used to analyze immuno-depleted serum samples from patients with PCa and BPH.
Results: The comparison between PCa (in the presence or absence of inflammation) and BPH (also in the presence or absence of inflammation) serum samples performed by SELDI-ToF-MS analysis, did not show differences in protein profiles. Differences became evident when the presence of inflammation was taken into consideration. When samples with histological sign of inflammation were excluded, 20 significantly different protein peaks were detected. Subsequent comparisons (PCa with inflammation vs PCa without inflammation, and BPH with inflammation vs BPH without inflammation) showed that 16 proteins appeared to be differently expressed in the presence of inflammation, while 4 protein peaks were not modified. With 2-DE analysis, comparing PCa without inflammation vs PCa with inflammation, and BPH without inflammation vs the same condition in the presence of inflammation, were identified 29 and 25 differentially expressed protein spots, respectively. Excluding samples with inflammation the comparison between PCa vs BPH showed 9 unique PCa proteins, 4 of which overlapped with those previously identified in the presence of inflammation, while other 2 were proteins, not identified in the previous comparisons.
Conclusions: This study indicates that inflammation might be a confounding parameter during the search of candidate proteomic biomarkers of PCa. The results indicate that inflammation represents a significant confounding factor, hence, only a well-selected protein pattern should be considered as a potential biomarker of PCa.

B-249

A Validated UPLC-MS/MS Method for Therapeutic Drug Monitoring of Sorafenib in Patients with Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) represents a global health problem and the incidence of this cancer in patient with cirrhosis is still increasing in several countries. There was no effective treatment available for patients diagnosed at advanced stage or who progressed into an advanced stage after other treatments failed. Sorafenib, an oral multikinase inhibitor with a potent antiangiogenic and proapoptotic activity, was approved with survival benefit. The concentration of sorafenib in the blood circulation is influenced by various physiological and pathophysiological effects in individual patients. A given dose of sorafenib can result in different plasma concentrations which may lead to sub-therapeutic drug exposure or increase adverse drug reactions at excessive plasma concentrations. The purpose for this study was to develop a method to quantitate sorafenib in plasma by using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).
Methods: The calibration curve samples were prepared by spiking drug free plasma with sorafenib tosylate. The stable isotope labeled sorafenib was used as an internal standard. After addition of the internal standard and protein precipitation, the supernatant was 10-fold diluted and injected into a chromatography system consisting of a UPLC BEH C18 (2.1 × 50 mm; particle size 1.7 μm) analytical column with gradient made of mobile phase (5 mM ammonium formate pH3.5 in water and acetonitrile containing 0.1% formic acid). The outlet of the column was connected to a triple quadrupole mass spectrometer with electrospray interface. Ions were detected in the positive multiple reaction monitoring mode. The concentration of analyte was calculated from the calibration curve and ion ratios between the analyte and the internal standard.
Results: The analytical range was linear with a correlation coefficient of over 0.99 in the range of 19.5 - 10279.5 ng/mL. The imprecision for within-run was less than 1.3 % and between-run was less than 3.6 %. The accuracy was evaluated by spike recovery and the mean recovery was 101%-104%. This assay showed no ion suppression or enhancement and no carryover. The chromatography run time was 4.5 min. The assay was applied to quantitate sorafenib in plasma samples from 82 advanced HCC patients administrated different dose of sorafenib. Our data showed that the sorafenib concentrations vary markedly individual patients after equal dose, this therapeutic drug monitoring of sorafenib is essential to optimized sorafenib efficacy.
Conclusion: A fast and accurate UPLC-MS/MS method to quantitated sorafenib was developed and successfully applied for routine therapeutic drug monitoring purposes in patients treated with sorafenib.

B-250

A Novel Derivatization-Based LC-MS/MS Method with High Sensitivity for Quantitation of Cannabinoids in Breath Samples

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Background:
Nowadays cannabis use monitoring is in urgent demand for both public health and safety concerns. To facilitate the clinical study of a prototype breathalyzer for real-time cannabis use monitoring, a high-sensitivity LC-MS/MS method was needed to detect low-level cannabinoids in breath samples. We report a novel derivatization-based LC-MS/MS method with high sensitivity (pg/ml level) for quantitation of cannabinoids. The derivatization is based on azotization reaction which is compatible with water, methanol, and acetonitrile. It is not required to dry down samples before carrying out analyte derivatization. The assay has been applied to breath samples and the serum samples obtained from test subjects in a clinical study.
Methods:
A stock solution containing 5 cannabinoids THC, THCCOOH, 11-OH-THC, CBN, CBD was diluted in methanol or drug-free serum to prepare a calibrator series from 1000 pg/ml to 0.10 pg/ml. An IS (internal standard) solution was prepared in methanol. Derivatization assay (breath or serum): 100 μl of breath sample or supernatant of protein-precipitated serum sample was mixed with 2.5 μl IS solution. To derivatize

analytes, 20 µl of a diazonium solution was added and the sample was incubated at room temperature for 0.5 hr.
Non-derivatization assay (breath): 50 µl of each breath sample was mixed with 70 µl diluent with IS. Gradient elution was employed in HPLC separation. Quantitative analysis was carried out using MRM in triple-quadrupole mass spectrometer.
Results:
Derivatization assay for breath samples:
Imprecision was determined by running replicates of QC samples. Accuracy was determined by trueness of average results of QC samples. For all analytes, accuracy: 90.9% ~ 112.2% at QC L1 (5.0 pg/ml), 94.5% ~ 108.3% at QC L2 (50 pg/ml), 95.3% ~ 105.6% at QC L3 (200 pg/ml); imprecision (CV): 3.5% ~ 9.9% at QC L1, 1.0% ~ 4.6% at QC L2, 1.5% ~ 7.7% at QC L3. Linear range is from LOQ to 1000 pg/ml for all analytes. LOQ: THC 0.50 pg/ml (CV 5.8%, Accuracy 96.0%); THCCOOH 1.0 pg/ml (CV 7.5%, Accuracy 84.5%); 11-OH-THC 2.5 pg/ml (CV 15.1%, Accuracy 106.1%); CBN 0.10 pg/ml (CV 8.1%, Accuracy 103.6%); CBD 1.0 pg/ml (CV 13.5%, Accuracy 116.3%).
Derivatization assay for serum samples:
The derivatization method was applied to serum samples to demonstrate its broad applicability. Linear range is from LOQ to 1000 pg/ml. LOQ: THC 10 pg/ml (CV 9.7%, Accuracy 103.3%); 11-OH-THC 10 pg/ml (CV 8.4%, Accuracy 113.3%); CBN 2.5 pg/ml (CV 11.7%, Accuracy 114.4%).
The derivatization assay is compared with the non-derivatization assay: THC level in 33 breath samples was analyzed using both assays, and excellent correlation was observed (Slope 1.12; R^2 0.997).
In a clinical study, 9 test subjects were recruited, and timed collection of breath samples was implemented. Maximum THC level appeared at around 15 min after marijuana administration in all test subjects.
Conclusion:
The derivatization-based LC-MS/MS method has been proved to significantly enhance LC-MS/MS assay sensitivity. In a pilot clinical study of a breathalyzer-type point-of-care device, the derivatization method was applied to analyze breath samples which contain cannabinoids at pg/ml level. The derivatization method was also successfully applied to serum samples.

B-251

Water Soluble Vitamins, metabolites and derivatives determination by LC-MS in Blood for clinical 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. A highly sensitive and specific LC/MS/MS analytical method has been developed for the quantitation of the water soluble vitamins, metabolites and derivatives in blood and include- vitamin B1 (thiamine, thiamine pyrophosphate), vitamin B2 (riboflavin), vitamin B3 (nicotinic acid and nicotinamide), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxal 5-phosphate, pyridoxine, pyridoxal), vitamin B7 (biotin), folic acid, 5-methyltetrahydrofolate and vitamin B12 (cyanocobalamin). A simple sample preparation technique that involved a simple protein crash and liquid-liquid extraction were utilized along with a one (1D) dimensional liquid chromatographic configuration. The described method achieves the required sensitivity and is capable of determining the water soluble vitamins, derivatives and metabolites over their dynamic range. Therefore, a simple and accurate quantitative analytical method was developed for the quantitatively measurement of water soluble vitamins, derivatives and metabolites in blood.
Method: A Thermo Scientific™ Quantis™ tandem mass spectrometer in positive Electrospray mode and a Thermo Scientific™ Dionex™ Vanquish™ Horizon HPLC system were initially utilized for this analysis. 200 µl of blood were used for the analysis of the water soluble vitamins, derivatives and metabolites in blood. Various columns were evaluated and an Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 1.5 µm with a water:methanol mixture containing 5 mM Ammonium Formate and 0.1% Formic Acid achieved baseline chromatographic separation for all the water soluble vitamins, derivatives and metabolites in serum 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 mode and the precision and accuracy of the method was verified using pooled quality control materials and serum samples.
Result: Good linearity and reproducibility were obtained with the concentration range of 1 to 50000 ng/ml for the respective water soluble vitamins, derivatives and metabolites in blood 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 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 of water soluble vitamins, derivatives and metabolites in serum. The sample preparation technique is quick and easily applied for high throughput analysis.

B-252

Hydroxytyrosol stability in urine and synthetic urine matrices

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Background: The Primary Prevention of Cardiovascular Disease with a Mediterranean Diet (PREDIMED) study found a significant decrease in cardiovascular disease in a high risk population with dietary supplementation of either extra virgin olive oil (EVOO) or mixed nuts compared to a control group. EVOO contains elevated levels of phenolic antioxidants. Specifically, oleuropein and one of its metabolites, hydroxytyrosol, are of increasing scientific interest because they are thought to have antioxidant and anti-inflammatory benefits. In order to monitor compliance of EVOO intake, the levels of hydroxytyrosol have been monitored in urine. Unfortunately, studies have found that hydroxytyrosol has poor stability under certain conditions, such as in the presence of multivalent cations. We monitored the stability of hydroxytyrosol in urine and synthetic urine for four weeks to determine optimal storage conditions. We also employed an internal standard, D3-hydroxytyrosol, synthesized by the University of Minnesota's Institute for Therapeutics Discovery and Development.
Methods: Hydroxytyrosol was spiked into both urine and synthetic urine at concentrations of 20 ng/mL, 150 ng/mL, and 350 ng/mL and aliquoted into microcentrifuge tubes. The urine was pH 5.0, and synthetic urine was pH 7.5. Specimens were stored either native or spiked with acetic acid to a final concentration of 0.15M. Storage conditions ranged from room temperature, 4°C, -20°C, or -80°C for four weeks, and samples were tested on day 0, 1, 2, 7, 14, and 28. Specimens were processed by dilution with 150 µL of diluent (1M acetic acid, pH 4.5, with 10 mM oxalic acid) and addition of internal standard, D3-hydroxytyrosol. LC-MS/MS (QTRAP 6500, AB SCIEX) was used for analysis with a C18 Kinetex column (Phenomenex) and water/methanol as mobile phases.
Results: Recoveries observed for hydroxytyrosol in synthetic urine were the following: 0-30% for room temperature on Day 2, 6-25% for refrigerated at 4°C on Day 14, and 20-73% for frozen at -20 °C at Day 28. Only the synthetic urine samples stored at -80°C were found to have recoveries of 94-99% at Day 28. However, with the addition of 0.15M acetic acid, hydroxytyrosol was stabilized in all temperatures examined in this study with recoveries ranging from 94-106%. Hydroxytyrosol was stable in the urine specimen at 4°C, -20°C, or -80°C, regardless of whether acetic acid was present or not (recoveries of 89-108%). The addition of the isotopically labeled internal standard assisted in normalization of hydroxytyrosol degradation during processing of the urine/synthetic urine specimens.
Conclusion: Our data suggests hydroxytyrosol has better stability at lower pH. Degradation can be mitigated by either storing the specimens at -80°C or by the addition of acetic acid as a urine preservative. Additionally, we have improved our method by incorporating a deuterium labeled internal standard of hydroxytyrosol during preparation of specimens.

B-253

An LC-MS/MS assay with online extraction for measurement of testosterone in serum or plasma

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Background: Measurement of testosterone in circulation is important for evaluation of androgen status in men, women, and children. The very low concentrations of testosterone in children, females, and males with androgen suppression therapies necessitate the use of mass spectrometry (MS) based methods. We aimed to develop a simple and cost-effective LC-MS/MS assay for the quantification of testosterone at very low concentrations in serum or plasma.
Methods: This method was developed on a QTRAP 5500 LC-MS/MS system (AB SCIEX, Framingham, MA) coupled to a Shimadzu LC 30A HPLC. Serum or plasma sample (200 µL) was mixed with 400 µL acetonitrile containing $^{13}C_3$-testosterone as internal standard. The resulting mixture was spun at 4000 xg for 10 min. The supernatant (50µL) was injected into a C18 trap column in-line with the LC-MS/MS system. Statistics were calculated using Excel (Microsoft, Redmond WA, USA) and EP Evaluator Release 10 (Data Innovations, South Burlington, VT, USA).
Results: The linearity of the assay was assessed by serial dilution and found to be 10 to 10,000 pg/mL with analytical recovery from 82 to 87%. Precision was evaluated based on EP10-A3 protocol. For spiked plasma samples (N=30) with mean concentrations of 145 pg/mL, 4,554 pg/mL, and 9,032 pg/mL, the within run coefficients of variation (CV) were 2.8%, 3.5%, and 3.7%, respectively and the total CV was 4.1%, 3.7%, and 4.0%, respectively. No significant carryover was observed from samples

with concentrations up to 26,748 pg/mL. No significant interference was observed from androstenedione (5,000 pg/mL), dehydroepiandrosterone (80 ng/mL), epi-testosterone (10,000 pg/mL), and estriol (28 ng/mL). To assess the accuracy of the assay, we analyzed 40 patient samples offered by CDC Hormone Standardization Program (HoSt) Phase 1. These samples had assigned value ranging from 72.7 to 7,460 pg/mL. Deming regression of the results by this method and the assigned values showed a slope of 1.061, an intercept of -35.63 pg/mL and an R of 0.998, with a mean bias of 0.08 %. **Conclusion:** We have developed an accurate LC-MS/MS method for measuring serum/plasma testosterone with online sample extraction. This assay has been fully validated for clinical use.

B-254

De Novo Amino Acid Sequencing of M-proteins by 21 Tesla FT-ICR MS Using Top-Down and Middle-Down MS/MS Techniques

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Background: The plasma cell disorders include multiple myeloma, AL amyloidosis, monoclonal gammopathy of undetermined significance, POEMS syndrome, and Waldenstrom's macroglobulinemia, which are characterized by a plasma cell clonal expansion. If there is clinical suspicion of one of these disorders, serum is tested for the presence of elevated levels of a monoclonal immunoglobulin (M-protein) secreted by clonal plasma cells. In contrast to typical cloning experiments, which are expensive, invasive and laborious, we report a non-invasive method for sequencing M-proteins from the blood which will enhance our ability to type the plasma cell disorders. **Methods:** Immunoglobulins were purified from patient serum with Melon Gel or camelid-derived nanobodies and digested with IdeS (FabRICATOR; Genovis). Disulfide bonds were then reduced with TCEP to produce antibody light, heavy Fd, and heavy Fc subunits (~25 kDa each), which were analyzed by reversed-phase LC-MS/MS. Mass spectra were acquired with our custom-built 21 T FT-ICR mass spectrometer, and MS/MS was performed with either electron transfer dissociation or collision-induced dissociation. Data were manually interpreted with Xcalibur 2.1 software (Thermo). Antibody isotype (constant region sequence) was determined by Xtract deconvolution, and fragments matched to the putative sequence by use of ProSight Lite (10 ppm fragment mass tolerance). Antibody variable region sequence characterization was performed by an in-house program, "AminoAcid Finder". **Results:** Nano-LC 21 T FT-ICR MS/MS was applied to analyze the M-proteins from AL amyloidosis patients sera (our group was blind to the M-proteins gene sequencing results). Based on our in-house developed top/middle-down *de novo* sequencing software, the M-protein light chain FR2-CDR2-FR3 was comprehensively characterized by MS/MS, and the assigned residues matched 100% to the corresponding gene sequence. The light chains were assigned to kappa KV1-33 germline sequence with seven amino acid mutations for the first sample and lambda LV3-21 germline sequence with five amino acid mutations for the second sample. The KV1-33 germline sequence is the most common kappa germline sequence identified in AL amyloidosis and is more likely to be associated with liver involvement, whereas the LV3-21 germline sequence is less commonly involved in AL renal patients. In another multiple myeloma sample, two M-protein light chains were detected with mass difference ~198 Da. MS/MS sequencing revealed that the two light chains belong to different germline sequences: kappa IGKV3-11 and kappa IGKV1-16. This example shows that our approach can simultaneously characterize more than one M-protein light chain, which is correlated with potential disease progression (e.g., malignant plasma cell mutation). Also, M-protein heavy chain glycoforms from multiple myeloma samples are well-known to differ from the glycoform profile in healthy human samples. **Conclusions:** We describe the first top/middle-down *de novo* sequencing of M-protein in serum with the advantages of ultrahigh mass accuracy and extensive sequence coverage. The results shown here serve as a blueprint for future characterization of endogenous M-protein in patients with a variety of immune system disorders. Work supported by the National Science Foundation through DMR-1157490 and DMR-1644779, and the State of Florida.

B-255

Assessment of Mass Spectrometry Teaching in Pathology Residency

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Background:
Mass spectrometry (MS) has seen growing adoption in routine clinical laboratory testing in recent years. Indirect oversight of this testing is largely performed by medical directors boarded in anatomic and/or clinical pathology (AP/CP or CP), who may have limited familiarity with these instruments in the clinical setting. In an effort to train the next generation of pathologists to have adequate insight into this methodology, we conducted a needs assessment to determine the current availability and interest in mass spectrometry teaching in pathology residency from both program directors and residents. The findings of this study will be used to design curriculum to provide appropriate MS training for pathology residents.
Methods:
Two separate surveys for program directors and pathology residents were constructed and managed using REDCap electronic data tools hosted at University of California, San Francisco. The questions for program directors were designed to assess current and idealized teaching format. The questions for residents were designed to assess interest in MS training, perceived utility of MS in pathology practice, and idealized learning format. Surveys were sent to clinical chemistry program directors nationwide and pathology residents from the University of California, San Francisco.
Results:
Of the program director respondents (n=12), 66.7% currently have mass spectrometry teaching incorporated into resident teaching. The topics they felt residents should have the most exposure to include: principles of mass spectrometry (100.0%), clinical method validation (91.7%), and endocrine testing (75.0%). The perceived least important topics for resident teaching were proteomics (33.3%), metabolomics (25.0%), and mass spectrometry tissue imaging (25.0%). Of the resident respondents (n=21), 81.0% felt MS teaching would 'very important' to the future practice of clinical pathology. Additionally, 81.0% felt that it would be at least 'somewhat important' to the future practice of anatomic pathology. When asked if they felt MS would be important to their personal future practice of pathology, 52.7% noted it would be 'somewhat important', 23.8% thought it would be 'very important', 12.5% had no opinion, and 9.5% did not think it would be important. Residents voted that the most important topics to have exposure to were: principles of mass spectrometry (85.7%), microbial identification (61.9%), and toxicology (47.6%). The topics perceived least important were metabolomics (4.8%), endocrine testing (4.8%), and inborn errors of metabolism testing (0.0%). Both groups surveyed were asked how they would prefer MS curriculum to be taught. The majority of both groups (≥80%) agreed the topics should be didactic (rather than bench or elective research) and residents preferred dedicated mass spectrometry methodology lectures over incorporation into clinical topics.
Conclusion:
Of those surveyed, a majority of pathology programs have MS teaching in their residency, largely focusing on LC-MS/MS and clinical method validation. Furthermore, a large majority of pathology residents felt MS would be at least somewhat important to their future practice of pathology, if not very important. Both groups agreed teaching should be lecture-based, with residents preferring an emphasis on methodology over incorporation into clinical topics.

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Association of Plasma Metabolites with Brain MRI Measures in the Atherosclerosis Risk in Communities-Neurocognitive Study (ARIC-NCS)

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Background: Cerebral small vessel disease (SVD) contributes to dementia, cognitive and physical function decline. Our previous studies measured plasma metabolites by a targeted metabolomics method and established cross-sectional relations between higher levels of six plasma metabolites (two plasma phosphatidylcholines [PCs]: PC aa C36:5 and PC aa 36:6 and four sphingomyelins [SMs]: SM C26:0, SM[OH] C22:1, SM [OH] C22:2, SM [OH] C24:1) and favorable cognitive or physical function. The primary objective of this study was to conduct a hypothesis-driven analysis to investigate the relation of these 6 plasma metabolites with magnetic resonance imaging (MRI) features of cerebral SVD (small subcortical infarcts and white matter hyperintensities [WMH]) and brain atrophy. Both brain pathologies are common causes of adverse cognitive and physical function. Additionally, we explored the relation between 131 additional plasma metabolites with WMH or brain atrophy. **Methods:** This study included 238 older adults participating in the Atherosclerosis Risk in Communities-Neurocognitive Study (2011-13, mean age [standard deviation (SD)]: 77.5 [5.6], 56.7% women, 21.9% African Americans). Individual plasma metabolite concentrations were log-transformed and modeled in SD units. Multivariable

linear regression was used to assess the association of each metabolite in separate models with neuroimaging measures except lacunar infarcts; for lacunar infarcts, binary logistic regression was used. All models performed accounted for demographics, *APOE* genotype, cardiovascular risk factors, comorbidities, and use of medications. **Results:** We found that higher concentrations of plasma PC aa C36:5 and SM C26:0 had adverse associations with MRI features of cerebral SVD (odds ratio of 1.69 [95% confidence interval (CI): 1.01, 2.83] with lacunar infarct, and β of 0.16 mm^3 [0.02, 0.30] with log[WMH] volume) and total brain volume (β of -0.41 mm^3 [-0.75, -0.08] with deep grey white cortical volume), respectively; higher levels of 3 plasma SM (OH)s were associated with favorable features of cerebral SVD: β of -0.21 mm^3 [-0.33, -0.09] between SM (OH) C24:1 and log[WMH] volume, and β of 12.0 mm^3 [5.5, 18.6], 11.8 mm^3 [5.0, 18.6], 7.3 mm^3 [1.2, 13.5] between SM [OH] C22:1, SM [OH] C22:2, and SM [OH] C24:1 with total brain volume, respectively. In exploratory analyses, after Bonferroni correction ($p < 0.00038$), we found that while plasma metabolites such as plasma PC aa C38:6 and PC aa C40:6 had adverse associations with MRI features of cerebral SVD (odds ratio of 2.98 [1.68, 5.32] and 2.75 [1.65, 4.58] with lacunar infarct, respectively), other metabolites such as arginine, glutamine, and 3 plasmalogens (PC ae C40:4, PC ae C42:4, and PC ae C42:5) had favorable associations (β [95% CI] of -0.20 mm^3 [-0.30, -0.11], -0.22 mm^3 [-0.32, -0.11], -0.25 mm^3 [-0.38, -0.12], -0.29 mm^3 [-0.42, -0.16], and -0.25 mm^3 [-0.39, -0.12] with log[WMH] volume, respectively). **Conclusion:** Higher concentrations of several circulating metabolites (SM [OH]s) previously associated with neurocognitive and physical function endpoints independently correlate with more favorable measures of brain structure and neuroimaging abnormalities of SVDs.

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Development and validation of cotinine in human urine by liquid chromatography electrospray ionization tandem mass spectrometry.

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Tobacco smoke is a widely recognized problem of public health due the risk factors leading to diseases as cancers and death worldwide. Determination of cotinine in urine is a good indicative of tobacco exposure. A simple, rapid and sensitive Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) was developed and validated for quantification of cotinine in human urine. In this process, 100 µL of urine were spiked with 25 µL of internal standard cotinine-d_3 and was treated with 200 µL of zinc sulfate 0.1M. Chromatographic separation was obtained using a UPLC System 1290 Infinity Agilent equipped with a Zorbax Eclipse Plus C18 RRHD column (2,1 X 50 mm 1,8 um) maintained at 35°C. Isocratic mobile phase consisting of methanol:water (98:2, v/v) with 0,1% of formic acid and 5 mM of ammonium formate at a flow rate of 300 µL.min[1]. The chromatographic run time obtained was 2.5 minutes. MS/MS detection was conducted a 6460 MS system (Agilent Technologies) by monitoring the fragmentation ions of 177→80 (*m/z*) and 177→98 (*m/z*) for cotinine and 180→80 (*m/z*) for cotinine-d_3. The linear range obtained for cotinine was 10.0-800.0 $ng.mL^{-1}$ and dilution was validated for samples that exceed the curve in 4 times. Limit of detection (LOD) was 0.6 $ng.mL^{-1}$ for cotinine. Within-day imprecision was less than 5.4% and between-day imprecision was less than 6.2 %. The medium range of recovery was between 94-109%. Cotinine was determinate with satisfactory sensitivity, precision, recovery and linearity. In conclusion, the method developed and validated has a quick and easy procedure for the measurement cotinine in human urine and can be applied to evaluation of the tobacco exposure.

B-258

Storage of urine specimens in POCT cups reduces concentrations of many drugs measured by confirmatory methods of urine drug testing.

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Background: Several pain management clinics use urine cups that contain point of care testing (POCT) urine drug strips to assess the presence of drugs in urine specimens. Some of the urine specimens that are received by clinical toxicology laboratory for confirmatory urine drug testing are in urine cups with POCT urine drug strips. We conducted this study to investigate the stability of drugs that are exposed to POCT urine drug strips before submitted for confirmatory urine drug testing. **Methods:** Drug free urine specimens in POCT urine drug test cups were spiked with common drugs that are tested in clinical toxicology laboratories. A dilute and shoot confirmatory liquid chromatography mass spectrometry method measured 87 drugs using Sciex 4500 instrument. The specimens were stored at room temperature and refrigerator conditions. The drugs concentration was measured after 30mins, 1hr, 2hrs, 5hrs, 6hrs, 24hrs, 48hrs and 96 hours when refrigerated and not measured until 24 hours when stored at room temperature. The concentration of drugs in urine specimen before exposure to POCT urine drug strips was used as the base line. Drug free urine spiked with the same drugs that were stored under the same condition in regular urine cups without POCT urine drug strips were used as a second control for the experiment. Alterations greater or lesser than 20% was defined as significant changes in drugs concentration. The drugs concentrations were selected at the cutoff concentrations. **Results:** Concentrations of amitriptyline, cyclobenzaprine, fentanyl, fluoxetine, flunitrazepam, nortriptyline, paroxetine and sertraline were significantly reduced within a range of 21-65% when urine specimen inside POCT cups were stored at room temperature for 24 hours. The spiked urine specimens that were stored in the same cups without POCT urine drug strips for 24 hours at room temperature did not show reduction in any of 87 tested drugs. The exposure of spiked urine to POCT urine drug strips for 24 hours in refrigerator significantly reduced the concentration of amitriptyline, cyclobenzaprine, paroxetine, propoxyphene, sertraline, duloxetine and buprenorphine. Exposure for 48 hours in refrigerator added, fentanyl, acetaminophen, duloxetine, haloperidol, atomoxetine, bupropion, desipramine and haloperidol, propoxyphene, fluoxetine, nortriptyline, dextromethorphan and doxepin to the list of affected drugs. Phencyclidine, methadone concentrations significantly reduced after 96 hours exposure of specimens to POCT urine drug strips in refrigerator. The reduction in concentration of drugs that were stored in POCT urine drug test cups in refrigerator varied between 22 to 45 %. When the spiked urine specimens were stored in refrigerator in urine cups without POCT urine drug strips for 96 hours, the concentration of all 87 measured drugs did not show any significant alterations. **Conclusion:** Exposure of urine specimens to POCT urine drug strips stored at room temperature or refrigerator reduces concentration of several drugs in urine drug testing performed by confirmatory liquid chromatography mass spectrometry method.

B-259

Determination of 7 Phosphatidylethanols in Blood using LCMS for clinical research use

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Background: Phosphatidylethanol (PEth) is an alcohol biomarker for alcohol use detection. PEth is a group of phospholipids containing 2 fatty acids of mainly palmitic and oleic acid (PEth 16:0/18:1 and 16:0/18:2) and a phosphoethanol. A simple, sensitive and specific LC-MS/MS analytical method was developed for the quantitation of the various phosphatidylethanols in blood using protein crash and a liquid-liquid extraction in blood sample preparation techniques. This easy method achieved good analyte recovery, post-extraction cleanliness and is capable of the sensitivities to quantitate the various phosphatidylethanols in blood over its dynamic range despite some challenging issues. Method: A Thermo Fisher TSQ Quantis tandem mass spectrometer in negative Electrospray mode and a Thermo Fisher Vanquish Horizon HPLC system were utilized. 100 ul of blood was used for the analysis of these compounds. Various columns were evaluated and initially a Thermo Fisher Accucore Phenyl-Hexyl 50 x 2.1 mm, 2.6 um with a water:acetonitrile/isopropanol mixture containing ammonium acetate achieved baseline chromatographic separation in less than 5 minute run time for PEth compounds. Quantitative analysis was performed using scheduled reaction monitoring (SRM) transition pairs for each analyte and internal standards of phospatidylpropanol and deuterated phosphatidylethanol were evaluated as to the most appropriate in negative mode. The accuracy of the method was verified using quality control materials and blood samples. Results: 7 various phosphatidylethanols were evaluated and it was determined that PEth 16:0/18:1 and 16:0/18:2 were the most prevalent in human blood. Good linearity and reproducibility were obtained with the concentration range from 0.05 to 500 umol/l for the various phosphatidylethanols with a coefficient of determination >0.995 for the sample preparation techniques employed. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range initially from 0.01 and 0.025 umol/l. Excellent reproducibility was observed for both compounds (CV < 10%) and all techniques and configurations. Conclusion: A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry method was developed and verified for the simultaneous measurement of the various phosphatidylethanols in blood. The sample preparation techniques are quick and easily applied for high throughput analysis and the degree of phosphatidylethanol formation was further determined over time and correlates linearly with ethanol concentrations. Thus PEth can be used to determine long-term exposure to ethanol better than other ethanol biomarkers such as Ethyl glucuronide or Ethyl Sulfate.

B-260

An Easy-to-use Automated Solid-phase Extraction Method for Quantification of Serum Nicotine and Metabolites using LC-MS/MS

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Background: Tobacco use has a major impact on outcomes after medical procedures, such as solid organ transplant for both donors and recipients. LC-MS/MS analysis has been used for simultaneous quantification of nicotine, cotinine, and 3-OH-cotinine to provide accurate measurement of recent nicotine exposure. Such analysis usually involves with analytes extraction from the specimen, for example, SPE, which can be labor-intensive. We developed and validated an easy-to-use automated method for simultaneous extraction of nicotine, cotinine and 3OH-cotinine for subsequent quantification by LC-MS/MS. Some of the advantages of the relatively small bench-top instrument used in the study include user-friendly programming for multi-step extractions; and various size-options for both cartridge format and plate format. These features allow its use for different assays varied in test volumes and specimen volumes. **Methods:** Patient serum was mixed with deuterated internal standards prior to extraction. The automated SPE extraction was performed on a Biotage Extrahera System using Oasis® HLB cartridges in a 24-cartridge format, which has multiple steps including cartridge pre-treatment, sample loading, cartridge washing and analyte elution. The eluent was directly collected in LCMS vials for subsequent LC-MS/MS analysis, which was performed on a Thermo Vanquish UHPCL system coupled with Endura QQQ mass spectrometer. Separation of the three analytes was achieved on a C18 column using a linear gradient with a total LC time of 3.5 minutes for each injection. Two MRN transitions (one quantifier and one qualifier) were set for each target analyte with one MRN transition set for its corresponding internal standard. Simultaneous peak integration and quantitation for all three target analytes were achieved automatically using the pre-installed TraceFinder™ Software. **Results:** For each analyte, good linearity across the analytical measurable range was obtained. The limit of quantification (LoQ) was established at 2 ng/mL. Precisions of < 5% was obtained for both within-run and between-day studies. The accuracy was assessed by comparing patient results to the previously established LC-MS/MS method, with correlation efficiency of > 0.99 achieved for each analyte. Different degrees of matrix effects were observed for nicotine (70% mean recovery), conitine (95% mean recovery) and 3OH-cotinine (89% mean recovery). No ion suppression was noted for nicotine or cotinine with slight ion suppression noted for 3OH-cotinine. No interference was detected from nornicotine, norcotinie, or anabasine for each analyte. **Conclusion:** The validated assay provides an easy-to-use automated sample extraction for simultaneous measurement of nicotine, cotinine and 3OH-cotinine in patient serum using LC-MS/MS, which reduces the need for manual labor and offers good precisions. The programming and operation of the method are straightforward, which makes it easily adaptable for uses with different sample extractions prior to LC-MS/MS analysis.

B-261

Mucopolysaccharides Quantitation in Blood and Urine by LCMS for clinical research use

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Background: Mucopolysaccharides (MPS) or glycoaminoglycans (GAG's) are sulfated polysaccharides that contain repetitive disaccharide units attached to a protein core. The mucopolysaccharides are highly polar and act as a lubricant and metabolic disorders of abnormal accumulations of the MPS occur because of enzyme deficiencies. A simple, sensitive and specific LC/MS/MS analytical method was developed for the quantitation of Hyaluronic Acid (HA), Dermatan Sulfate (DS), Heparan Sulfate (HP), Keratan Sulfate (KS S1 and S2) and Chondroitin Sulfate (CS) and are enzymatically digested to disaccharides by the various MPS enzymes blood and using an acid digest and methanolysis in urine. This easy method achieved good analyte recovery, post-extraction cleanliness and is capable of the sensitivities to quantitate the MPS's in urine and blood over their dynamic range in both matrices despite some challenging issues. Method: A Thermo Fisher TSQ Quantis tandem mass spectrometer in positive and negative Electrospray mode and a Thermo Fisher Vanquish Horizon HPLC system were utilized. 100 ul of urine and blood were used for the analysis of these compounds. Various columns were evaluated and initially a Thermo Fisher Accucore C18 100 x 2.1 mm, 2.6 um with a water:acetonitrile mixture containing 5mM Ammonium Acetate achieved baseline chromatographic separation in less than 8 minute run time for all compounds. Quantitative analysis was performed using scheduled reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive mode and accuracy of the method was verified using quality control materials and blood and urine samples. Results: Good linearity and reproducibility were obtained with the concentration range from 1 to 2000 nmol/l for the various MPS's with a coefficient of determination >0.995 for both sample preparation. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range initially from 0.2 and 0.5 nmol/l. Excellent reproducibility was observed for both compounds (CV < 10%) and all techniques and configurations. Conclusion: A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry method was developed and verified for the simultaneous measurement of MPS in urine and blood. The sample preparation techniques are quick and easily applied for high throughput analysis and included enzymatic and acid digestion in blood and urine. The identification of MPS-I, II, III, IV and V could easily be achieved.

B-262

A Clinical Research LC-MS/MS Method for the Measurement of Serum Estrogens

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Background: The two major biologically active estrogens in non-pregnant humans are 17β-estradiol (E2) and estrone (E1). E2 is produced primarily in the ovaries and testes by the aromatization of testosterone, whereas, E1 is primarily derived from androstenedione. E2 can be metabolized to E1 and conversion of E1 to E2 is also possible, making the measurement of both compounds desirable. Some immunoassay techniques lack analytical sensitivity and selectivity for E2, whilst published LC-MS/MS methods often use large sample volumes with complex sample preparation, including derivatization to reach the analytical sensitivity and selectivity requirements. Here we describe a simple method for the measurement of E2 and E1 by LC-MS/MS for clinical research. **Methods:** Samples (250µL) were spiked with $^{13}C_3$ labeled internal standards and a liquid/liquid extraction performed using a mixture of hexane and ethyl acetate. Following centrifugation, the top layer was transferred to glass vials, evaporated to dryness and reconstituted using methanol/water prior to analysis. Chromatographic separation was achieved, in less than 5 minutes, using a Waters® CORTECS® Phenyl column (2.1 x 50mm, 2.7µm) with a water/methanol/acetonitrile/ammonium fluoride gradient on the Waters ACQUITY UPLC® I-Class system. E2 and E1 were detected using electrospray negative ionization on a Waters XEVO® TQ-XS mass spectrometer. In-house calibrators (2-1000pg/mL) and quality controls (3, 30 and 175pg/mL) containing both E2 and E1 were prepared using reference material from Cerilliant (Round Rock, TX) and MSG4000 stripped serum from Golden West Biologicals (Temecula, CA). **Results:** Total precision and repeatability assessments for E2 and E1 in samples spiked across the calibration range 2–1000pg/mL were ≤8.4% CV, except for the lowest calibrator level at 2pg/mL (≤12.5% CV). Analytical sensitivity of the method allows for E2 and E1 measurement at 2pg/mL, determined over 5 occasions (n=5 extractions), where the precision was ≤20%CV and signal to noise ratio (ptp) was >10:1. Accuracy of the method for E2 was determined by analyzing 40 Phase 1 samples from the CDC Hormone Standardization Program (Atlanta, GA); good agreement was observed with a Deming fit of y=1.14x – 1.85 and a mean bias of ≤10.9 % (Altman-Bland). The method was shown to be linear over the range 0.43–1108pg/mL for E2 and 0.65–1113pg/mL for E1, with no significant carryover up to 2000pg/mL. Quantitative matrix factor was evaluated using six individual serum donors. Matrix factors for both analyte peak area and analyte:internal standard response were calculated. Ion enhancement was observed but compensated for using the internal standard (E2 range -13.2% to 2.0% and E1 range -14.7% to 9.1%). Endogenous and exogenous interferences were assessed and the mean recovery observed was between 85–115%. **Conclusions:** A simple LC-MS/MS method has been developed for the measurement of serum estradiol and estrone. This clinical research method demonstrates good precision, linearity, analytical sensitivity and accuracy. For Research Use Only, Not for use in diagnostic procedures.

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Targeting Production of a Fast-Forming Proteotypic Peptide for Rapid Quantification of Apolipoprotein A1 in Plasma by LC-MS/MS

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Background: Apolipoprotein A1 (apoA1) is the major protein component of high-density lipoprotein particles in plasma, comprising up to 70% of the total protein mass. Clinically, concentrations of apoA1 in serum or plasma are used in risk assessment of cardiovascular events. Clinical laboratories commonly use nephelometric or turbido-

metric methods to measure apoA1, which can be costly, require a relatively large volume of sample (e.g. upwards of 150 µL of serum or plasma) and prone to interferences common to immunoassays (i.e. lipemia, cross-reactivity, etc.). An alternative methodology, liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been previously applied to the measurement of apoA1; however, uptake of these methods for routine clinical testing has not been realized due to complex and time-intensive sample preparation workflows. Toward the design of an assay suitable for implementation in a clinical laboratory, we simplified the sample preparation workflow including eliminating reduction and alkylation, and using only additive steps. By optimizing digestion conditions and monitoring digestion profiles of nine different apoA1 proteotypic peptides, we identified a peptide (THLAPYSDELR, residues 185-195) demonstrating rapid and stable digestion kinetics. Herein we describe the design and validation of a simple and rapid quantitative apoA1 LC-MS/MS assay targeting this fast-forming peptide. **Methods:** For the external calibrators, the peptide sequence THLAPYSDELR (unlabeled) was synthesized with concentration assigned by HPLC and amino acid analysis. We used a C-terminal $^{13}C/^{15}N$-Arg labeled peptide as the internal standard (IS), and pooled human EDTA plasma as quality controls (QC_1 = 1.00 g/L; QC_2 = 1.53 g/L). Samples were diluted in phosphate-buffered saline and added to a 50 mmol/L ammonium bicarbonate buffer containing the IS. Samples were mixed briefly and denatured by heating at 99 °C for 10 min. Samples were then cooled to room temperature and N-tosyl-L-phenylalanine chloromethyl ketone treated trypsin was added for digestion. Samples were incubated at 37 °C for 20 min. Note that all steps are additive and no centrifugation or separation is required. The method validation followed Clinical & Laboratory Standards Institute guidelines, which, briefly, includes assessment of sensitivity, precision, accuracy, linearity, interferences, and stability. **Results:** Using our rapid digest protocol requiring a brief 20 min digestion, proteotypic peptide THLAPYSDELR was selected for quantitation of apoA1. The amino acid analysis-assigned calibrators ranged from 0.005 – 0.300 g/L. With plasma specimens subjected to a 10-fold dilution, the clinically reportable range was 0.05 – 3.00 g/L. QCs were stable through at least 4 freeze-thaw cycles and could be left at room temperature for at least 4 days. Method comparison (n = 40) of our LC-MS/MS method to the Siemens BNII Chemistry system immunonephelometry apoA1 assay revealed the following linear regression: LC-MS/MS = 0.70 × immunonephelometry – 0.09, R^2 = 0.9148, CI_{slope}: 0.63,0.78. The intra-assay precision was 3.30% (QC_1) and 6.70% (QC_2) and the inter-assay precision study is ongoing. **Conclusions:** By streamlining sample preparation and optimizing conditions of denaturation and digestion, we were able to develop a simple and rapid LC-MS/MS method for quantitation of apoA1 in human plasma.

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A rapid and simplified LC-MS/MS workflow for the analysis of pain management drugs for clinical research.

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Background: Comprehensive pain management and drug panels for clinical research analysis often include such commonly used substances such as opioids, benzodiazepines and stimulants. Other classes of compounds can include muscle relaxants, anti-epileptic drugs such as gabapentin, synthetic cathinones (“bath salts”) and other substances. Often, multiple methods are used to obtain a comprehensive view of the various drug classes. Key workflow considerations include the use of fewer, more comprehensive analyte panels and rapid sample preparation and analytical techniques, all of which must be balanced against the need for sample integrity and data quality. The objective of this study was to develop a comprehensive LC-MS/MS analysis strategy for a large drug panel (80 compounds from 22 drug classes) using a simplified solid phase extraction (SPE) protocol that incorporates in-well hydrolysis and pre-treatment of the urine sample. **Methods:** Calibration curves were prepared by spiking the compounds into urine covering the appropriate measurement range for each compound, from 2-200 ng/mL for 6-MAM and fentanyl, to 25-2,500 ng/mL for many opiates and amines. Urine samples (spiked calibrators, QCs and independent QC material from UTAK) were extracted using mixed-mode cation exchange polymeric SPE plates. All pre-treatment steps were conducted within the SPE plate wells. Analytes were extracted using a modified procedure designed to extract all components in a single protocol. LC-MS/MS analysis was conducted using a Waters ACQUITY I-Class UPLC system coupled to a Xevo TQ-S micro mass spectrometer under reversed-phase conditions. **Results:** The method was evaluated for linearity, precision, accuracy (recovery), extraction efficiency and matrix effects. All analytes eluted within 3.1 minutes while maintaining baseline separation of all isobaric compounds. Calibrator linearity was assessed and QC results were accurate and precise for all compounds over the measurement range. Precision performance over five days was acceptable both within and between runs (%CV<12%). Accuracy was assessed using commercially available control materials from UTAK (mean bias < 6.2 %) and all spiked QC sample determined concentrations ranged from -7.8% to 16.2% bias of the target values at the lowest QC level and within ±10% bias of target values at all other levels, with an overall mean bias of <3%. Extraction efficiencies were high and consistent for all compounds, averaging >80% with %RSDs under 15% for all analytes. Internal standard corrected matrix effects were less than 20% for all but 3 compounds, with %CVs <20%. **Conclusion:** This method enables the rapid extraction and analysis of a diverse panel of drugs for clinical research. A single, rapid SPE method is used to extract 80 compounds with high efficiency. The use of 96-well plates, combined with in-well sample pre-treatment eliminates sample transfer steps, minimizing the risk of cross-contamination or sample transfer errors. The combination of sample preparation, chromatography and tandem MS analysis results in a complete and comprehensive workflow. For Research Use Only, Not for use in diagnostic procedures

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Evaluation of the Shimadzu CLAM 2000 fully integrated, automated sample preparation system for LCMS in the routine clinical laboratory.

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Background: Lack of availability of fully automated LC-MS/MS systems has significantly impeded adoption of the technology into the routine clinical laboratory. We evaluated the analytical performance and workflow of the Shimadzu Clinical Laboratory Automation Module 2000 (CLAM-2000)/Nexera XR LCMS-8050 for analysis of total 25-OH Vitamin D (25-OHD) in a clinical laboratory setting. Because many methods for 25-OHD do not resolve the 3-epi isomer of 25-OHD3 (which is thought to be an inactive form of vitamin D), we specifically developed the CLAM 2000/Nexera XR LCMS-8050 method to enable quantification of 3-epi 25OHD3. **Methods:** The method was calibrated using commercially available Chromsystems 6PLUS1 calibrators for 25-OHD3 and 25-OHD2. 3-epi 25-OHD3 calibrators were prepared by spiking 3-epi 25-OHD3 into stripped serum to final concentrations of 0, 5, 10, 20, and 40 ng/mL. Sample preparation was fully automated using the Shimadzu CLAM-2000 module. Briefly, a 1% Zinc Sulfate/95% MeOH mixture containing internal standard for 25-OHD3, 25-OHD2 and 3-epi 25-OHD3 was added to 30µL of patient serum. After automated mixing and onboard incubation for 15 min, the precipitated solution was filtered through a 0.45µm PTFE membrane by vacuum filtration. 15 µL of the filtrate was automatically injected and analyzed using the Nexera XR LCMS-8050 system. A MeOH/dH20 mobile phase gradient was used to obtain separation on a 2.7µm Raptor FluoroPhenyl LC Column (Restek) with guard column. MS analysis was performed using APCI in positive ion mode. Method validation experiments appropriate for a Laboratory Developed Test (LDT) were performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines. **Results:** The method was linear from 2 ng/mL - 150 ng/mL for 25-OHD3 and 25-OHD2, and 2 ng/mL - 40 ng/mL for 3-epi 25-OHD3. For between-run imprecision, coefficients of variation were < 6% at 5.2 - 60.9 ng/mL and < 15% at 2 ng/mL. Patient sample comparisons were performed using a routine LC-MS/MS method used in our clinical laboratory that employs manual preanalytical processing. The CLAM 2000/Nexera XR LCMS-8050 method agreed with the routine laboratory method and exhibited an average systematic error of < 6%. Bias vs. National Institutes of Standards and Technology (NIST) SRM 972a Vitamin D reference materials was ≤ 10.9% for all four levels of materials. 13/94 patient samples had 3-epi 25-OHD3 ≥ 2ng/mL, however the concentrations were not clinically significant. Use of the CLAM-2000 Nexera XR LCMS-8050 system saved approximately 80 minutes of labor vs. our routine laboratory method. **Conclusions:** The CLAM-2000 Nexera XR LCMS-8050 system exhibited similar analytical performance to our validated laboratory method for total 25-OHD, decreased labor requirements and improved workflow efficiency for LC-MS/MS testing in the clinical laboratory. The method also enabled quantification of 3-epi 25-OHD.