Tuesday, August 2, 2016

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

Mass Spectrometry Applications

A-341

A Novel LC-MS/MS Method for the Quantitation of Abiraterone Metabolites in Patients with Castration-Resistant Prostate Cancer: Innovation of Separation of Diastereoisomers Without Using a Chiral Column

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Background: Prostate cancer tumor progression depends on androgen receptor (AR) presence and function. Testosterone (T) and 5α-dihydrotestosterone (DHT) are the endogenous ligands for fueling the growth of malignant cells. In most advanced cases, the cancer initially responds to treatment with medical or surgical castration, but after time, castration-resistant prostate cancer (CRPC) develops, and patients eventually die from their disease. In 2011, the US FDA approved abiraterone acetate (AA) in combination with prednisone for the treatment of CRPC. Abiraterone (Abi) is a steroidal compound; it is administered as the prodrug AA. Abi inhibits CYP17A1, an enzyme required for androgen biosynthesis. We have recently reported that a novel Abi metabolite (3-keto-Δ^4-Abi (D4A)) is more potent than the parent drug (Abi) in blocking enzymes essential for DHT synthesis and also more potent in inhibiting tumor growth in xenograft mice (Li et al, Nature. 2015). The D4A structure is similar to that of testosterone and androstenedione, which may enable further metabolism of D4A. **Methods**: A Liquid chromatography mass spectrometry method was developed and validated to detect Abi and its metabolites in human serum using an AB Sciex Qtrap 5500 mass analyzer coupled with a Shimadzu Nexera UPLC station. The mass spectrometer was operated in positive ion mode using electrospray ionization (ESI) source. Tuning parameters were optimized for the analytes by infusing a solution containing 200 ng/mL of each. The separation of the analytes was achieved using a Zorbax Eclipse Plus C18 150*2.1mm, 3.5µm column at 40°C, and isocratic mobile phase 35% A (H_2O), 65% B (methanol:acetonitrile; 60:40) with 0.1% (formic acid) in both and flow rate 0.2 ml/min. Analytes and the IS (Abiraterone-d4) were extracted from 100µl human serum (collected from CRPC patients treated with AA) with 2 ml methyl tert-butyl ether. After evaporation, the residue was reconstituted using 300 µl 50% methanol and 10µl was injected. A 7-point calibration curve for all analytes was constructed, and six quality control samples; 2 (low, mid, and high) were injected with the samples. The analytes were quantified using multiple reaction monitoring (MRM). **Results**: Data were processed using Analyst 1.6.2 software (AB Sciex). All the analytes were separated with the developed method despite their closely related structures. Analysis in human serum showed that Abi was linear in the range of 2.0-400 ng/ml, and all the metabolites were linear in the range of 0.1-20 ng/ml the analysis also show that the samples were free from interference. Intra and inter day precision and accuracy results were within the 15% CV for the three QC levels, the results also show that the analytes were stable in serum at different conditions and also in solution; no matrix effect was found in all analytes. In all samples obtained from CRPC patients, Abi and its metabolites were all detected. **Conclusion**: The validated LC-MS/MS method resolved and quantitated all the metabolites despite the similarity in their structures, including resolving diastereoisomers, which precludes analysis of co-eluting isomers based solely on their MRM transitions. Reversed-phase chromatographic conditions were identified to accomplish the separation of all metabolites and their subsequent accurate quantification.

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Simple and Sensitive Method for Quantitative Measurement of Methylmalonic Acid by Turbulent Flow Chromatography and Tandem Mass Spectrometry

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Background: Methylmalonic acid (MMA) is elevated in patients with inherited defects in methylmalonyl CoA mutase or due to inherited or acquired deficiencies of vitamin B12. MMA levels are measured in serum to evaluate individuals with deficiencies of vitamin B12 or to assess children with symptoms of methylmalonic acidemia. Although methods to measure MMA by mass spectrometery have been previously developed, we utilized turbulent flow liquid chromatography (TFLC) to simplify the sample preparation procedure. The sample was prepared by precipitating serum proteins using methanol and directly injecting the supernatant into the turbulent flow column after centrifugation. The objective was to develop an MMA assay performed by TFLC-MS/MS with the following characteristics: analytically sensitive with a clinically useful dynamic range; good specificity with no cross-reactivity with succinic acid (SA); and suitable analytical transferability for a high volume clinical laboratory.

Methods: Serum samples were prepared by protein precipitation using methanol containing deuterated MMA as an internal standard. TFLC-MS/MS analyses were performed on a Thermo Scientific TLX-2 HPLC system (TurboFlow® technology) interfaced to a TSQ Quantum Ultra mass spectrometer operated in the negative ion ESI mode. Chromatographic separation was achieved using a Cyclone MAX TurboFlow®column (50 X 0.5 mm) and an Allure Organic Acids analytical column (150 X 3 mm). The HPLC elution occurred with a mobile phase composition of 50% methanol that was held for 1.3 minutes. Calibrators (6) were prepared in blank human serum.

Results: The analytical measurement range (AMR) for MMA was 30-1000 nmol/L with a CV less than 20% at the lower limit of quantitation (LLOQ); the calibration curves were linear over the AMR with correlation coefficients $R^2 \geq 0.995$. Dilutions of 1:2, 1:10 and 1:20 were validated giving a clinically reportable range of 30-10^5 nmol/L. The accuracy of the MMA assay was evaluated by comparing results of 63 residual patient specimens to the results obtained from a national reference laboratory utilizing LC-MS/MS methodology. The accuracy was further evaluated through recovery experiments. The slopes of the linear regression curves comparing the assays was +/- 1% with excellent correlation coefficients. MMA recoveries at concentrations spanning the AMR were between 96 and 111%. Within-day and between-day (N=20) CVs at concentrations spanning the AMR were less than 12%.

Conclusion: We have developed an accurate and sensitive assay to measure MMA levels in serum by TFLC-MS/MS. The method showed excellent correlation with previously established mass spectrometry-based methods and has been fully validated for imprecision, accuracy, linearity, recovery, carryover, specificity and matrix effects. The assay is more simple to perform than previously published methods and has proven very accurate and robust and cost effective.

A-343

Validation of a rapid liquid chromatography tandem mass spectrometry method for serum 25OHD &evaluation of the necessary to separate 3-epi $25OHD_3$

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Background: Increasing clinical implementation of liquid chromatography tandem mass spectrometry (LC-MS/MS) for measuring serum 25-hydroxyvitamin D (25OHD) has revived interest in separating and measuring 3-epi $25OHD_3$, which was neglected because routinely used LC-MS/MS could not separate it (called NEPI-LC-MS/MS) from $25OHD_3$. However, the necessary to separate 3-epi $25OHD_3$ in clinical practice is controversial. **Methods:** We developed and validated a rapid LC-MS/MS method to separate 3-epi $25OHD_3$ (called EPI-LC-MS/MS) and compared the results with those from routine NEPI-LC-MS/MS. And 982 clinical samples were analyzed by both the methods. Results: Both methods showed a linearity coefficient correlation exceeding 0.999 in the 2.5-200 ng/mL concentration range for $25OHD_2$ and $25OHD_3$. Moreover, they showed between run coefficient variation (CV) and total CV of <5% for $25OHD_2$ and $25OHD_3$. Accuracy test results showed that the accuracy bias was below 3.5% in the absence of 3-epi $25OHD_3$. Comparing the25OHD results obtained by the two methods for 982 patients (age 1 -100 years) showed excellent clinical agreement (Cohen's kappa = 0.875) and correlation (R2 = 0.973). Our data showed that among the 982 patients, only 73 patients had 3-epi $25OHD_3$ (>2.5 ng/mL); out of these 73, the 3-epi 25OHD3 level in 58 patients was between 2.5 and 5 ng/mL. In patients with less than 150 ng/mL 25OHD (25OHD2+25OHD3), only 8 had 3-epi $25OHD_3$ exceeding 5 ng/mL (ranging from 5.3 to 11.0 ng/mL). Among samples containing 3-epi $25OHD_3$, only three were separated into different 25OHD-deficiency groups using the above methods. **Conclusion:** A rapid and precise EPI-LC-MS/MS method with efficient separation of 3-epi $25OHD_3$ for measuring 25OHD was developed. Our results showed that 3-epi $25OHD_3$ had little effect on routinely used NEPI-LC-MS/MS.

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Validation of Posaconazole Quantification Using LC-MS/MS

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Background: Posaconazole is a triazole antifungal agent used for prophylaxis & treatment of invasive fungal infections in severely immunocompromised patients. Large variations in inter-individual posaconazole pharmacokinetics may be associated with decreased efficacy leading to breakthrough infections and, therefore, monitoring of posaconazole levels is highly recommended. The objective of this study was to validate the performance of a newly developed method for measuring posaconazole concentrations using LC-MS/MS according to CLSI EP5 A standards.

Material and Methods: Serum samples from patients receiving posaconazole therapy were collected according to our institution standards protocols. A 100 µL aliquots of patient sera, calibrators (0, 0.190, 2.85, and 5.54 µg/mL posaconazole), and controls (0.465, 1.84, and 4.61 µg/mL posaconazole) were each deproteinized with 300 µL of methanol containing d3-voriconazole (50 ng/mL) as an internal standard, vortexed for 1 minute and centrifuged at 4,000 rcf for 10 minutes. Following centrifugation, 10 µL of the supernatant was transferred to an autosampler tube and diluted with 990 µL of water. The deproteinized samples (10 µL injection) were analyzed by a Shimadzu Nexera LC with a ThermoFisher "Cyclone" (50 x 0.5mm) guard column and Thermo "Accucore" C18 (3x50mm) separation column heated to 30°C. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Total elution time was 4 minutes and the starting mobile phase consisted of 20% B at a 0.65 mL/min flow rate, transitioned to 60% B by 0.6 minutes, 100% B by 0.75 minutes, and returned to 20% B by 3.10 minutes. Posaconazole was monitored by a Sciex API 5500 triple quadrapole MS/MS. The multiple reaction monitoring scan was conducted in positive polarity mode with a total scan time of 0.63 s and a dwell time of 100 ms. Fragment peaks were detected at 683.1 m/z (quantifying) and 614.1 m/z (confirmation) from the parent posaconazole 701.2 m/z. The internal standard d3-voriconazole (353.15 m/z) yielded fragments 284.2 m/z and 130.0 m/z. Multiquant software was used to quantify posaconazole concentration in serum based on a calibration curve generated from a ratio of the 683.1 m/z fragment over the 284.2 m/z internal standard peak. Performance of the LC-MS/MS method for detecting posaconazole levels in three control samples was compared with the manufacturer's reference values for the control material (RECIPE Chemicals).

Results: The LC-MS/MS method for posaconazole was linear over the analytical range of 0.1 to 5.54 µg/mL and R^2= 0.9988. This study established that the LC-MS/MS offered acceptable precisions with an intra- and inter-assays coefficients of variation of <5% and <6%, respectively. The correlation between samples (n=20) analyzed on the Sciex and the concentrations verified by RECIPE Chemicals samples was acceptable with R^2= 0.9662 (y= 0.9964x + 0.2634). The correlation with serum pool samples (n=10) spiked with posaconazole that were run by LC-MS/MS on the Sciex and by HPLC at an outside reference laboratory were also adequate with R^2= 0.9930 (y= 1.291x + 0.069).

Conclusions: The LC-MS/MS method offered a rapid and reliable method for monitoring posaconazole concentrations in serum samples from patients receiving posaconazole therapy.

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Evaluation and Validation of LC-MS/MS for Quantification of 25-Hydroxyvitamin D2 and D3 Compared to a Chemiluminescent Immunoassay

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Background: The importance of monitoring 25-Hydroxyvitamin D2 and D3 concentrations (25(OH)D2 and D3) has increased recently as more physicians prescribe Vitamin D supplements because of their extra-skeletal roles in preventing cancer progression, autoimmune diseases, cardiovascular disease, hypertension, aging, diabetes, and infectious diseases, but high concentrations may be toxic. This study was designed to validate and compare the performance of an LC-MS/MS method to quantify 25(OH)D2 and D3 concentrations to a chemiluminescent immunoassay (CLIA) by DiaSorin.

Materials and Methods: Serum samples were analyzed by CLIA using DiaSorin Liaison XL analyzer and reagent. In lieu of utilizing an automated liquid handling station, an electronic pipette was used in the LC-MS/MS assay to improve the ergonomics, precision, and throughput of ≈135 samples-per-day. For the LC-MS/MS, an 80µL aliquot of patient sera, calibrators and controls from ChromSystems were deproteinized with 160µL of acetonitrile containing internal standard d6-25(OH)D3 (9.33 ng/mL) in a 96-well plate, sealed, rocked for 5 minutes, and centrifuged at 4,000 rcf for 6 minutes. The microplate was loaded onto a Shimadzu Nexera LC and the deproteinized supernatants (25µL) were injected onto heated (40°C) ThermoFisher "Cyclone" (50x0.5mm) and Thermo "Accucore" C18 (3x50mm) columns using a mobile phase of 18 ohm water (A) and methanol (B) both with 0.1% formic acid. The 25(OH)D2 and D3 fractions were eluted during a 4.1 minute gradient analysis starting with 30% B, transitioning to 95% B at 1.5 minutes, and re-equilibrating to 30% B at 3.1 minutes. 25(OH)D2 and D3 were detected and quantified on a Sciex API 5500 MS/MS in MRM APCI positive mode. Fragment peaks were detected for 25(OH) D2 (395.2 m/z) at 209.2 m/z (quantifying) and 269.2 m/z (qualifying), 25(OH)D3 (383.2 m/z) at 211.2 m/z (quantifying) and 229.2 m/z (qualifying), and d6-25(OH)D3 (389.2 m/z) at 371.3 m/z. Multiquant software was used to quantify 25(OH)D2 and D3 concentrations in serum based on internal standard-corrected calibration curves of the 25(OH)D2 and D3 quantification fragments and reported automatically to the hospital's LIS. LC-MS/MS and CLIA method performance were compared in patient serum samples. Method validation of the LC-MS/MS protocol was also conducted.

Results: There was a poor correlation between LC-MS/MS and CLIA (n=68) methods: standard error estimate (8.375), average error index (0.50), and regression equation (y=1.187x – 2.265; r^2= 0.7893). However, there was excellent correlation between LC-MS/MS results and CAP samples (n=6): standard error estimate (1.36), average error index (0.24), and regression equation (y=1.072x – 0.88; r^2= 0.9996). The summary of the LC-MS/MS method validation for total Vitamin D was as follows: linear range (1.0-68.0 ng/mL), intra-assay precision (<9.1%), inter-assay precision (3.7-7.2%), limit of detection (0.3 ng/mL), limit of quantitation (1.0 ng/mL), carry-over (0.3%), lipemia interference recovery (104%), hemolysis interference recovery (99%), icterus interference recovery (99%), ion suppression (95.3%). The average cost-per-billable-test was approximately 7-10 times lower than immunoassay methods.

Conclusions: The LC-MS/MS method using an electronic pipette and deproteinizing sample preparation is a rapid, accurate, and cost-effective method for measuring 25(OH)D2 and D3 concentrations given the excellent CAP sample correlation, 4.5 minute analysis, LIS instrument interfacing, and low cost-per-test.

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A sensitive LC-MS/MS method for the quantification of urinary 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) including pediatric reference values

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Background

Oxidative stress has been implicated in numerous diseases, including arthritis, atherosclerosis, Alzheimer's disease, cancer, diabetes, hypertension, and inflammation. Adducts generated from free radicals during oxidative stress could damage nucleic acids (DNA and RNA), proteins, and lipids, and contribute to disease initiation and acceleration. Therefore, early detection of oxidative stress is crucial in disease prevention and health management.

Mayo Clinic is currently offering a LC-MS/MS based clinical test to measure urinary 8-*iso*-$PGF_{2\alpha}$ with established reference interval for adults. Pediatric population represents a special group of people whose reference interval could be different from adult population.

We herein report the development and validation of an ultra-sensitive LC-MS/MS test for the measurement of urinary 8-*iso*-$PGF_{2\alpha}$ in infants and children. Reference interval for pediatric population was also established.

Method

Each urine sample was spiked with internal standard (8-*iso*-$PGF_{2\alpha}$-d_4) and subjected to solid phase extraction with Phenomenex Strata X-AW cartridge. The extracted sample was analyzed with a Thermo Ultimate 3000 UHPLC system coupled with a Thermo Quantiva triple quadruple mass spectrometer equipped with a HESI probe. Quantitation was performed with Multiple Reaction Monitoring mode under negative ionization mode. Calibrators were prepared by spiking various amount of 8-*iso*-$PGF_{2\alpha}$ into synthetic urine with no detectable 8-*iso*-$PGF_{2\alpha}$. Quality control samples were made from pooled pediatric urine samples. Left over urine samples (n=136) with normal urine analysis results from in- and outpatients from Children's Hospital Los Angeles were used to establish reference intervals for children age 2m - 18y. Tukey's method was used to exclude outliers, and EP evaluator was used to calculate reference interval.

Result

The liquid chromatography method is highly selective, separating 8-*iso*-PGF2α from other isomers. No peak was identified that could interfere with 8-*iso*-PGF2α quantitation in all the urine samples analyzed (n=136). The assay was linear from 0.024 nM to 20 nM (R^2 = 0.999). Recoveries were above 85% and matrix effects were below 5%. The variability (CVs) was determined at nM level: the intra-day variability ranged from 4.0 % to 4.5 % (n = 20); and the inter-day CVs ranged from 4.3 % to 5.7 % (n = 20). The accuracy of our laboratory developed test was evaluated with a clinical reference laboratory (n=40), and a correlation coefficient of 0.96 was observed. Reference interval for pediatric population was established to be < 0.5 ng 8-*iso*-PGF2α/mg creatinine, lower than the reference interval established by Mayo Clinic (<1 ng *iso*-PGF2α/mg creatinine) for adult population.

Conclusion

Overall, an ultra-sensitive LC-MS/MS assay was developed and validated to measure urinary 8-*iso*-$PGF_{2\alpha}$ for pediatric population with satisfactory selectivity, precision, and accuracy. The assay is very precise and accurate, and can be readily used for the assessment of oxidative stress for translational research and clinical usage in pediatric population.

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A novel biomarker (UCN3) for sleep apnea measured by mass spectrometry using multiple reaction monitoring

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Background: Obstructive sleep apnea (OSA) is a common disorder affecting adults and children. Three percent of children (approximately 2.3 million) in the US are diagnosed annually with OSA. Polysomnography is the gold standard procedure for diagnosing this condition, but it is expensive ($1,500 to $2,000 per procedure), involves an overnight stay in a sleep laboratory, and requires the placement of numerous sensors. Having a rapid non-invasive urine-based assay able to assist in the diagnosis of OSA would be a major advance in respiratory medicine. Urocortin 3 (UCN3), a stress-induced 4.1 kD peptide, has been measured in the urine of children with OSA versus those with only primary snoring. A preliminary report based on ROC analysis showed a diagnostic sensitivity of 93.7% and specificity of 78.7% to predict OSA. Urine concentrations of UCN3 have been reported in the range of 1.0 to 1.2 ng/mL. Currently, there are no commercially available immunoassays for UCN3 which meet the limit of quantitation (LOQ) of <1.0 ng/mL and has been rigorously validated to meet CLIA '88 standard. We hypothesized that a mass spectrometry method using multiple reaction monitoring (MRM) and stable isotope dilution would enable quantification of UCN below 1.0 ng/mL.

Method: UCN3 precursor peptides generated from chymotrypsin, Asp-N, or trypsin were evaluated in-silico using Skyline and determined experimentally by nanospray-UPLC-MS using a Waters Synapt-G2-Si Q-TOF system. A heavy isotope form of a precursor peptide sequence (NIAK^AKNL) incorporating $^{13}C_6{}^{15}N_2$ L-lysine was synthesized (New England Peptide; Gardner MA) to serve as an internal standard for quantification; the concentration was determined by amino acid analysis. Carboxymethyl cellulose (CMC) resin was used to enrich UCN3 spiked in human urine; UCN3 concentration was determined using the bicinchoninic acid assay. Chymotrypsin digested peptides were desalted using solid phase extraction with a C18 loaded tip.

Results: Survey scans of precursor peptide from chymotrypsin digest showed three transition masses with strong intensities. The LOQ using a 1:1 mixture with a heavy isotope was 0.31 ng/mL (CV=5.3%, S/N>10), and linear up to 6 ng/mL. Ion quantifier/qualifier ratio was 3.76 (CV=9.88%) and 3.85 (CV=6.52%) for light and heavy precursor peptides respectively over the linear range. UCN3 bound to CMC at pH 8.5 is resistant to elution with sodium chloride up to 1.4 M; addition of 30% acetonitrile was required to elute the peptide. Recovery of spiked UCN3 from the CMC enrichment was 94-98% (n=3).

Conclusion: We have developed a mass spectrometry method for measuring UCN3. The method has a lower limit of detection <1.0 ng/mL and a dynamic range suitable for measuring the estimated normal reference range of UCN3 in human urine. We also developed a novel enrichment method for concentrating and purifying UCN3. Taken together, these results will enable more accurate quantitation of UCN3 in both adult and pediatric populations.

A-348

The Analysis of C3-Epimers of 25-Hydroxyvitamin D in Serum by LC-MS/MS

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Background: Vitamin D analysis has increased dramatically in clinical practice due to its association with multiple human diseases and the prevalence of vitamin D deficiency worldwide. Vitamin D exists in two forms, vitamin D2 and vitamin D3; each undergoes metabolism to form 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3] which are used as the biomarkers for the assessment of vitamin D status. The epimeric forms of 25(OH)D, 3-epi-25-hydroxyvitamin D2 and D3, have been identified and may contribute to a large portion of the total 25(OH)D concentration, particularly in infant populations. Studies have shown that the C3 epimers have much lower bioactivity than the primary metabolites; therefore, a specific quantitation of these epimers is necessary for a proper clinical assessment of vitamin D status. Since these epimers are isobaric, chromatographic separation is necessary for accurate quantitation. In this study, the Raptor™ FluoroPhenyl column was used for chromatographic separation of 25(OH)D and their C3-epimers. The established chromatographic method was able to accurately quantitate the 3-epi-25-hydroxyvitamin D2 and D3 metabolites in fortified beagle serum. **Methods:** Serum was fortified with four analytes, 25(OH)D2, 25(OH)D3, 3-epi-25(OH)D2, and 3-epi-25(OH)D3, and extracted using a liquid-liquid extraction (LLE) method. Serum (400µL) was mixed with 15µL of internal standard solution (1µg/mL of d6-25-OH-D3 in methanol), 0.2 M $ZnSO_4$ (400µL) and methanol (400µL) in a 4-mL glass vial. A 2mL aliquot of hexane was added, mixed for 90 seconds, and then centrifuged for 10 minutes at 4300rpm. The hexane layer was removed and evaporated to dryness under nitrogen at 55°C. The dried extract was reconstituted with 100µL of a 50:50 water:methanol solution and injected (10 µL) for analysis on a Shimadzu Nexera XR UHPLC coupled to a Sciex API 4000™ mass spectrometer. **Results:**The calibration standards were prepared in synthetic human serum, SeraFlex LCMSMS, and subjected to the LLE procedure. Good linearity was obtained for all analytes with a concentration range of 1 to 100ng/mL (with 1/x weighting). Standard deviations were ≤10% (the lowest concentration was ≤20%) and R-squared values were 0.996-0.999 for all compounds. The quantitative results of 3 QC levels of fortified synthetic serum samples showed acceptable method accuracy with percent recovery within 10% of the nominal concentration for all QC levels. The %RSD values ranged from 0.9-6.6% and 2.2-4.5% for intra-day and inter-day analyses, respectively, indicating an acceptable method precision. The validated method was used to analyze the 8ng/mL fortified beagle serum which showed acceptable accuracy and precision. **Conclusion:**

It was demonstrated that the Raptor™ FluoroPhenyl column can provide unique selectivity for accurate and differential quantitation of 25-hydroxyvitamin D and C3-epimers in serum. The chromatographic analysis was performed using 0.1% formic acid in water and methanol as mobile phases with a 7-minute analysis time. The analytical method is applicable to the clinical analysis of total 25-hydorxyvitamin D concentration and provides the option to report the C3-epimer concentrations separately.

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Maximizing the LC-MS Output by Using a New Four Channel HPLC with Multichannel Optimization

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Background: In a single channel LC-MS workflow, a mass spectrometer may only be utilized in a small portion of time during a HPLC run. Typically, the idle time of the mass spectrometer includes the elution time before the first analyte peak and the equilibration time after the last analyte peak. A new HPLC is designed to bring the productivity of up to four HPLC channels to a single mass spectrometer, four times the samples can be run, maximizing the LC-MS output. With built-in multichannel optimization software, up to four identical or different test methods can be run, and simplify workflow. We evaluated three units of prototype of a new four channels HPLC with a new Tandem-MS. **Methods:** This HPLC instrument has four LC channels, which are synchronized to a single mass spectrometer. Each channel operates independently, allowing four identical or different tests to run simultaneously. In addition, identical or different analytical columns and mobile phases can be used with each channel, providing method flexibility. Multichannel optimization ensures the maximum performance of the mass spectrometer with little to no idle time. When four channels run simultaneously, the data collection times are scheduled with staggered starts so that the data collections do not occur at the same time. In this

study, calibration standards, stable isotopically labeled internal standards, QC sample and test sample were spiked in synthetic urine. Tandem MS was operated in selected reaction monitor (SRM) mode with heated electrospray ionization (HESI) positive ion mode and specific SRM transitions of precursor ion to product ion were selected for identification and quantitation of each compound. For identical method, a test mixture of four example compounds (Atenolol, Warfarin, Lidocaine, Imipramine) in synthetic urine were analyzed (dilute and shoot) in four HPLC channels. Each HPLC channel used the same liquid chromatographic elution method, the same composition of binary mobile phases from its own solvent bottles, and the same type of analytical column. A total of 1976 samples were run unattended and continuously for about 60 hours. QC samples were inserted in every 30 samples for each channel. For different methods, a precision study was conducted with four different methods using this four channel HPLC (n=40 for each channel; 10 replicates per run, 4 runs per channel). In this study, identical mobile phases and identical analytical columns were used for each channel. Each HPLC channel ran with a different HPLC elution method and a different set of example compounds (Channel 1: Atenolol, Warfarin, Lidocaine, Imipramine; Channel 2: Amphetamine, Methamphetamine; Channel 3: Alprazolam, α-hydroxyalprazolam; Channel 4: Oxycodone, Noroxycodone). **Results and Conclusion:** Within-instrument precision (n=40), all test compounds showed the RSD's of less than 5% in both concentration and retention time; Between-instrument precision (n=40X3 units), showed RSD's of less than 10%. The test results demonstrated that this four channel HPLC can continuously run tests unattended for about 60 hours. In addition to pre-processed samples, samples of less complex matrices can be injected directly with a dilute-and-shoot process. For in vitro diagnostic use. Not available in all countries.

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Development of Liquid Chromatography-Tandem Mass Spectrometry Method for Measuring Plasma Free Metanephrines

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Background: Pheochromocytomas are rare catecholamine-producing tumors of the chromaffin cells of the adrenal medulla. The secretion of catecholamines from pheochromocytomas are episodic, leading to sustained or paroxysmal symptoms, including hypertension, sweating, flushing, and tachycardia. Untreated pheochromocytomas are frequently lethal. The metanephrines (metanephrine, normetanephrine, and dopamine) are metabolites of catecholamines, and are continuously released from chromaffin granules, independent of the episodic secretion of catecholamines. This contributes to the higher sensitivity of metanephrines in screening for pheochromocytomas. Measurement of plasma free metanephrines are recommended as the first-line test in screening for pheochromocytomas. A negative result can exclude a pheochromocytoma. In this study, we aimed to develop a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measuring free metanephrine and normetanephrine in plasma.

Methods: d3-metanephrine and d3-normetanephrine were used as the internal standards (IS). Calibrators and quality controls were prepared by spiking metanephrine and normetanephrine standards into blank serum. 20µL of IS solution containing 12 nmol/L of each IS and 1 mL distilled H_2O were added into 500 µL of calibrators, quality controls and samples, respectively. Mixed samples were subjected to solid phase extraction (SPE) using Phenomenex Strata X-CW polymeric weak cation cartridges. Eluent from the extraction was evaporated under nitrogen at 37°C, and the dried residues were dissolved in 150 µL of 90% acetonitrile/10% H_2O. Injected samples were separated on a Phenomenex Kinetex HILIC Column (50 x 2.1 mm, 2.6 µm) with a flow rate of 0.5 mL/min and a total run time of 7 min using the Shimadzu Nexera X2 UHPLC. The LC gradient started with 95% mobile phase B (Acetonitrile) and 5% mobile phase A (100 mM ammonium formate in H_2O with pH adjusted to 3.5 by formic acid). Mobile phase B was decreased to 85% over 4.25 min and the metanephrines eluted out with good separations in this gradient. Analytes and ISs were detected by the Sciex QTrap 5500 mass spectrometer in positive mode using the following transitions: Metanephrine: 180.2/148.0 (quantifier), 180.2/120.0 (qualifier), 183.1/151.1 (IS); Normetanephrine: 166.1/134.1 (quantifier), 166.1/121.1 (qualifier), 169.1/137.1 (IS).

Results: Our method demonstrated robust linearity for metanephrine and normetanephrine over a concentration range of 0.1 nmol/L – 5 nmol/L with R^2 > 0.999. The total precision displayed CVs < 10% for all QC levels. Limits of quantitation (LOQ) for both analytes were defined at 0.1 nmol/L with CVs < 20%. The SPE extraction recovery for metanephrine was 81-112%, and 43-90% for normetanephrine. About 50% ion suppressions were observed for both analytes due to matrix effect, but the addition of ISs completely compensated for the suppression. This method demonstrated good correlations with the LC-MS/MS method used in ARUP laboratories: Metanephrine: ARUP LC-MS = 1.065 HMH LCMS - 0.018, bias = 0.002 nmol/L, R^2 = 0.96; Normetanephrine: ARUP LC-MS = 0.930 HMH LCMS + 0.023, bias = -0.103 nmol/L, R^2 = 0.99. Assay interference, carryover, sample stability and post-extraction stability were also tested.

Conclusion: We developed a sensitive LC-MS/MS method for plasma free metanephrines measurement. This method showed good analytical performances and is ready for clinical use.

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A Novel Dilute and Shoot LC-MS/MS Method for the Measurement of Nicotine, Cotinine, Nornicotine, and Anabasine in Human Urine.

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Background:

Nicotine from cigarette smoking rapidly diffuses into the blood stream and elicits its desired effect in the brain. The liver then metabolizes nicotine into multiple metabolites which have significance related to nicotine consumption. Cotinine is the primary metabolite of nicotine and is an indicator of exposure to tobacco smoke. If anabasine and nornicotine are present, this is a strong indicator that the patient recently smoked tobacco. Thus, testing for nicotine and its metabolites can be useful for monitoring tobacco usage and nicotine patch effectiveness for patients enrolled in tobacco cessation clinics. Herein, we have developed a simple yet robust dilute and shoot LC-MS/MS method for the quantitation of nicotine and its metabolites.

Methods:

A 25 µL aliquot of internal standards containing deuterated versions of nicotine, cotinine, anabasine, and nornicotine were added to a 100 µL aliquot of sample. This milieu was then diluted by 100 µL of mobile phase (85 % of 20 mM ammonium bicarbonate and 15 % acetonitrile). A 10 µL injection was carried by the mobile phase to the columns, 100 x 4.6 mm EVO C18 (Phenomenex, Torrance, CA) using an LC system from Thermo Fisher Scientific (Waltham, MA). The separated constituents were then analyzed by a 6500 triple quadrupole mass spectrometer (SCIEX, Framingham, MA). The MRM transitions for each analyte were as follows: nicotine 163.1/132.1, nicotine-d4 167.1/136.1, cotinine 177.1/80.1, cotinine-d3 180.1/80.1, anabasine 163.1/120.1, anabasine-d4 167.1/134.1, nornicotine 149.1/130.1, and nornicotine-d4 153.1/134.1. To prevent carryover, a second mobile phase which consisted of 45 % acetonitrile, 45 % isopropanol, 10 % acetone, and 0.1 % ammonium hydroxide flowed through the LC system lines and columns after each analysis.

Results:

The following metrics were used to evaluate the performance of the method: precision, linearity, recovery, and accuracy. Testing for interferences such as bilirubin, hemolysis, drugs of abuse, and common over the counter drugs was also performed. Precision was evaluated on an inter- and intra-run basis. Both series produced a coefficient of variation (% CV) of less than or equal to 10%. A total of 5 concentrations which spanned the analytical measurement range and run in triplicate were used for the linearity study. For each analyte, no measured value exceeded 15% from the expected value and all slopes for each analyte were all well within the 0.9 - 1.1 range. A total of 40 specimens previously analyzed at Mayo Clinic Rochester were used for reference for the accuracy study. All analytes had mean percent differences within ±10% and produced slopes well within the 0.9-1.1 range. Samples ranging from low levels to gross amounts of bilirubin and hemolysis as well as samples with low to high levels of drugs of abuse and common over the counter drugs did not interfere with the analysis.

Conclusion:

The dilute and shoot LC-MS/MS method for nicotine and its metabolites is a robust method with quick sample preparation and can be used clinically to evaluate samples from patients in need of nicotine replacement therapy.

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Development of a Simplified Extraction and High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) Method for Plasma Propofol Quantitation

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Background: Propofol remains a key sedative and amnestic agent utilized in general anesthesia. Owing to its lipophilicity, propofol has a rapid onset allowing expedient induction of deep sedation in emergent procedures. Administration of propofol requires a delicate balance between depth of sedation and the primary side effects of hypotension and respiratory depression taken into the context of its short duration of action. A closed-loop infusion device that adjusts the dose through continuous monitoring of plasma propofol levels to achieve the appropriate depth of sedation has

been developed and tested in rabbits. In order to validate the quantitation accuracy of the device, we developed a liquid chromatography-tandem mass spectrometry assay to serve as a gold-standard for measurement of plasma propofol. Current applications for measuring propofol require significant sample pretreatment, derivatization, or both to achieve high sensitivity and low background interference.

Methods: A total of 6 calibrators and 3 levels of quality controls were prepared by spiking sterile filtered heparinized rabbit plasma with propofol over a concentration range of 0.1-20.0 µg/mL. Samples (500 µL) were combined with propofol-d17 internal standard and diluted 1:1 with de-ionized water before applying to Phenomenex Novum™ 6cc Simplified Liquid Extraction (SLE) cartridges. Analytes were eluted with 2 x 2.5 mL aliquots of methyl tertiary butyl ether. Prior to evaporating the samples to dryness under nitrogen at 25°C, eluents were spiked with 0.5% tetrabutylammonium hydroxide in methanol to prevent loss of propofol. Samples were reconstituted in 75 µL of 50:50 acetonitrile:DI water and injected onto a Nexera X2 UPLC coupled to a AB Sciex 5500 Q-Trap Mass Spectrometer with electrospray ionization in negative ion mode. Separation was achieved using a reversed-phase Hypersil Gold C18 column (100x2.1 mm i.d., 1.9 µm particle size) under isocratic flow of 80:20 acetonitrile:DI water at 0.500 mL/min. Analytes were detected in multiple reaction monitoring mode with the following ion transitions: propofol (177→161 m/z) and propofol-d17 (194→174 m/z).

Results: Propofol and propofol-d17 were eluted within 2.0 min. Propofol calibration curve was linear over the measuring range of 0.1-20.0 µg/mL (R^2>0.996). The LOQ was 0.1 µg/mL with a CV <15% and the LOD was 0.08 µg/mL. The intra- and inter-day precision of the 3.0, 8.5, and 17.0 µg/mL QC was 7.4, 9.5, and 14.2%, and 8.2, 9.4, and 15%, respectively. Accuracy was assessed using spike and recovery experiments at 6 concentrations over the measuring range with recovery ranging from 90-120%. Carryover for the assay was 0.17%. In post-column matrix effect infusion studies both analytes displayed a global reduction in signal of <10% compared to mobile phase alone. SLE cartridges provided significant increases to precision and recovery with a cleaner background in comparison to traditional liquid-liquid extraction. The addition of 0.5% tetrabutylammonium hydroxide to the eluents was essential to increasing sensitivity by raising the signal for both analytes by 3-fold.

Conclusion: We have developed a simple, accurate, and sensitive method for detecting propofol in plasma without the need for derivatization. This method will be used to assess the accuracy of a closed-loop infusion device for propofol during procedural sedation studies.

A-353

Optimization of derivatization reaction used in sample preparation method in analysis of methylmalonic acid in plasma for clinical research.

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Background: Methylmalonic acid (MMA) is small polar molecule that poses challenges for the development of quantitative LC-MS methods. Two analytical approaches have been described in the literature: analysis of methylmalonic acid in negative ionization mode and analysis of derivatized MMA in positive ionization mode. The product of the derivatization reaction with n-butanol shows improved retention to reverse phase chromatographic column and higher ionization efficiency than underivatized MMA, making this method a preferred solution for quantitative analysis. The derivatization reaction parameters described in the literature resulted in variable, up to 100 fold, reaction efficiency demonstrated by large variability in internal standard signal. Several reaction parameters were investigated and optimized to ensure reproducible and efficient butylation reaction of MMA.

Methods: The sample preparation method included protein precipitation followed by derivatization reaction, evaporation and reconstitution. The chromatographic separation was performed using a 2.8-minute isocratic LC method. Methylmalonic acid and its deuterated analog (internal standard) were detected on a hybrid quadrupole-orbitrap mass spectrometer using APCI ionization probe. The mass spectrometry method collected MS/MS spectra for each analyte in a PRM experiment at a resolution of 70K.The following derivatization reaction parameters were optimized: reaction time, temperature, n-butanol additives, and other additives which could catalyze the reaction.

Results: We found that reaction temperature does not have significant effect on reaction efficacy and reproducibility so we recommend that reaction is carried on at room temperature. We also found that extending the reaction time up to 1 hour did not improve reaction performance. The main reaction parameter found to play significant role in reaction efficacy and reproducibility was an additive. Addition of appropriate salt increased reaction efficacy and significantly improved reaction reproducibility, resulting in reproducible internal standard signal (within 10% difference). Implementation of new derivatization reaction parameters allowed us to develop a quantitative method with a limit of quantitation of of 25 nM, precision and accuracy within 10%, and negligible matrix effects.

Conclusion: We demonstrated improved performance of derivatization reaction allowing cost efficient sample preparation method for LC-MS analysis of methylmalonic acid in plasma.

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Development and validation of riboflavin in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry.

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Background: The Riboflavin (vitamin B2) serves as a precursor for coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes participate in a range of reactions of reduction and oxidation. Vitamin B2 is critical for metabolism and energy production. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has high analytical specificity and enables analysis in short period of time. **Objective**: Development a simple, rapid and sensitive LC-MS/MS method to determination of riboflavin in human plasma using deuterated internal standard (IS) and precipitation for prepare the samples. **Experimental**: 100 µL of plasma spiked with 75 µL of deuterated internal standard in acetonitrile was treated with zinc sulfate 0.1M. Chromatograph separation was obtained with a Poroshell 120 EC-CN column (100 mm x 2.1mm x 2.7µm) on an Agilent 1290 HPLC and 6460 Mass Spectrometer system in the positive-ion mode. The MS/MS detection was conducted by monitoring the fragmentation ions of 377.2→243.0 (*m/z*) for riboflavin quantifier, 377.2→172.0 (*m/z*) for riboflavin qualifier and 383.2→249 (*m/z*) for riboflavin-dioxopyrimidine-$^{13}C_4$, $^{15}N_2$ quantifier (internal standard). **Results**: The chromatographic run time was approximately 3.0 min. The linear range obtained for riboflavin was 0.5-50.0 ng.mL^{-1} and dilution was validated for samples that exceed the curve in 2 times. Limit of detection was 0.2 ng.mL^{-1}.The precision intra-day was less than 10% and inter-day was less than 11%. **Conclusion**: A rapid method has been developed successfully for the quantitative analysis of riboflavin in human plasma using a simple prepare of samples without derivatization and with a short run time.

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Development and validation of pyridoxal 5′-phosphate in human plasma by liquid chromatography electrospray ionization tandem mass spectrometry.

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The Pyridoxal 5′-phosphate (PLP) is the active biologically form of vitamin B6. PLP is a cofactor in several enzyme-catalyzed reactions and its deficiency can cause neurological disorders. A rapid LC-MS/MS method was developed for quantitative determination of PLP in human plasma 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* 247.9→150.0 for PLP quantifier, 247.9→94.1 for PLP qualifier and 250.2→152.0 for PLP-d3. Chromatographic separation was performed on an Eclipse plus C18 RRHD column (100 mm x 2.1 mm, 1.8 µm) and isocratic mobile phase water:methanol (98:2, v/v) with 0.1% de formic acid at 350 µL/min. The analyte was measured within 2.1 minutes instrumental run time. The extraction procedure is a simple protein precipitation with trichloroacetic acid 5% using only 100 µL of sample and 25 µL of deuterated internal standard (PLP-d3). The linear range was achieved for PLP at 3.0 - 120.0 µg/L. The medium range of recovery was between 91 and 109% for PLP. Intra-day and inter-day precision ranged were between 1.6-9.6% and 6.6-9.5%. The tests of quantification limits, linearity, precision and recovery were adequate for clinical evaluation. In conclusion, the LC-MS/MS method has been developed and validated successful.

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Matrix Effects Identified and Addressed for Urine Copper, Zinc, and Magnesium Performed by an Inductively Coupled Mass Spectrometry Method

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BACKGROUND Urine trace metal analysis is used for various purposes such as to help diagnose diseases, to monitor for malabsorption or dietary deficiencies, and to monitor exposure. Our laboratory runs an inductively coupled mass spectrometry (ICP-MS) method for quantifying copper, zinc, and magnesium in urine. Recently a significant bias was noticed via proficiency testing program. The investigation started with analyzing a certified NIST sample in nitric acid and no bias was observed. We then investigated whether matrix effect was the root cause for the bias and what modifications to the method could mitigate this problem. **METHODS** The assay was run on a Thermo Fisher X Series2 ICP-MS with a collision/reaction cell controlled by PlasmaLab (ver. 2.6.2.337). The original method employed a 1:10 dilution of patient specimens with 0.1% nitric acid prior to analysis. Additional experiments with dilutions at 1:15, 1:20, 1:25, 1:50, 1:100 were performed and analytical measurement range (AMR) was determined for each. Each AMR determination consisted of analyzing in triplicate a series of samples which included a spiked patient pool and serially diluted samples using 0.5% nitric acid. The acceptable criteria included a signal-to-noise ratio >10, accuracy 80-120%, and CV <20%. The purpose of these experiments was to identify the maximal sample dilution that would reduce the bias but still offer acceptable sensitivity. Statistics were calculated using Excel (Microsoft, Redmond WA, USA). **RESULTS** The 1:50 dilution allowed for a similar sensitivity compared to the original 1:10 dilution while significantly reducing the bias. The original average bias in the proficiency samples was -13% for magnesium, -11% for copper, and -15% for zinc. The average bias using 1:50 dilution was -6.9% for magnesium, 3.5% for copper, and -1% for zinc. **CONCLUSION** Matrix effect for ICP-MS methods could cause significant bias and increased dilution can be used to reduce the matrix effect while maintaining the sensitivity.

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Determination of Whole Blood Selenium by an Inductively Coupled Plasma Mass Spectrometry Method

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BACKGROUND Selenium concentrations in human blood are variable, depending on the selenium content of foods consumed and the regional availability of the element. The richest sources of selenium include seafood, meat, cereals and grains. Both excessive and insufficient intake of selenium can have health implications. Low selenium intake may increase some forms of cancer, is implicated in increased incidence of cardiovascular disease (Keshan), weakening of the immune system, impaired growth, osteoarthritis in children, and fertility. Conversely, selenium toxicity includes gastrointestinal upsets, hair and nail loss, tooth decay, liver failure, skin lesions, fatigue and damage to the nervous system. The primary goal of this study was to develop a high-sensitivity inductively coupled plasma mass spectrometry (ICP-MS) method for quantification of Se in whole blood. **METHODS** This method was developed on a Thermo Fisher X Series2 ICP-MS with a collision/reaction cell controlled by PlasmaLab (ver. 2.6.2.337). Whole blood (1 mL) was added to 9 mL of 0.1% nitric acid, vortex mixed then centrifuged at 3400 g for 5 min. Statistics were calculated using Excel (Microsoft, Redmond WA, USA) and EP Evaluator Release 10 (Data Innovations, South Burlington, VT, USA). **RESULTS** Full technical validation was performed and the assay met the institutional requirements. The linearity of the assay was 13.3 to 563.3 µg/L with analytical recovery from 83.8 to 118.1%. Precision was evaluated based on EP10-A3 protocol. For spiked whole blood samples (N=30) with mean concentrations of 68 µg/L, 127 µg/L, and 190 µg/L, the within run coefficients of variation (CV) were 1.0%, 1.1%, and 1.6%, respectively and the total CV was 1.5%, 2.0%, and 2.5%, respectively. No significant carryover was observed from samples with concentrations up to 723 µg/L. Deming regression was performed using 30 patient samples ranging from 81 to 242 µg/L showed a slope of 0.977 (95%CI: 0.911-1.043), an intercept of 4.2 µg/L (-4.9 to 13.4) and an r of 0.9846 in comparison with a commercial ICP-MS method at NMS Labs (Willow Grove, PA). **CONCLUSION** The high sensitivity and accuracy make this ICP-MS methodology suitable for clinical monitoring of Se.

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Quantification of Plasma Total Testosterone and Dehydroepiandrosterone by LC-Q-Exactive MS

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BACKGROUND: Total testosterone (TT) and dehydroepiandrosterone (DHEA) are measured in adults for androgen abnormalities and in pediatrics for cases of delayed or precocious puberty. The very low concentrations of TT in pediatrics and females made it essential to use mass spectrometry (MS) based methods for high specificity and sensitivity. The high resolution accurate mass (HRAM) capability of the quadrupole-Orbitrap (Q-Exactive) MS can be used to improve specificity, however; for some isobaric compounds separation must occur before the MS. This study aimed to develop a highly sensitive and specific assay using the LC-Q-Exactive MS system for the quantification of TT and DHEA at very low concentrations in plasma. **METHOD:** A Q Exactive quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific) coupled with Thermo Scientific Accela HPLC pump were used. TT and DHEA were extracted with methyl tert-butyl ether (MTBE) from plasma (500µL) after precipitation by mixing the plasma with 0.1M zinc sulphate (200µL) in water and testosterone-d3 (10µL; 225 ng/mL) in methanol. The supernatant was evaporated at 37°C under a stream of nitrogen then derivatized with hydroxylamine (100µL; 100 mg/mL) for 30 min. The resulting solution was extracted again with MTBE, dried down at 37°C under a stream of nitrogen and reconstituted with 125µL of (1:1 MeOH:H2O). The final solution (25µL) was injected onto an Accucore C18 (50 x 2.1mm, 2.6µm; Thermo Fisher Scientific, Waltham, MA). The mass spectrometer was set at positive heated electrospray ionization in the parallel reaction monitoring (PRM) mode. The MRM transitions (m/z) were 304.23>124.07 and 304.23>112.07 for TT, 307.23>124.07 and 307.23>112.07 for d3-Te, and 304.23>253.19 for DHEA. Statistics were calculated using EP Evaluator v10 and Microsoft Excel. **RESULT**: The LC run time was 5.5 minutes per injection. Testosterone and DHEA were separated both chromatographically and with unique transitions post-derivatization. TT was linear from 50-15240 pg/mL using transition 307.3>124 and DHEA was linear from 0.11-60.70 ng/mL using transition 304.2>253 with analytical recovery ranging from 86-118% for all compounds. Within-run and total CVs were < 5.5% and 7.5%, respectively for TT and < 26% and 21%, respectively for DHEA. Method comparison with an immunoassay was completed for TT. The Deming regression statistics for the comparison were as follows: range 11.80-980.80 ng/dL, slope 1.090, intercept 26.39, SEE 62.77, and correlation coefficient 0.9820. **CONCLUSION**: An accurate and precise LC-Q-Exactive method for total testosterone and DHEA was developed.

A-360

High-Throughput LC-MS/MS Measurement of Pregnenolone in Human Blood Serum for Research Purposes

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Background: Pregnenolone is a biosynthetic precursor to other steroids such as corticosteroids, androgens, and estrogens. It is converted to progesterone by 3-beta-hydroxysteroid dehydrogenase or to 17-OH-pregnenolone by 17-alpha-hydroxylase. Researchers investigating how these enzymes function need to quantify pregnenolone within an analytical range of 10 to 500 ng/dL (0.3 to 1.5 nmol/L) in blood serum. Since pregnenolone does not ionize well by either atmospheric-pressure chemical-ionization (APCI) or electrospray ionization (ESI), derivatization with hydroxyl amine was necessary to reliably achieve the desired analytical range.

Methods: Pregnenolone was measured in blood serum using a multi-channel ultra high-performance liquid chromatography (UHPLC) coupled to a triple-quadrupole mass spectrometer (MS) with heated electro-spray ionization (HESI). Sample preparation involved extracting specimens with methyl-t-butyl ether after spiking them with pregnenolone-D_4 internal standard (IS). The extracts were evaporated and the residues were reacted with hydroxyl amine to form positive-ion oxime derivatives. The preparations were dried and reconstituted with water and methanol (1:1). Injections were made into a 4-channel UHPLC system. A 4.5-minute water-to-methanol gradient eluted the analyte and IS through a heated column, packed with solid-core silica with phenyl groups bonded to its surfaces, into the HESI source. Selective-reaction monitoring (SRM) within a 1-minute data window produced quantitation and conformation chromatographic peaks.

Results: Confirmation/quantitation ion ratios among calibrators, QCs, and specimens were within 20% of averages calculated from the calibrators. Method precision, assessed as percent coefficient of variation (%CV) of peak areas from 20 replicate injections of two pools of test specimens, were better than 5% and 8% for intra- and inter-batches, respectively. Carryover, measured in blanks immediately following injections of the highest calibrator, never exceeded 0.2%. Specimen IS peak areas averaged 65% relative to the averaged IS peak areas in calibrators and QCs, indicating moderate ion-suppression by matrix. However, the IS in each sample successfully compensated for matrix effects as proved by method comparison results. The desired analytical range from 10 to 500 ng/dL (0.3 to 1.5 nmol/L) was achieved and was consistently linear ($r^2 \geq 0.999$ with 1/X weighting). For method comparison, 40 donor samples were analyzed and results were compared with those from a reference lab. Pregnenolone values among these samples ranged from 13 to 130 ng/dL and the percent difference between two analytical methods did not exceed 20% for 93% of the samples. Sample throughputs were 13, 26, 39 or 52 injections per hour when multi-channeled across 1, 2, 3 or 4 channels, respectively.

Conclusion: We developed a sensitive, robust, high-throughput quantitation assay for pregnenolone which can measure 10 to 500 ng/dL (0.3 to 1.5 nmol/L) in blood serum. The LC-MS/MS method can be multi-channeled with other HESI-MS/MS methods.

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High-Throughput LC-MS/MS Measurements of Estrone (E1) and Estradiol (E2) in Human Blood Serum for Research Purposes

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Background: Estrone (E1) and estradiol (17-β-estradiol or E2) are two steroid hormones involved in the development and function of female anatomical and physiological characteristics and processes such as the menstrual cycle. Researchers studying the effects of E1 and E2 on such things need to quantify them within an analytical range of 5 to 500 pg/mL (18.5 to 1,850 pmol/L) in blood serum. E1 and E2 form negative ions by deprotonation in both electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) sources of mass spectrometers with low efficiency. In order to robustly achieve the needed quantitation limits, most researchers use dansyl chloride to form positively charged derivatives of these and other estrogens.

Methods: E1 and E2 were measured in blood serum using a multi-channel ultra high-performance liquid chromatography (UHPLC) coupled to a triple-quadrupole mass spectrometer (MS) with heated electro-spray ionization (HESI). Sample preparation involved extracting specimens with methyl-t-butyl ether after spiking them with estradiol-D_5 internal standard (IS). The extracts were evaporated and the residues were reacted with dansyl chloride to form positive-ion dansylated derivatives. The preparations were dried and reconstituted with water and acetonitrile (1:1). Injections were made into a 4-channel UHPLC system. A 5.5-minute water-to-methanol gradient eluted the analyte and IS through a heated column, packed with solid-core silica with hydrocarbon groups bonded to its surfaces, into the HESI source. Selective-reaction monitoring (SRM) within a 1-minute data window produced quantitation and conformation chromatographic peaks.

Results: Confirmation/quantitation ion ratios among calibrators, QCs, and specimens were within 20% of averages calculated from the calibrators. Method precision, assessed as percent coefficient of variation (%CV) of peak areas from 20 replicate injections of two pools of test specimens, were better than 6% and 7% for intra- and inter-batches, respectively. Carryover, measured in blanks immediately following injections of the highest calibrator, never exceeded 0.5%. Specimen IS peak areas averaged 37% relative to the averaged IS peak areas in calibrators and QCs, indicating high ion-suppression by matrix. However, the IS in each sample successfully compensated for matrix effects as proved by method comparison results. The desired analytical range from 5 to 500 pg/mL (18.5 to 1,850 pmol/L) was achieved and was consistently linear ($r^2 \geq 0.995$ with 1/X weighting). For method comparison, 40 donor samples were analyzed and results were compared with those from a reference lab. Values among these samples ranged from 16 to 156 pg/mL for E1 and 11 to 356 pg/mL for E2 and the percent difference between two analytical methods did not exceed 20% for 95% of the samples. Sample throughputs were 10, 21, 32 or 43 injections per hour when multi-channeled across 1, 2, 3 or 4 channels, respectively.

Conclusion: We developed a sensitive, robust, high-throughput quantitation assay for estrone and estradiol which can measure 5 to 500 pg/mL (18.5 to 1,850 pmol/L) in blood serum. The LC-MS/MS method can be multi-channeled with other HESI methods.

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Analytical and preanalytical validation of simultaneous measurement of estrone and 17beta-estradiol in serum by LC-MS/MS

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Background:MALDI-TOF MS is increasingly used for routine bacterial and fungal identification in Japan, whereas application of LC-MS/MS in clinical chemistry laboratories has remained very limited. Although immunoassays are often used for measurement of serum estradiol (E2) when fast turnaround time is required, more sensitive and specific measurements are needed for determination of menopausal status, estrogen deficiency and in the diagnosis of sex hormone related disorders. Furthermore, simultaneous measurement of estrone (E1) and E2 is often requested particularly from gynecologic oncologists. Indeed, increased risk of endometrial cancers has been shown in subjects with high serum estrogen levels. The aim of this study is to develop and validate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous measurement of E1 and E2 in human serum.

Methods:For sample preparation, 100μL of calibration solution, QC sample or patient serum were diluted, spiked by 20μL of internal standard (10ng/mL) and were loaded on the supported liquid extraction (SLE) plate and were then extracted by using 1.8 mL of extraction solution. The extracted samples were dried under nitrogen and were derivatized by dansyl chloride acetone solution. An aliquot for 40μL was then subjected to LC-MS/MS.The analytes were separated on a CAPCELL CORE C18 column (Shiseido) that was attached to the Bruker UPLC. A water/acetonitrile solvent gradient was used to achieve chromatographic separation of the E1 and E2 in 10 minutes.The selected reaction monitoring (SRM) was performed with a Bruker EVOQ Elite in electrospray ionization (ESI) and positive ion mode. The SRM transitions were m/z 504>171 for E1 and 506> 171 for E2. Serum E2 levels were also determined by immunoassay routinely used in our clinical laboratory (Architect CLIA, Abbott).

Results:The lower limit of quantifications of E1 and E2 were 6.2pg/mL and 7.3pg/mL, respectively. The analytical mesurement range for E1 was 6.2-1200pg/mL and 7.3-1600pg/mL for E2. Intra-assay CVs (n=20) were 2.7% (at 52.6pg/mL) for E1 and 6.4% (at 33.5pg/mL) for E2 , and inter-assay CVs (n=20) were 5.5% (at 52.6pg/mL) for E1 and 6.2% (at 33.5pg/mL) for E2. The recoveries were 98.6-100.8% for E1, 99.7-99.8% for E2. The accuracy was found to be within specified limits of BCR576, BCR577 and BCR578. There were no significant interference by the marked hyperbilirubinemia, hemolysis and chye. There were no effects by the up to 10 times of freeze-thaw cycles and there were no differences among the E1 and E2 values obtained using 6 different types of blood collection tubes.Method comparison studies for samples with E2 300pg/mL showed: [CLIA E2] = 0.8598[LC-MS E2] +9.9988 (n= 19).

Conclusion:We have developed and validated simultaneous measurement of estrone and 17beta-estradiol in serum by LC-MS/MS. We are now planning to use this method for E1 and E2 measurement on a routine basis in our university hospital.

A-363

Noninvasive prenatal diagnosis of fetal RhD status from RhD negative pregnant women using MALDI-TOF Mass Spectrometry

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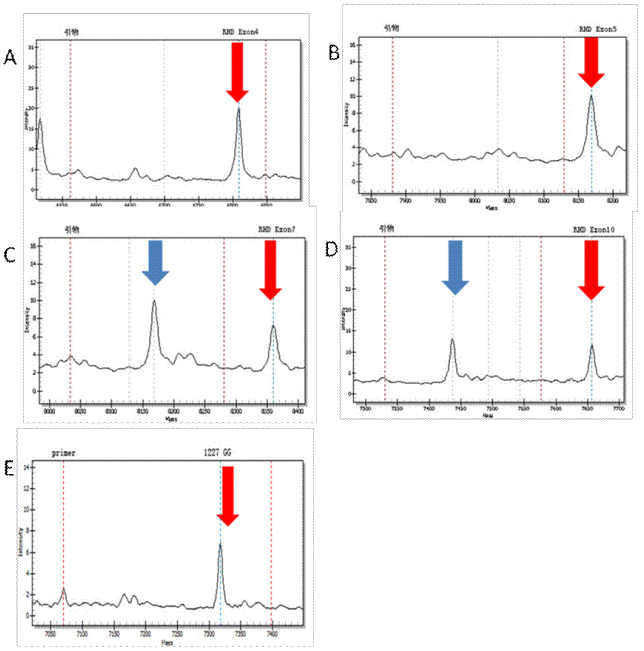
Background: Noninvasive prenatal genotyping of fetal RHD can prevent the unnecessary administration of prophylactic anti-D to women carrying RhD-negative fetuses. We establish a method for noninvasive prenatal diagnosis of fetal RHD genotyping from Chinese RhD-negative women based on matrix-assisted laser ionization time of flight mass spectrometry(MALDI-TOF MS).

Methods: RhD negative pregnant women with single fetus (20~40 gestational weeks) were recruited from August 2013 to March 2015. The existence of fetal DNA was confirmed by SNPs. Fetal RhD genotype was detected by MALDI-TOF MS targeting exon 4, 5, 7, 10 and RHD 1227A to predict the fetal RhD status. A double blind trial was carried out to compare the results of fetal RhD detected by MALDI-TOF MS and serological tests on cord blood.**Results:** A total of 40 plasma samples were collected. Fetal RhD genotype was detected by MALDI-TOF MS targeting RHD exon 4, 5, 7, 10 and RHD 1227A. 38 cases were identified RhD positive when one case was typed RhDel and one was RhD-negative. Five neonatal blood samples could not be obtained

due to loss to follow up, and the remaining 35 genotypings were in concordance with newborn D phenotypes at delivery.

Conclusion: These preliminary results demonstrate the feasibility of noninvasive prenatal diagnosis of fetal RhD status from RhD-negative maternal plasma in Chinese population using MALDI-TOF MS.



A-366

IntrinsiX™ - A novel calibration approach for performing quantitative LC-MS/MS analysis of serum methotrexate for clinical research

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Background: Batch mode analysis has limited the utility and throughput of quantitative LC-MS/MS assays. Here we describe the novel IntrinsiX™ workflow applied to the analysis of methotrexate in serum using ^{13}C-labeled analogs of methotrexate as internal calibrators. An accurate and precise quantitative result is generated in a single injection, eliminating the need to analyse a traditional set of external calibrators.

Methods: Cerilliant (Round Rock, Texas, USA) supplied four ^{13}C-labeled analogs of methotrexate that were designed to minimise isotopic interference. The analogs were used to prepare a 4-point IntrinsiX calibration curve over the range 0.025 - 10 µmol/L. IntrinsiX calibrators were added to each serum sample (50µL) and proteins precipitated using methanol. Following centrifugation, the supernatant was diluted and injected onto a Waters HSS-SB C18 UPLC column (2.1x30mm, 1.8µm) using a Waters ACQUITY UPLC® I-Class and quantified with a Xevo® TQD mass spectrometer.

EQA samples supplied by NEQAS (Nottingham, UK; n=14) and WEQAS (Cardiff, UK; n=9) were analyzed using an in-house conventional LC-MS/MS method, in which six non-zero calibrators are used for quantification, and the results compared with the new IntrinsiX approach.

Results: Following CLSI EP6-A the calibration range was shown to be linear from 0.0175 - 13.0µmol/L, with no detectable carryover up to 100 µmol/L. Coefficients of variation for inter- and intra-method imprecision for 0.1µmol/L, 1.0µmol/L, 2.5µmol/L and 7.5µmol/L samples were all ≤ 6.8% (n=25, days=5).

The agreement between the new IntrinsiX approach and the conventional LC-MS/MS method for the analysis of the EQA samples was described by the Deming equation y=-0.99x-0.02 (n=23, range 0.025 –2.18µmol/L), demonstrating significant constant bias with no proportional bias (p>0.05). The correlation between the new IntrinsiX approach and the all laboratory trimmed mean (ALTM) for the EQA results was described by the Deming equation y=0.94x+0.03 (n=14, range 0.030 - 2.14µmol/L), again demonstrating significant constant bias with no proportional bias (p>0.05).

Interference testing demonstrated a mean recovery of 101% for both endogenous compounds and metabolites tested. Following CLSI EP7-A2, recovery of samples containing 0.1 and 1.0µmol/L methotrexate (n=3) were unaffected (mean 101.0%, range 95.5 - 106.3%) when co-spiking with high concentrations of endogenous compounds (albumin, bilirubin, cholesterol, triglycerides and uric acid) and Intralipid®. Similarly, recovery was unaffected (mean 101.1%, range 98.2 - 104.9%) when methotrexate pools were supplemented with 5 and 50µmol/L 7-OH methotrexate (n=3) and 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA; n=3), showing absence of interference from these metabolites.

Conclusions: We have successfully implemented a novel calibration approach for performing quantitative LC-MS/MS analysis of serum methotrexate for clinical research. Incorporating the calibrators into each test sample allows improved throughput, shorter time to first result and the possibility of a workflow that does not require samples to be grouped into batches. Additionally, each sample is perfectly matrix-matched as demonstrated by the excellent results of the interference testing (mean bias 101%).

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

A-367

Determination of Estetrol in Human Plasma by a Validated LC-MS/MS Method

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Background: Estetrol (E_4), or 15α-hydroxyestriol, is an estrogen steroid hormone, found in detectable levels in maternal plasma at around week 20. It is produced by the fetal liver during pregnancy and reaches the maternal circulation through the placenta. E_4 is detected from the 9-th week of pregnancy in maternal urine and after the second trimester levels in maternal plasma rise steadily with concentrations of unconjugated E_4 to about 1 ng/mL towards the end of pregnancy. So far the physiological function of E_4 has not been studied and is unknown. The possible use of E_4 as a marker for fetal well-being has been studied quite extensively. This paper presents the development and validation of LC-MS/MS determination of E_4 with the aim to be applied in the course of a food effect study.

Methods: E_4 and Estetrol-d_4, internal standard (d_4-E_4) were extracted from human plasma with Ethyl acetate and derivatized with dansyl chloride. Chromatographic separation was performed on C18 analytical column with gradient elution utilizing mobile phases consisting of acetonitrile (different proportions), water and formic acid Positive electrospray ionization and multiple reaction monitoring were used to follow the predominant transitions: collision energy 36, m/z 538→171 for E_4, and m/z 542→171 for d_4-E_4. Raw data of mass chromatograms were collected and processed by specialized software, and weighted ($1/X^2$) linear regression was performed to determine the concentration of E_4. Validation strategy was strictly adhered to current industrial guidance.

Results: Selectivity was assessed with 8 individual sources of human plasma (including one lipemic and one hemolyzed) and confirmed with matrix effect (ME) averaging 94-102% for E_4, 93-105% for d_4-E_4, and relative ME of 97-102%. Accuracy ranged from -3.90 to 1.84 % within runs and from 1.04 to 3.86 % between runs. Precision was up to 6.84 % within-runs, and up to 9.99% between-runs. Linearity was assured with 8 point calibration curve in the range 0.0259 ÷ 25.9480 ng/mL, $R^2 > 0.99$, y=3.906x + 0.021. Lower limit of quantification was set at 0.0259 ng/mL with accuracy and precision of less than 10% for both within runs and between runs. Freeze-thaw stability was determined for five cycles each lasting 24 h, post-preparative stability was documented for 48 h at 10ºC, short-term stability at room temperature was proven for 4h at daylight; stock solution stability and long term stability in plasma were documented for 154 days at -20ºC. With run time of 2.0 min, a throughput of over 170 samples per working day was achieved.

Conclusion: The method was validated according to current industrial requirements and allows the accurate and precise determination of E_4 in human plasma.

Key words: Estetrol, LC-MS/MS

Word count: 427

A-368

A Rapid LC-MS/MS Method for the Quantification of Lacosamide, Desmethyl Lacosamide, Gabapentin, Clozapine, and Topiramate

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Background: Lacosamide (LCM), Gabapentin (GAB), and Topiramate (TOP) are antiepileptic drugs approved by the FDA for treatment of epilepsy. Lacosamide is metabolized to the O-desmethyl lacosamide (ODL), the major metabolite in human. Clozapine (CLZ) is an atypical antipsychotic medication primary used for the treatment of schizophrenia. Measurement of these drugs has been performed by liquid chromatography-ultraviolet (HPLC-UV), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and in some cases enzyme immunoassay. However HPLC-UV methods can suffer from longer run times and potential interferences while enzyme immunoassays can be relatively expensive. LCM, ODL, GAB, and CLZ have a high ionization efficiency in positive electrospray ionization mode, while TOP has a poor ionization efficiency in positive mode which necessitates negative electrospray ionization. Our objective was to develop a rapid, accurate, and sensitive LC-MS/MS assay for the quantification of LCM, ODL, GAB, CLZ, and TOP. **Methods:** Serum (25µL) and IS solution (150µL; lacosamide-$^{13}C,D_3$, Gabapentin-D_{10}, Clozapine-D_4, and Topiramate-D_{12} in methanol) were vortex mixed and centrifuged. Supernatant (10µL) was added to 1000µL of 0.1% formic acid in water and vortex mixed then 3µL was analyzed on Accucore C18 column in an LC-MS/MS system. Total chromatographic time was 1.90 minutes with a ionization polarity switch at 0.90 minutes. A quantifier and a qualifier transition were monitored for all analytes. **Results:** No differential matrix effect or interferences were observed. Analytical Measurement Range (AMR) date for all 5 analytes is presented in Table 1. The total coefficient of variation was <4.7% for LCM, <5.6% for ODL, <4.4% for GAB, <5.5% for CLZ, and <7.2% for TOP at three levels tested. **Conclusion:** This rapid and sensitive LC-MS/MS assay meets the sensitivity, accuracy, and precision requirements for clinical use.

Table 1: AMR Data

Analyte (Units)	Analytical Measurement Range	% Recovery
Lacosamide (µg/mL)	0.5 - 48.1	84.4 - 100.8
Desmethyl Lacosamide (µg/mL)	0.6 - 52.3	87.5 - 106.1
Gabapentin (µg/mL)	0.5 - 47.6	87.1 - 99.5
Clozapine (ng/mL)	20.7 - 2113.2	82.7 - 105.7
Topiramate (µg/mL)	1.0 - 50.8	80.8 - 101.6

A-369

Determination of Monosialogangliosides in Human Plasma by a Novel UPLC/MS/MS Assay in Combination with Chemical Derivatization

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Objective To develop a LC/MS/MS method and quantitatively monitor the plasma level of monosialogangliosides in patients affected by GM3 Synthase Deficiency (GSD), an inherited neurological disorder characterized by seizure and profound developmental stagnation, for clinical diagnosis and therapeutic evaluation of an ongoing clinical trial. **Clinical Relevance** Gangliosides are a large subfamily of glycosphingolipids that present abundantly on the plasma membrane of neuronal and glial cells of vertebrates. These molecules are structurally characterized by a distinctive oligosaccharide moiety being attached to a ceramide portion with variable length and saturation degree on the fatty acid chains. Physiologically, they play critical roles in the regulation of various receptor-mediated cell signaling pathways and essential cellular events. Disruption in their metabolic pathways is pathologically implicated in the development of numerous neurodegenerative disorders, such as Parkinson disease, Alzheimer disease, and ganglioside GM3 synthase deficiency (GSD). In order to more comprehensively understand the disease etiologies, a reliable LC/MS/MS method with enhanced sensitivity is urgently demanded for relevant biomedical studies. **Methodology** In this study, a novel reverse phase UPLC/MS/MS method for determination of monosialogangliosides, GM1, GM2, and GM3, in human plasma has been developed and validated. This assay employed DMTMM & PAEA chemical derivatization for signal enhancement and deuterium-labeled monosialogangliosides as internal standards (IS). The analytes and ISs were extracted from plasma using protein precipitation procedure, cleaned up with liquid-liquid partition, dried under nitrogen purging, and derivatized with 2-(2-Pyridilamino)-ethylamine (PAEA) & 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM). Thereafter, the samples were injected into a Shimadzu Nexera UHPLC system interfaced to an AB Scix Qtrap 5500 mass spectrometer that operated in ESI positive and Multiple Reaction Monitoring (MRM) mode to achieve detection with superior sensitivity and specificity. **Validation** Considering that the m/z from singly charged molecular ions of monosialogangliosides were mostly beyond the detection range of our Qtrap 5500 mass spectrometer, and they showed low preference to be doubly charged by both positive and negative ESI, we introduced a novel DMTMM & PAEA chemical derivatization method to increase the abundance of their doubly charged molecular ions in positive ESI. The sensitivity of monosialogangliosides in positive ESI was observed to undergo a 15-20 fold enhancement after derivatization. In addition, more than 15 different components were chromatographically resolved from each other within an 11min run. Moreover, calibration curves ranging from 10~2000, 10~2000, and 80~16000 ng/ml with correlation coefficients of 0.9981, 0.9989, and 0.9977 were established for plasma measurements of monosialogangliosides GM1, GM2, and GM3, respectively. Thereafter, we validated this assay based on the FDA guideline for bioanalytical method validation on precision, accuracy, stability, and extraction recovery. The relative percent error (R.E.) and coefficient of variation (CV) from measurements were below 11 and 11% for each monosialoganglioside species. The extraction recovery was found to be above 80% for each monosialoganglioside species. The loss of derivatized analytes from storage was found insignificant (<10%) under studied conditions. **Conclusion** In summary, we developed and validated a novel quantitative assay for determination of monosialogangliosides in human plasma using LC/MS/MS, which has been successfully applied to the ongoing clinical study.

A-370

Development, Optimization, and Evaluation of an Ultra Performance Liquid Chromatographic-Tandem Mass Spectrometric (UPLC-MS/MS) Method for the Quantification of the Anti-Malarial Atovaquone in Plasma

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Background: Malaria is a life-threatening parasitic disease primarily spread through the bites of infected mosquitoes. Malarial parasites are released into the bloodstream, resulting in infection of both liver cells and erythrocytes. A primary modality in disease treatment is the administration of anti-malarial agents. One prophylactic drug, atovaquone (ATQ), has been used both in single- and multi-drug applications for disease treatment. Early pharmacokinetic studies have demonstrated high inter-individual drug variability. Thus, there is a significant need to monitor plasma drug concentrations to fill in pharmacological gaps. With the scarcity of analytical methodologies available in the literature, we have developed and optimized an ultra-performance liquid chromatographic-tandem mass spectrometric (UPLC-MS/MS) method for the robust quantification of ATQ in human plasma.

Methods: ATQ and its deuterated standard, atovaquone-d5 (ATQ-d5), were acquired from Toronto Research Chemicals. Calibrators and quality control solutions were prepared by spiking both compounds into drug-free K_2EDTA human plasma (Biological Specialty Corporation). Following protein precipitation, samples were evaporated to dryness and reconstituted in 20:80 water:acetonitrile containing 0.1% formic acid. Samples were separated on a Synergi 2.5 µm Polar-RP 100A (100 x 2 mm) column (Phenomenex). ATQ was detected over 1.3 minutes on an API 4000 mass analyzer (SCIEX) using an ESI source operated in negative ionization and selected reaction monitoring (SRM) modes. The method was validated in accordance with the FDA Guidance for Industry Bioanalytical Method Validation recommendations. Validation metrics included the assessment of precision and accuracy, linearity, stability, and matrix effects.

Results: Parent to product ion transitions for both ATQ and ATQ-d5 were identified through the direct infusion of the compounds into the mass analyzer. Transitions identified for monitoring in SRM mode for ATQ and ATQ-d5 were 365.1-198.9 and 370.0-204.1 *m/z*, respectively. Due to pharmacokinetic parameters associated with ATQ, two calibration curves were generated to quantify the drug at both lower and higher (therapeutic) concentrations. Thus, two analytical measuring ranges were established at 5-1000 ng/mL and 1000-25000 ng/mL, respectively. Linearity was assessed from the slope of $1/x^2$ (sub-therapeutic range) and 1/x (therapeutic range) weighted least squares-fitted linear regression analysis. Representative calibration curves yielded regression equations with r^2 values of 0.9971 and 0.9978, respectively. Each curve required 0.05 mL samples of calibrator, quality control (QC) or unknown. For higher concentrations, samples were diluted 10-fold with drug-free plasma prior to protein precipitation. QC materials for both lower and higher ranges were prepared at low (15 ng/mL, 3000 ng/mL), mid (150 ng/mL, 8750 ng/mL) and high (850 ng/mL, 22500 ng/mL) concentrations, respectively. Pre-validation intra-assay precision and accuracy studies demonstrated values within expected thresholds defined by regulatory guidelines.

Conclusion: This work describes the development, optimization, and analytical evaluation of a UPLC-MS/MS method for ATQ quantification in plasma. The assay can be run in a reflexed fashion by which samples with ATQ concentrations not detectable by the higher, therapeutic range calibration would be subsequently run using the lower, sub-therapeutic calibration. In contrast to previous methods, the presented method requires low sample volumes, has a limit of quantitation of 5 ng/mL, and an analytical run time of 1.3 min.

A-371

Validation of an LCMS Method for Chiral Determination of Methamphetamine

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Background: Methamphetamine is a highly addictive and abused stimulant drug due to its potent stimulation of the central neural system. Several prescription formulations of d-methamphetamine are available. Patients who are on a drug monitoring program may test positive for methamphetamine during their drug confirmation. Physicians need to ensure their patient is not using over-the-counter inhaler formulations which contain l-methamphetamine. l-methamphetamine is a vasoconstrictor intended as a decongestant. d-methamphetamine is a major drug trafficking concern due to illegal illicit drug sales. There is a need for robust methodology with simple sample preparation in the clinical laboratory to support care-provider decisions. Isomeric chirality determination by separation of "l" and "d" stereoisomers may support putative decisions regarding licit or illicit drug. While GCMS is known for its high chromatographic resolution and ability to separate isomers through a derivatization process, labs may not have access to this instrumentation. Furthermore, a simple sample preparation process is desired for routine testing. **Methods:** An LCMS method was validated using an Agilent (Santa Clara, CA) 1200 liquid chromatograph coupled with a 6400 tandem mass spectrometer using electrospray ionization in positive mode. Our laboratory has evaluated several methodologies for isomeric determination of methamphetamine. With that experience, it has obviated the need for simple sample preparation, along with a rugged analytical testing methodology. To that end, we developed a crash-dilute-shoot (CDS) sample preparation method compatible with a chiral stationary phase analytical column. Sample preparation involved a 50 microliter aliquot of patient urine with 50 microliters of internal standard solution. This was homogenized with equal parts methanol for precipitation, followed by 30 minute centrifugation. The final dilution step resulted in an overall 1:20 sample:dilution. The column is an Astec Chirobiotic Supelco (Sigma-Aldrich, St. Louis, MO) dimensions of 150 mm x 4.6 mm with 5 µm particle size. A 5 mm C18 guard column (Agilent) was installed to preserve the column from patient specimen testing. An isocratic chromatograph (flow rate 1 milliliter/minute) resulted in elution of both isomers under 9 minutes. Mobile phase was 95% methanol with 0.1% acetic acid and 0.04% ammonium hydroxide. Mass spectrometric conditions were in multiple reaction monitoring for methamphetamine precursor 150.1 *m/z* and transitions 119.1, 91.1 *m/z*. The methamphetamine internal standard precursor was 155.1 *m/z* with transition 121.1 *m/z*. The dwell time was set to 200 milliseconds for each transition. A five level calibration curve was prepped in the same manner as patient samples for quantitation. **Results:** The calibration yielded linear quantitation curves for both isomers of methamphetamine. Controls were indicated at 50% below LOQ for both isomers, along with a +50% control for d methamphetamine and +50% for l methamphetamine. A negative urine control is included with each batch. Internal validation results gave excellent correlation versus the online/validated method of isomer separation by chiral selective cyclodextrin by capillary electrophoresis tandem mass spectrometry. The method has been qualified for clinical testing of patient urine specimens for isomeric determination of methamphetamine. **Conclusion:** This method is a robust and practical assay for the determination of methamphetamine isomer in the clinical laboratory.

A-372

Development and Validation of a Novel LC/MS/MS Method for the Quantification of Red Cells Methotrexate Polyglutamates

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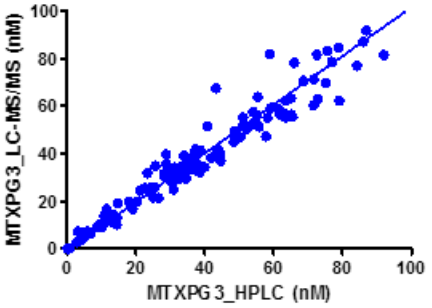
Objective: Therapeutic drug monitoring of red blood cells (RBC) methotrexate polyglutamates (MTXPG) is recognized as a valuable tool to optimize exposure to methotrexate in autoimmune rheumatic diseases. Our objective was to develop and validate a novel LC/MS/MS and establish its concordance with our reference method that uses HPLC with post column photo-oxidation and fluorimetric detection.

Methods: The LC/MS-MS consisted of a TSQ Quantiva (Thermofisher, Uppsala, Sweden) system. The chromatographic method used a pentafluorophenyl 2.1x50mm, 2.6 µm particle column, with mobile phase consisting of 0.1% formic acid and 0.01% trimethylamine with acetonitrile gradient. The sample treatment procedure used 100 µl RBC and consisted of a deproteinization step with perchloric acid in the presence of deuterated MTXPG3 as the internal standard. A 10 µl perchloric acid extract was injected onto the LC/MS/MS system following centrifugation. Sample analysis was performed in positive ionization with an m/z 713→308 transition for MTXPG3, the preponderant MTXPG in RBC. The run time was 6 minutes (vs 30 minutes for the reference method). The LC/MS/MS method was compared to our reference using RBC lysates obtained for routine testing in our clinical laboratory. Analysis consisted of Deming's regression slope and linear regression correlation coefficient.

Results: The analytical performance of the LC/MS/MS method consisted of intra-day and inter-day coefficient of variation below 15% at three different concentrations ranging from 5 to 100 nmol/L RBC. Detection limit was 1 nmol/L RBC. Using a total of 130 RBC lysates, there was a good concordance between MTXPG3 levels determined using the LC/MS/MS when compared to those determined using the reference method (Deming's slope = 1.026; Regression coefficient = 0.9525).

Conclusion: The LC/MS/MS method developed is equivalent to our reference method and can be used in clinical practice to optimize MTX dosing.



A-373

Development and Validation of Simultaneous Measurements of Four Vitamin D Metabolites in Serum by LC-MS/MS for Clinical Laboratory

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Background: Mass spectrometry (MS) is a powerful analytical tool in clinical laboratories. MALDI-TOF MS is used for bacterial and fungal identification on a routine basis in an increasing number of microbiology laboratories in Japan, whereas application of LC-MS/MS in clinical chemistry has remained very limited. The measurement of serum 25-hydroxyvitamin D [25(OH)D] as an indicator of vitamin D status is still widely conducted by immunoassays. Compared to other techniques, the advantages of LC-MS/MS include ability to simultaneously measure multiple analytes and the need for small sample volume for analysis, which in turn enhances the diagnostic capabilities of LC-MS/MS. The aim of this study is to develop and validate LC-MS/MS method to simultaneously measure four vitamin D metabolites in serum for application in clinical laboratories.

Methods: For sample preparation, 280µL of internal standard solution was added to 20 µL of calibration solution, QC sample or serum and were vortexed for one minute. The samples were applied to 96 well supported liquid extraction plate and were incubated for five minutes. The analytes were eluted three times with 700uL of ethyl acetate:hexane mixture (50:50, v/v). After elution, solvent was evaporated to dryness under nitrogen at 45 °C and was derivatized by the Cookson-type reagent (DAPTAD) that rapidly and quantitatively reacts with the s-cis-diene structure of vitamin D metabolites, and can markedly enhance the ionization efficiency. The derivatized samples were evaporated to dryness and then, the residue was dissolved in 23 µL of 30% v/v CH3CN containing 0.1% v/v HCOOH, and 20 µL sample was injected to LC-MS/MS.

Reconstituted samples were introduced from Shiseido HPLC to Thermo Fisher TSQ Vantage. Vitamin D metabolites were separated on a Shiseido core shell column

(CAPCELL CORE C18, 2.1×75mm). The column temperature was maintained at 40 °C and run time was 5.5-min. Samples were ionized using an electrospray ionization (positive-ion mode) and ions were detected by selected reaction monitoring.

Results: The LLOQs were 0.091, 0.020, 0.013, 0.024 ng/mL and the analytical measurement range was up to 59.4, 25.8, 66.1, 200 ng/mL for 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2 and 24,25(OH)2D3, respectively. The accuracy was found to be within specified limits of NIST SRM 972a. The intra-assay CVs were 0.9%, 3.0%, 4.4% and 3.8% for the low level 25(OH)D3 samples (7.16 ng/mL) and 1.9%, 3.1%, 3.5% and 1.6% for the medium level 25(OH)D3 samples (18.3 ng/mL) for 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2 and 24,25(OH)2D3. The inter-assay CVs for 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2 and 24,25(OH)2D3 were 2.7%, 5.7%, 5.6% and 6.6% for the low and 2.6%, 5.8%, 6.1% and 3.2% for the medium, respectively. The extraction recoveries were 97.5%-99.0%, 93.3%-103.9% and 92.8%-100.5% for 25(OH)D3, 3-epi-25(OH)D3 and 25(OH)D2, respectively.

Conclusion: We have developed and validated simultaneous measurements of the four vitamin D metabolites 25(OH)D3, 3-epi-25(OH)D3, 25(OH)D2 and 24,25(OH)2D3 in serum by LC-MS/MS. This method will be useful to obtain accurate quantitative data of these four vitamin D metabolites for clinical samples.

A-376

Amino acid analysis by mass spectrometry for concentration determination of C-reactive protein in candidate reference material SRM 2924

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Background: The National Institute of Standards and Technology (NIST) is developing a standard reference material (SRM) - SRM 2924, C-Reactive Protein Solution. This material consists of recombinant C-reactive protein (CRP) in aqueous buffer intended to serve as a "pure substance" reference material to provide traceability to SI units when used as a calibrant in the analysis of future reference materials containing CRP in biological matrices such as serum. The objective of this study was to determine the concentration of CRP using a higher order method. Analysis of this candidate material will include concentration assignment by amino acid analysis (AAA) using isotope dilution liquid chromatography tandem mass spectrometry (ID-LC-MS/MS).

Methods: The material was received from the manufacturer in 12 boxes each containing 100 vials with 1 mL of aqueous buffer with a concentration of 0.49 g/L of CRP as measured by ultraviolet absorption with an extinction coefficient of 1.70 at 280 nm (A_{280}). A stratified random sampling plan was executed whereby two vials were selected from each box and further divided into four analysis groups to be performed in different weeks using independently prepared calibration and LC buffers. AAA was performed by vapor phase hydrochloric acid hydrolysis (118 °C for 48 hr) of dried samples spiked with isotope labeled free amino acids (isoleucine, leucine, phenylalanine, proline and valine) and calibrated by similarly processed unlabeled amino acids in a five point calibration curve. A control material, NMIJ CRM 6201-b, C-reactive Protein Solution, was included in duplicate to assess accuracy and reproducibility. The control material was diluted 2-fold with equivalent buffer to match the expected levels in SRM 2924. The five calibrants, six samples and two controls were hydrolyzed within the same hydrolysis vessel for each separate analysis group.

Results: Linear regression conducted for each of the five individual amino acids within a sample yielded similar slopes, intercepts and regression coefficients with values close to 1, 0 and greater than 0.999, respectively. The five values obtained for each amino acid in each sample were likewise very similar and were averaged to obtain a single result for each sample. The values determined for each analysis group were 20.7 µmol/kg, 20.3 µmol/kg, 19.8 µmol/kg, 21.1 µmol/kg with coefficient of variation being 2.4%, 1.9%, 4.4% and 1.8%, respectively. The value of CRP in NMIJ CRM 6201-b was found to be 38.9 µmol/kg (cv = 0.9%) comparing well to the certified level of 40.0 µmol/kg (uncertainty = 1.6 µmol/kg) which was also value assigned via AAA. The combined average concentration was 20.5 µmol/kg (cv = 2.7%) which corresponds to a value of 0.47 g/L, within 4% of the A_{280} value.

Conclusion: The result of the AAA on SRM 2924 show that the analysis is repeatable within each separate run and across multiple analysis groups spanning several weeks in time indicating a robust method. Assignment of the CRP level in SRM 2924 will be based on this study but will also include an estimate of uncertainty. Release of this material is anticipated during 2016.

A-378

Development of a high throughput hemoglobinopathies workflow using high resolution accurate mass analysis

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Background: Hemoglobin profiling research focuses on detecting, characterizing, and performing relative quantitation of all hemoglobin chains, including sequence variants and PTMs. Recently, mass spectral detection has emerged as a suitable method to perform hemoglobinopathies due to the advances in resolution at fast mass spectral acquisition rates for both full scan and tandem mass spectrometry while maintaining accurate mass determination over large dynamic ranges. To satisfy the analytical requirements for a high-throughput assay, routine sample preparation is needed along with automated data processing capabilities are needed.

Methods: All experiments were performed on a Q Exactive Focus mass spectrometer. Hemoglobin samples were created directly from whole blood or dried blood spots and diluted with 90:10:0.1 (H_2O/MeOH/acetic acid) and loaded into a separate well of a cation exchange plate. Bovine hemoglobin was spiked into some samples to increase the complexity. The plate was washed to remove salts and small molecules, and then targeted MW range was displaced using an ammonium formate solution. Each sample was loaded directly onto an SEC column for on-column washing and elution into the mass spectrometer. HR/AM MS and tandem MS was acquired using a 70,000 resolution setting and all subsequent data was processed using the Pinnacle software for automated qualitative and quantitative analysis.

Results: The primary challenge associated with hemoglobinopathies is to detect and confirm hemoglobin sequence variants with mass shifts as small as 1 Da in a high throughput method. The MW range of both hemoglobin chains makes intact profiling feasible with mass resolution around 50,000. The approach presented here combines rapid sample preparation to isolate the targeted MW range covering hemoglobin (ca. 4 minutes per plate), with on-line clean up and introduction into the mass spectrometer for subsequent profiling. Multiple data acquisition methods were used, including alternating HR/AM MS and all ion fragmentation (AIF), as well as modified data dependent acquisition (DDA) on selected mass ranges. Data acquisition was performed in two minutes and the injection cycle time was six minuntes. Automated data processing was performed using known base sequences for human alpha and beta hemoglobin chains, and targeted searching routines in the Pinnacle software. To increase the complexity of the sample, bovine hemoglobin was spiked into some samples at known ratio covering 7:3, 5:5, and 3:7 (human:bovine) as well as neat samples for each species. Reproducibility and robustness was evaluated by preparing all at least 25 replicates per hemoglobin mixture.

Conclusion: Research results were able to demonstrate accurate MW determination and sequencing from reported hemoglobin chains, identifying sequence truncations as compared to the Uniprot sequences. In addition, PTMs such as deamidation, deoxydation, and oxidation was evaluated for both human and bovine hemoglobin chains. The measured variance for each chain in all samples was less than 20% despite using a SEC for introduction into the mass spectrometer and the ratios between species was less than 15%.

A-379

A high-resolution accurate mass (HRAM) mass spectrometry method to assist in identification of hemoglobin variants

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Background:

Hemoglobinopathies are some of the most common hereditary disorders in the world. Depending on hemoglobin mutation, patients may present with anemia, polycythemia, or persistent cyanosis. Correct variant identification aids in treatment choice and understanding disease severity as well as possible consequences of family planning. Typical screening methodology is based on HPLC (high performance liquid chromatography) and CZE (capillary zone electrophoresis). While of great utility, these methods cannot separate some clinically significant rare variants. Since mass spectrometry does not depend on differential mobility of hemoglobins, variants that are otherwise silent can be detected if they exhibit sufficient mass difference from normal subunits. With high-resolution instruments more accessible to clinical laboratories, even variants with subtle mass shifts are able to be identified. An HRAM MS method was developed to investigate the ability to detect abnormal hemoglobin variants in order to aid hemoglobinopathy diagnosis.

Methods:

10 uL of EDTA whole blood samples were lysed in 20% acetonitrile followed by solid phase extraction (SPE). The samples were then injected onto a nanoLC column (EasySpray C18 Pepmap) coupled to a quadrupole-orbitrap hybrid mass spectrometer (ThermoScientific Q-Exactive). An isolation window of 920-1200 m/z range ensured that hemoglobin was the major observed compound reducing potential interferences from other proteins. After LC-MS analysis, samples were deconvoluted using an automated method in Protein Deconvolution 3.0 (ThermoScientific) utilizing Xtract mass algorithm to produce monoisotopic masses. Selection criteria of a minimum of 3 charge states and 10 signal-to-noise (S/N) cut-off reduced interference from low abundance proteins. In order to screen mass difference with possible variants, an update to HbVar database was implemented allowing for difference in mass searches.

Results:

Normal masses for alpha (15116.898±0.004 Da) and beta (15856.258±0.007 Da) subunits were verified in our patient population. Analysis of over 40 hemoglobin samples included Hb D (Δm (*beta*)= -0.991±0.006 Da), Hb E(Δm (*beta*)= -0.987±0.011 Da), Hb SC(Δm (*beta*)= -30.771±0.453 Da), and Hb-Philadelphia (Δm (*alpha*)=14.04 Da) variants. Despite known difficulty deconvoluting hemoglobin subunits with a small mass change, we demonstrated that it is possible to resolve 1 Da subunit mass differences and even extend the method to heterozygous samples without chromatographic separation as a result of high resolution and Xtract deconvolution algorithm.

Conclusion:

The combination of monoisotopic mass reports for patient hemoglobin subunits with a newly-updated HbVar database allows for straightforward variant searching based on reported mass difference. This methodology should lead to better identification of hemoglobin variants when used in combination with conventional hemoglobin identification techniques.

A-380

Advantages of Standardized/Kitted Methods for Protein Digestion; a Monoclonal Antibody Example

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Background: Monoclonal antibodies (mAb) are becoming a more common place treatment for cancers, autoimmune diseases, and other diseases thought to be untreatable in the past. Therapeutic drug monitoring of these biologics is starting to be considered of clinical significance, especially as these therapeutics are lifelong treatments with great expense. Method development to monitor drug concentrations in serum encompasses immunoassays or mass spectrometry pathways; including the intact light chain quantitation or the use of tryptic peptides unique to the mAb. The latter is a highly complex assay with sample clean-up, reduction, alkylation, and trypsin digestion. Many of these steps are manual, with long incubation times and can be prone to error in a clinical laboratory. As future mAb peptide methods are implemented into the clinical laboratory setting, the standardization and improved traceability of pre-analytical steps for parts or the entire assay could be very advantageous. **Objective:** The goal of this study was to perform a method comparison between a laboratory developed manual sample preparation protocol using in-house reagents and a kitted digest method for the analysis of tryptic peptides from infliximab. **Methods:** Residual waste serum samples with physician-ordered infliximab testing were obtained from the clinical laboratory (n=39). Samples were processed per standard operating procedure. Briefly, a protein level standard is added to serum, and the mixture undergoes a protein crash using saturated ammonium sulfate (SAS). The reconstituted pellet is then denatured, reduced, alkylated, and digested as previously published; with DTT, IAA and trypsin weighed out and reconstituted daily. Side by side, the SAS mixture was prepared using a kitted digest (Waters ProteinWorks Digest Kit, Waters Inc.) that incorporates denaturation, reduction, alkylation, and digestion steps, following manufacturer's directions. Analysis for both methods was performed by LC-MS/MS on an API 5000 triple quadrupole mass spectrometer (ABSciex). **Results:** Sample processing using the standard operating procedure has a turn-around-time (TAT) >10h, from the initial denaturation to the digestion quenching. The kitted digest method allowed completion of the protocol within 4 hours, which would allow sample preparation to be performed in one single shift. The accuracy of infliximab using Waters' ProteinWorks Digest Kit was measured using ordinary linear regression; y=1.02x+1.10; R^2=0.99. Additionally, quantitation using tryptic peptides *m/z* unique to the light chain or the heavy chain of infliximab yielded similar results; y=0.97x-0.21, R^2=0.96, and may be used as a different measure of accuracy and complete digestion. **Conclusions:** The kitted method is amiable to any upstream enhancement/enrichment steps that are employed with mAb workflow, and results in equivalent quantitation of infliximab in residual serum samples. Although precision and cost still need to be fully evaluated, this proof-of-concept initial comparison showed important advantages such as lot traceability, ready-to-use reagents and a significant improvement in TAT compared to the current method, what can allow better follow up and accommodate an eventual increase in test volumes without a substantial method change and adding efficiency to the sample pre-analytical processes.

A-381

A Single-Injection LC-MS/MS Method for the Assessment of Impaired Glucose Tolerance

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Background: Early diagnosis of prediabetes is crucial for delaying and preventing its progress to type 2 diabetes. Millions of people are diagnosed with type 2 diabetes every year, and the total costs of diagnosed diabetes in the US is more than $245 billion per year. Impaired glucose tolerance (IGT) can be an important indicator of prediabetes. Current tests to diagnose IGT, such as the OGTT, are cumbersome and undesirable to the patient. We developed and clinically validated a test for impaired glucose tolerance in 2014 (Quantose™ IGT), utilizing biomarkers discovered on our metabolomics platform. Due to the varying polarity of the analytes, the method was performed in two LC-MS/MS injections, one traditional reverse-phase and one ion-pair injection for the more polar analytes. Because using a two-injection method significantly limited throughput, it was desired to develop a single-injection LC-MS/MS test for IGT.

Methods: Plasma samples are mixed with isotopically labeled internal standards and extracted via protein precipitation. The extracts are analyzed by a single reverse-phase, negative MRM LC-MS/MS method for the quantitation of 2-hydroxybutyric acid (0.500 to 40.0 µg/mL), 3-hydroxybutyric acid (1.00 to 80.0 µg/mL), 4-methyl-2-oxopentanoic acid (0.500 to 20.0 µg/mL), 1-linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (2.50 to 100 µg/mL), oleic acid (10.0 to 400 µg/mL), pantothenic acid (0.0100 to 0.800 µg/mL), and serine (2.50 to 100 µg/mL). An IGT risk score is calculated from the analyte results using a multivariate algorithm that also incorporates glucose.

Results: Reverse-phase chromatography was chosen to accommodate all seven biomarkers, which showed significant differences in polarity and had previously been analyzed in two separate LC-MS/MS injections. Analytical method validation was performed on four identical LC-MS/MS systems with five runs on each instrument for five separate days. Acceptable linearity (R^2>0.99) was observed for all the analytes over the ranges. Inter-assay imprecision of all analytes (n=100 per level: low, mid, and high levels) was less than 5.8%. Inter-assay imprecision of the IGT scores was less than 2%. Inter-assay accuracy at the lower limit of quantitation was within +/-10% of the nominal value and imprecision at the LLOQ was less than 9%, for all analytes (n=100). Relative analytical recovery was determined to be between 96.3% and 103% for all analytes. Short term stability evaluation indicated that plasma samples were stable after 6 hours at room temperature, 4 days in a refrigerator, or 5 cycles of freeze/thaw. Specificity and potential interference were assessed during the validation, and their impacts reasonably addressed. Method comparison to the two previously-used clinically validated methods was performed and the correlation between the old and new methods was greater than 0.95 for all analytes, with most analytes correlating above 0.99. The overall IGT score correlation was >0.999 with less than 1% average bias.

Conclusions: The new method reduces the time per test and cost by about 50% compared to the previously used two methods. The assay shows excellent precision, linearity, specificity, and accurate comparison to previously-used methods. This method for assessing IGT has now been used on over 3000 patients, and has performed robustly.

A-382

Automated mass spectrometric method for identification and quantitation of wild-type and familial variants of amyloid-beta peptides in cerebrospinal fluid

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Background: Until recently, quantitative analysis of amyloid-beta (Aβ) peptides in CSF has relied almost exclusively on the use of immunometric assays. LC-MS/MS methods to date have focused on wild-type Aβ peptides; however, known familial

mutations that occur within the Aβ42 region could result in a falsely low Aβ42 by LC-MS/MS and subsequently a disease risk misclassification. To account for specimens containing familial variants, we sought to develop a strategy to detect and identify variants along with wild-type Aβ peptides.

Methods: After incubation of CSF with denaturant, peptides were concentrated by solid phase extraction; extracts were then analyzed by high performance LC coupled to a triple quadrupole mass spectrometer. ^{15}N-labeled synthetic Aβ42 and Aβ40 peptides were used as internal standards. Sample processing steps were automated on a liquid handler. The method was evaluated following clinical guidelines including assessment of sensitivity, selectivity, linearity, precision, accuracy, stability and interferences. In addition to wild-type peptides, representative mutant Aβ42 synthetic peptides D23N (Iowa), A21G (Flemish), E22G (Artic), E22Q (Dutch), and E22K (Italian), were studied. A method comparison between the Innogenetics ELISA and the LC-MS/MS method was completed using 155 CSF specimens from the biobank at the University of British Columbia's Clinic for Alzheimer's Disease and Related Disorders.

Results: The analytical measurement range was 100-3000 ng/L for Aβ42 and 100-20000 ng/L for Aβ40. For the diagnostically relevant Aβ42 peptide, the total coefficient of variation near the medical decision limit was 7.9%. Acceptable recovery (85-115%) was observed for up to 5% whole blood contamination and no significant interference was observed with bilirubin or intralipid. The method comparison yielded the following regression: LC-MS/MS = 2.64*ELISA - 247.4, r^2 = 0.63. All peptide variants studied were resolved chromatographically from wild-type using our LC method. Linear regression analysis between CSF specimens (n=40) processed by manual and automated sample prep yielded r^2 = 0.97 for both Aβ42 and Aβ40.

Conclusion: Using instrumentation common to hospital labs, we developed an automated method for quantitation of Aβ peptides in CSF, inclusive of familial Aβ variants. The method was validated following established clinical guidelines and a data analysis procedure was established to identify the presence of variant Aβ peptide sequences without a priori knowledge of the isoform(s) present in specimens submitted for analysis.

A-383

Ethyl Glucuronide and Ethyl Sulfate in Urine: LC-MS/MS Method Evaluation and Assessment of Total Excretion Levels Post Alcohol Intake

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Background: Ethyl glucuronide (ETG) and ethyl sulfate (ETS) are metabolites of ethanol that are excreted in urine and considered specific biomarkers for alcohol consumption.

Objectives: To evaluate the performance characteristics of Q-Exactive mass spectrometer coupled with liquid chromatography for the quantification of ETG and ETS in urine and to assess the total levels of ETG and ETS excreted in the urine following low and moderate alcohol consumption.

Methods: Linearity, precision, and functional sensitivity were assessed using synthetic urine spiked with ETG and ETS. For ETG and ETS excretion study, 36 healthy volunteers (18 males and 18 females) consumed either 0.2g or 0.4g of ethanol per kg body weight. Nine timed urine specimens were then collected over the next 72 hours. Urine (20 µL) was diluted in 380 µL of diluent containing ETG-D5 and ETS-D5 before 10 µL of which was injected. The analysis was carried out on an LC-Q-Exactive system using a Kinetex C18 column (100 x 2.1 mm, 2.6 µm); total run time was 5 minutes. Additionally, urine creatinine (creat) concentrations were measured using Jaffe reaction on Beckman Olympus to calculate the ETG/creat and ETS/creat ratios (µg/g). All values are expressed as mean±SD.

Results: Assay was linear between 50 and 50,000 ng/mL with R^2 values of 0.9984 and 0.9997 for ETG and ETS, respectively. Functional sensitivities at a CV of 10% were 23 ng/mL and 35 ng/mL for ETG and ETS, respectively. Between-day imprecisions (CV) ranged between 3 and 5% at low, medium and high levels of ETG and ETS. Concentration of ETG and ETS peaked in urine either 6 hours or 18 hours following alcohol intake. At 0.2g/kg intake level, average peak ETG and ETS concentrations were 4485±8550 ng/mL and 992.0±1546 ng/mL, respectively. At 0.4g/kg intake level, average peak ETG and ETS concentrations were 8195±5543 ng/mL and 1647±1093 ng/mL, respectively. In the first 24 hours following 0.2g/kg ethanol intake (day 1), 4482±5423 µg of ETG (ETG/creat =35±52) and 1244±1199 µg of ETS (ETS/creat =10±11) were excreted. In the second 24 hours (day 2), excretion dropped to 174.0±146.0 µg of ETG (ETG/creat =1.2±1.1) and 77.00±51.00 µg of ETS (ETS/creat =0.47±0.35). Day 3 levels were 91.00±75.00 µg of ETG (ETG/creat = 0.60±0.42) and 64.00±53.00 µg of ETS (ETS/creat =0.38±0.15). At 0.4g/kg intake level, day 1 excretions were 12129±6039 µg of ETG (ETG/creat =78±40) and 2740±1064 µg of ETS (ETS/creat =18±6.8). Excretions on day 2 dropped to 118.0±64.00 µg of ETG (ETG/creat =0.82±0.47) and 82.00±45.00 µg of ETS (ETS/creat =0.52±0.16). On day 3, 65.00±42.00 µg of ETG (ETG/creat =0.54±0.37) and 52.00±33.00 µg of ETS (ETS/creat =0.38±0.23) were excreted.

Conclusions: The validated LC-MS/MS method is highly precise and offers sensitive quantification of ETG and ETS in urine. 24-hour ETG and ETS excretion levels following low and moderate ethanol intake are reported. Total levels and peak concentrations of excreted ETG and ETS are highly variable with no significant differences between males and females but with significant differences between the two levels of alcohol intake tested.

A-384

Agreement and validation of reliable scores for identification of microorganisms using MALDI-TOF

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Background: Identification of microorganisms using MALDI-TOF (MT) changed substantially standard procedures and workflows in clinical microbiology laboratories. Rapid identification, high accuracy and reduced costs and workload figure among the main benefits of this technology. However, as all new method, some improvements can only be reached along expertise and practice. The aim of this study is to evaluate the accuracy of the identification by MT using the standard protocol for protein extraction and direct identification (without extraction). We also intended to compare and validate the lower reliable score at genus and species level using the direct identification. **Methods:** We performed the identification using the Microflex LT® (Bruker Daltonics, Bremen, Germany) platform and the software Biotyper 3.2. For the interpretation of results we followed the manufacture's recommendations as following: 2.300 – 3.00 (highly probable species identification); 2.000-2.999 (secure genus identification and probable species identification); 1.700-1.999 (probable genus identification) and 0-1.699 (no reliable identification). **Results:** We performed the study in two steps. At the first step we selected 160 isolates previously identified in the routine and we processed at the MT with and without tube extraction to compare the agreement between the results. Twenty strains of the following isolates were selected: *S. aureus*, *S. pyogenes*, *Enterococcus faecalis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Gardenerella vaginalis* and *C. albicans*. We found 100% of agreement between both protocols despite lower scores according to direct protocol as expected. At second part of our study we selected more 160 strains randomly to compare not only the accuracy but also the lowest reliable scores obtained with the identification without extraction. Different species were tested, among them *Staphylococcus* spp. *Streptococcus*, *Enterococcus* spp., *E. coli*, *Proteus* spp., *Pseudomonas* spp. *Klebsiella* spp. and *Gardnerella vaginalis*. A 100% of agreement was observed at specie level. Comparing the scores obtained we noted that using direct identification, a break point of 1.750 is the lowest score for a reliable identification for all isolates tested at genus and species level. **Conclusion:** we conclude in our study that despite recommendations of manufacturers, scores under the established may be reliable for releasing genus and species identification in the routine laboratory using the protocol without tube extraction.

A-385

Mass spectrometry imaging workflow in clinical research

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Cancer incidence in Europe was recently estimated at 3.45 million cases, with 1.75 million deaths, and costing the EU 124 billion Euros every year. With such incidence rates, fundamental research and understanding of cancer biology is required to prevent (prognosis), identify (diagnosis) and treat cancer. Mass spectrometry imaging (MSI) is now increasingly used for clinical research applications due to significant technological improvements that have made the technique more accessible. Matrix-assisted laser desorption/ionization (MALDI), initially introduced by Caprioli et. al [1], is the dominant MSI technique used today, due to the ability of MALDI to analyse intact proteins directly from tissue. Furthermore MALDI is widely available and has been commercially developed by a number of vendors.

In the last few years, several alternative ambient ionization techniques have been developed that can ionize clinically important molecules, such as lipids, directly from tissue. One of these techniques, desorption electrospray ionization (DESI),

is a surface analysis technique incorporating an electrospray probe, that can be utilized as a spatially resolved imaging technique by rastering a surface under the spray using a high precision X,Y stage. As the electrospray droplets impact upon a surface, chemical constituents are desorbed and transferred into the atmospheric inlet of the mass spectrometer source. Ionization occurs due to the charge imparted onto the droplets. No modification to the sample such as matrix addition is necessary and therefore minimum sample preparation is required to run a DESI imaging experiment, making this technique more compatible within a clinical research environment

Here we describe the workflow for clinical research where MALDI and DESI imaging techniques were employed to characterize the molecular profiles which were significantly different between the normal and the tumour part of the tissues. The tissue sections analyzed were frozen clinical human tissue sections used for research puroposes, from liver and colorectal biopsies. For the MALDI MSI experiments, matrix solution was sprayed automatically using a nebulizing spray device, making the sample preparation step more reproducible than manual spraying. Consecutive or similar tissue sections were also analysed by DESI imaging. However in these experiments, no sample preparation step was required. Solvent solutions mainly used for the desorption and ionisation step were a mixture of Methanol-Water, which proved ideal for lipids and/or small molecule metabolite detection. By managing solvent and gas flow rates appropriately, in combination with the optimised voltages, the DESI technique resulted in negligible destruction of the tissue surface and therefore the same tissue sample can be histologically stained. In this case the molecular distribution was compared with the tissue's microscopic structure obtained from the H&E stained image.

A-386

Quantitative Testing for Polychlorinated Biphenyls (PCBs) in Human Serum Utilizing Gas Chromatography Tandem Mass Spectrometry (GC-MS/MS)

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Background:

PCBs consist of a class of compounds produced for commercial use from 1930 to 1977 that were ultimately banned in 1979. Aroclors, named by the manufacturer consists of mixtures of PCB congeners in certain ratios that contain desirable characteristics with applications in plasticizers, adhesives, sealants, electrical transformers, capacitors, and wiring, among others. Although banned almost 40 years ago, PCBs continue to pose serious health risks to humans through diet and other environmental exposures. Varying volatility, fluctuating environmental decomposition rates, varying metabolic rates once adsorbed, and possible exposure to multiple Aroclors with overlapping congeners often make Aroclor testing in humans difficult to interpret. The objective of this study is to present a method for measuring 11 individual PCB congeners in human serum by GC-MS/MS.

Methods:

Sample preparation involves adding a mixture of 8 fluorinated analogue internal standards to 0.5mL of specimen, followed by the addition of urea, 2-propanol, water, methanol and petroleum ether. Samples were then mixed, centrifuged and the organic supernatant brought to dryness. Next, the extracts were reconstituted in hexane, then poured into solid phase extraction cartridges containing Florisil® and sodium sulfate. Additional hexane was added to each cartridge for elution, which was collected, concentrated and transferred to autosampler vials for analysis. Agilent's GC-MS/MS platform utilizing Retention Time Locking (RTL) and Multiple Reaction Monitoring (MRM) time segments were used for acquisition to enhance selectivity and sensitivity. Analysis was performed on a DB-5MS capillary column (15mx0.25umx0.25mm) using an oven ramp ranging from 80°C to 270°C, over a 10 minute run time.

Results:

The scope contains reporting limits ranging from 10 pcg/mL to 80 pcg/mL and a linear dynamic range spanning two and a half orders of magnitude for quantitation. Precision data were obtained by calculating the %CV from target for both the high and low QC tested five times per batch over the course of three batches. With-in run precision ranged from 2.8% to 7.0% and total precision ranged from 2.6% to 6.1% for all target compounds. Accuracy was determined using five replicate samples spanning the linear range. The slope values ranged from 0.992 to 1.051, y-intercept values ranged from -5.3 to 2.41, and systematic error values ranged from 3.7% to 6.2% for all target compounds. The correlation coefficient for calibration curves ranged from 0.999 to 1.000 for all target compounds.

Conclusions:

In conclusion, we were able to accomplish all of the project goals by successfully developing, validating and implementing a congener specific PCB assay by GC-MS/MS. The method is robust meeting all validation acceptance criteria including, precision, accuracy, selectivity, sensitivity and linearity. Other notable aspects include small volume of samples required for analysis, simple and cost effective sample preparation, quick run time and easily interpretable data.

A-387

Automating Liquid Chromatography-Tandem Mass Spectrometry (LC-MSMS) testosterone analysis for the small (or large) clinical laboratory - Tecan® AC Extraction Plate™ (AC Plate) used on a Tecan Freedom EVO® 100 automated liquid handler (ALH).

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LC-MSMS is recommended for quantifying serum testosterone in female and pediatric samples. We sought to implement a robust, highly automated LC-MSMS method also suitable for testosterone in adult male samples.

The proposed simplicity of the AC Plate is a desirable feature. Pipetting and orbital shaking are the only functions required for AC Plate extraction. Traditional sample preparation for testosterone requires additional ALH accessories or manual steps. We evaluated extraction reagent pH, solvent, and metal salt content and then validated the optimized method.

An ALH with an orbital mixer performed all extraction steps. The LC-MSMS was a Waters Acquity LC-XEVO TQS in positive ESI mode. The column was a Waters HSS C18, 2.1x150mm, 2.5 µm. Mobile phases A and B (MP-A/B) were 2 mmol/L ammonium acetate, 0.1% formic acid and acetonitrile, 0.1% formic acid. The LC gradient was 90:10 to 10:90 MP-A:MP-B, flow rate 0.4/0.6 mL/min, run time 6.3 min. MRMs acquired were 289/97, 289/109, 292/100, 294/97. The $^{13}C_3/^{2}H_5$-mixed working internal standard (I.S.) was 50/800 ng/dL. Injection volume was 3-10 µL.

Reagents, samples and barcoded plates are placed on the ALH deck. The user enters the number of samples to be tested and the starting well in the plate. Barcodes on sample tubes and plates are scanned by the robot. The plate map is transferred by MS VBScript into a Sample Table template on a shared directory. The sample table is imported to MassLynx by scanning the barcode on the injection plate.

The EVO robot extraction protocol is: add 100 µL serum, 25 µL I.S., and 175 µL of protein releasing reagent (0.33 mmol/L LiCl, 24% acetonitrile, 0.1% NH_4OH), shake for 10 min, discard the residue. Wash twice with 250 µL of 0.1% NH_4OH, shake for 5 min and discard the residue after each wash. Elute with 100 µL of 35:65 H_2O:acetonitrile, shake for 5 min and transfer to a 700 µL glass insert plate.

Mean recovery and decrease from matrix effect were 46% and 24% respectively. No interference was observed from epi-testosterone, DHEA, or other drugs and steroids tested. No interfering peaks were found from BD SST tubes and the mean bias was -4% between samples collected with or without SST gel. The validated analytical measurement range (AMR) was 2-1,000 ng/dL. S:N at the lower limit of quantitation ranged from 23-48. Within and between run precision (n=5) using BioRad LiquiChek Immunoassay-Plus and CAP Accuracy Based survey materials at means of 18, 87, 279, 508 and 987 ng/dL had CVs <5% and <12% respectively. No carryover was observed between 3,000 ng/dL and < AMR samples. Mean bias for CAP ABS samples was -4%. Deming regression

statistics for patient samples ranging between 12 and 566 ng/dL versus a CDC-HoST certified reference laboratory were slope 0.92, intercept 2.4, SEE 5.9, bias -6.9%.

We conclude that the AC Plate meets the vendor's claims for ease of use and implementation with good analytical performance across the desired AMR. This extraction consumable appears suitable for small or large laboratories desiring to simplify and automate sample preparation for serum testosterone.

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Glycated hemoglobin in normal (AA) and variant hemoglobins (AC, AE, AD) using mass spectrometry: comparison with boronate affinity HPLC

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Background:

HbA1c is a well-established marker of glycemic control and an aid to diabetes diagnosis. There are many methods for measuring HbA1c based on a variety of principles including immunoassay, capillary electrophoresis, ion-exchange and boronate affinity chromatography (BAC). Hemoglobin variants can affect HbA1c results from some methods, which can negatively impact clinical interpretation of

results. BAC is generally considered to be immune to interference from Hb variants due to the fact that separation is based on binding specifically with cis-diol groups of glucose bound to hemoglobin and not on the structure of the hemoglobin chain. We evaluated the relationship between the glycated and non-glycated beta chains of normal and variant hemoglobins using LC/MS.

Methods:

Hemolysates from non-diabetic and diabetic subjects with normal HbAA or with three common heterozygous variants (HbAE, HbAC, or HbAD) were prepared. Measurements of the percentage of glycated globin chains were done using an LC-MS system operated in positive ESI mode. The quantitation of glycated forms was based on comparison of the areas under the curves in the extracted-ion chromatogram of the 17+ charged forms of glycated β vs. non-glycated β and the 16+ charged forms of glycated α vs. non-glycated α globin chains. The percentages of glycation of intact α and β globin chains were calculated. The ions β17+ and α16+ were chosen for quantitation because of their high signal intensities and absence of interferences in MS. Due to the fact that glycated and non-glycated globin chains may have different ionization efficiencies, we checked the validity of the method by analyzing mixtures of purified glycated and non-glycated normal globin chains in different known ratios.
Results:

Results from this LC/MS method showed a strong linear correlation of measured β glycated ratios with β glycated values from IFCC Reference Material ($y = 1.075x - 0.016$, $R^2 = 0.9998$). Results obtained by LC-MS for normal hemoglobin and three common heterozygous variants (HbAE, HbAC, HbAD) showed very similar linear correlations (i.e. comparable slope and intercepts) with Trinity Ultra^2 boronate affinity HPLC.

Conclusions:

The fact that all variants produce results that are comparable to that observed for normal HbAA suggests that the evaluated variant globin chains have glycation rates similar to that of HbAA. These results support the use of boronate affinity as a comparison method in Hb variant interference studies.

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Validation of a Sensitive Method for the Simultaneous Quantification of Six Endogenous Anabolic Steroids Using GC-MS/MS

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Background: Endogenous anabolic androgenic steroids are listed by World Anti-Doping Agency (WADA) as prohibited performance enhancing substances. Accurate measurement of anabolic steroids is required for the detection of doping abuse. **Objective:** To validate a GC-MS/MS method for the quantification of androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), testosterone (T), and epitestosterone (E) in urine. **Methods:** For linearity and functional sensitivity, synthetic urine spiked with anabolic steroids was used. For precision, three urine samples containing low, medium or high levels of anabolic steroids were tested in triplicates for five days. Samples were spiked with deuterated internal standards and treated with β-glucuronidase followed by solid-phase extraction of steroids using 3M Empore C18 columns. Samples were derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Analysis was carried out on Thermo Trace 1310 gas chromatographer coupled with TSQ Quantum XLS Ultra mass spectrometer. **Results:** Assay was linear between 200 ng/mL and 7500 ng/mL for A and Etio, between 1 ng/mL and 200 ng/mL for 5αAdiol, between 1 ng/mL and 500 ng/mL for 5βAdiol, and between 0.25 ng/mL and 200 ng/mL for T and E. R^2 values were >0.99 for all steroids. Assay imprecision (CV) ranged from 3% to 11% for A and Etio, from 1% to 3% for 5αAdiol and 5βAdiol, and from 2% to 7% for T and E for all three levels of steroids tested. Functional sensitivities at 10% CV was 10 ng/mL for A and Etio, 1.5 ng/mL for 5αAdiol, 1.7 ng/mL for 5βAdiol, 0.25 ng/mL for T, and 0.23 ng/mL for E. **Conclusion:** The validated method for endogenous anabolic androgenic steroids quantification in urine using GC-MS/MS is sensitive and demonstrates excellent analytical performance.