Tuesday, July 28, 2015

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

Mass Spectrometry Applications

A-242

Development and Validation of a Liquid Chromatography Tandem Mass Spectrometry Bioanalytical Method for 21-Deoxycortisol, 11-Deoxycortisol and Corticosterone in Serum or Plasma

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Background:

Congenital Adrenal Hyperplasia (CAH) is most often caused by mutation of the 21-hydroxylase gene (CYP21), which results in underproduction of cortisol with overproduction of precursor steroids and their metabolites by the adrenal glands. Similarly, the second most common cause of CAH is a defect in 11-Hydroxylase (CYP11B1), for which the screening tests are 11-Deoxycortisol or 11-Deoxycorticosterone. Historically the most common biomarker used for detecting CAH in pediatric patients is 17-Hydroxyprogesterone (17OHP). In recent years the use of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) has afforded the clinical laboratory the opportunity to simultaneously quantify multiple additional biomarkers such as Androstenedione, 11-Deoxycortisol, Cortisol, DHEA and Testosterone to improve the specificity and sensitivity of clinical diagnosis. Another less commonly used biomarker for 21-Hydroxylase deficiency is 21-Deoxycortisol, which increases from very low levels in normal patients to high levels in affected patients as 17OHP rises to very high levels. In this study we modified our existing multiplex assay for 11-Deoxycortisol and Corticosterone to include 21-Deoxycortisol, thus screening for three important markers.

Method:

An analytical method was developed using a TX-4 HPLC system (Thermo-Fisher) with Agilent® 1100 pumps (Agilent Technologies, Inc.) and a Sciex® API5000 (Danaher) triple quadrupole mass spectrometer. Independent calibration curves were prepared for each analyte in depleted serum (Golden West Biologicals). Sample preparation consisted of isotope dilution using a cocktail of three heavy isotope internal standards followed by Liquid-Liquid Extraction. A Fluophase® RP (Thermo-Fisher) analytical column (2.1x50mm, 5 um) was used with a water/methanol solvent gradient to achieve chromatographic separation of all isobars in under 6 minutes. Positive mode atmospheric pressure chemical ionization (APCI) was used for detection in Multiple Reaction Monitoring (MRM) mode.

Validation Data:

Analytical sensitivity was 10 ng/dL for each analyte and the analytical measurement range was up to 2,000 or 5000 ng/dL (up to 20,000 ng/dL with dilution). Inter-assay precision ranged from 2.4-9.8% for 11-Deoxycortisol, 2.4-11% for Corticosterone and 6.1-7.0% for 21-Deoxycortisol. Accuracy ranged from 92.6-97.8% for 11-Deoxycortisol, 96.5-98.5% for Corticosterone and 91.6-102.7% for 21-Deoxycortisol. Reference intervals for both adults and pediatric patients were developed for 11-Deoxycortisol, Corticosterone and 21-Deoxycortisol. Reference intervals were also developed for 11-Deoxycortisol to support Metyrapone stimulation testing.

Clinical Significance:

The use of 21-deoxycortisol may be beneficial in reducing the rate of false positives in CAH diagnosis when used in concert with other steroid hormones, including 17-Hydroxyprogesterone and Cortisol.

A-244

MALDI-TOF MS analysis of HbA1c in Diabetes Mellitus

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Background: Optimal diagnosis and monitoring of diabetes mellitus relies on measurements of HbA1c, the relative amount of glucose attached to the N-terminal valine of the beta-subunit of hemoglobin. Here we report a novel approach to determine HbA1c using MALDI-TOF mass spectrometry. MALDI-TOF analyses of diluted, whole blood samples determine the ratio of hemoglobin subunit(s) with CVs of ≤ 3%.

Methods: Whole blood samples at a final dilution of 1:2000 in 5 mg/mL sinapinic acid were analyzed on a SimulTOF 200 (SimulTOF Systems, Sudbury, MA) from m/z 5000-20,000 in positive, linear mode. Each spectrum was the average of 20,000 laser-shots evenly distributed across the surface of the sample spot. Spectra were calibrated using the M1+ and M2+ ions of hemoglobin alpha- and beta-subunits. HbA1c was calculated from glycated alpha- or beta-subunit (M+162) using the following formula: [(M+162)/ (M + (M+162))]. Results obtained by MALDI-TOF assay are compared with those obtained in a clinical laboratory used clinically accepted HPLC methodology.

Results: Analytical standards and clinical patient samples for quantitation of total glycation on the beta-subunit of hemoglobin exhibited linearity (y = 0.79x + 1.4; R2 = 0.99) from 1.36% to 17.94% with CVs <1.66% (Figure 1).

Conclusion: While MALDI-TOF MS detects all glycation of the alpha and beta-subunit of hemoglobin, we have established the direct relationship between the MALDI-TOF method for the determination of the glycated beta chain and the HPLC method for determination of HbA1c. Additionally, the strong correlation between glycation of the alpha- and beta-subunits allows their ratio to be used as a quality control check for the determination of the glycated beta chain.

A-245

Simultaneous Sensitive Quantitation of Testosterone and Estradiol in Serum by LC-MS/MS Without Derivatization

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Background: Very sensitive measurements of serum estrogens and testosterone are important in adult and pediatric endocrinology and oncology. Sensitive measurements of estradiol (E_2) are needed for determination of menopausal status, estrogen deficiency, estrogen measurements in men, during antiestrogen treatment and in the diagnosis of other sex-hormone-related disorders. E_2 levels in postmenopausal women, men and children are typically <20 pg/mL. Ultrasensitive testosterone (Te) measurements are needed for adult women, whose values are routinely <50 ng/dL, in children, and men undergoing antiandrogen therapy whose values are usually less than 10 ng/dL. Many automated immunoassays lack the required sensitivity and specificity for the measurement of these very low concentrations of E_2 and Te. Our objective was to develop a very sensitive LC-MS/MS assay for both Te and E_2 in serum in a single analysis without the need for chemical derivatization and extended extraction protocols.

Methods: Serum samples (500 microL) were prepared by the addition of deuterated internal standards of both compounds followed by a liquid-liquid extraction using hexane:ethyl acetate (9:1, v/v). The supernatant was evaporated at 35°C under a stream of nitrogen and reconstituted in methanol:water (1:1, v/v). 80 microL was injected into the LC-MS/MS system. Chromatographic separation was performed on a Thermo Scientific TLX-2 HPLC system interfaced to an ABSciex 6500 mass spectrometer operated in both positive and negative ion ESI mode. Chromatographic separation was achieved using an Accucore C18 (50 X 3 mm i.d.) analytical column. Mobile phase comprised of A: 0.02 mM ammonium fluoride in water and B: acetonitrile. The HPLC gradient elution was 15-50% of mobile phase B over 2 minutes which was then held for 1.5 minutes. MRM transitions were as follows: Te 289.2>97.1 and 289.2>109.1 m/z; E_2 271.0>145.1 and 271.0>143.1 m/z. Calibrators and controls were purchased from Chromsystems using their multilevel calibrators and tri-level controls.

Results: The LOQs of Te and E_2 were 1 ng/dL and 5 pg/mL, respectively. The analytical measurement range (AMR) for Te was 1-1,170 ng/dL and 5-600 pg/mL for E_2. The calibration curves were linear over the AMR with correlation coefficients $R^2 \geq 0.998$. Assay accuracy was determined both by comparison with a LC-MS/MS method performed at a national laboratory and through recovery studies. Comparison with

samples analysed by LC-MS/MS at the reference laboratory showed the following: Te had a regression slope of 1.01, R^2=0.996; E_2 had a regression slope of 0.92, R^2=0.996. Te recoveries at three concentrations spanning the AMR were between 101.4 and 105.4% and E_2 recoveries were between 100.3 and 105.2%. Within-day (N=10) and between-day (N=20) CVs at concentrations spanning the AMR were less than 5% for both analytes. A solution containing 13 naturally-occurring steroids with the potential for isobaric interferences or with similar retention times were analyzed and showed no interferences.

Conclusion: We have developed an accurate and highly sensitive assay to simultaneously measure Te and E_2 levels in serum by LC-MS/MS without the need for chemical derivatization. Unlike many immunoassays this method is free of cross reactivity from structurally similar analogs.

A-246

Tandem mass spectrometry-based molecular networking to detect drugs of abuse and analogues

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Background: There is a need to quickly identify newer generations of illicit drugs such as "bath salt" analogs since routinely used multiple reaction monitoring (MRM) LC-MS/MS techniques are by definition targeted approaches. The development of MRM based methods requires a primary standard, which does not exist for most designer drugs when they first appear. In order to shorten the duration between the appearance of and the ability to identify a new designer drug analog, we propose the application of a novel approach that combines LC-MS/MS with molecular networking. Molecular networking exploits the premise that molecules with similar structures fragment similarly and computationally connects related molecules based on pair-wise comparisons of MS/MS spectra. The primary goal of this study is to evaluate the ability of LC-MS/MS and molecular networking to identify structurally related drugs from mixtures.

Methods:MS/MS reference spectra were generated on an AB SCIEX QTRAP 6500 by direct infusion of eleven standards purchased from Cerilliant diluted in methanol to 10 ng/mL. Data were converted to mzXML universal format using MSConvert from ProteoWizard, and MS/MS spectra were compared and analyzed using computational algorithms according to gnps.ucsd.edu. The computational algorithms simplify the data by generating consensus spectra from identical MS/MS spectra with a cosine similarity score of 0.97 or higher, where a score of 1 indicates identity. Then the consensus spectra, which are merged scans from multiple files, were compared pairwise and cosine similarity scores were assigned per pair. The results from molecular networking (via gnps.ucsd.edu) were imported into Cytoscape and displayed as a network of nodes and edges. The nodes labeled with precursor mass represent MS/MS consensus spectra, and the edges indicate cosine similarity score above 0.7, with the thicker lines reflecting higher scores. The mass difference between nodes or precursor masses may be used to predict the differences in functional groups.

Results: In the network, the nodes that represented consensus spectra for individual standards corresponded to methylone (m/z 208.1), ethylone (m/z 222.13), 4-methylephedrine (m/z 180.14), N-ethylcathinone (m/z 178.13), 3,4-DMMC (m/z 192.15), and R- and S-cathinone (m/z 149.19). Mass spectrometry detects m/z and not stereochemistry; it was surprising that R- and S-cathinone were represented by independent nodes. R- and S-methcathinone shared two nodes. There were five nodes representing methedrone (m/z 193.24), two nodes for diethylproprion (m/z 205.26), and the node for mephedrone (m/z 178.12) was combined with spectra from N-ethylcathinone.

The spectral patterns for methylone, ethylone, 4-methylephedrine, and N-ethylcathinone all show a strong initial loss of 18. Methylone and ethylone both have subsequent losses of 30 then 28, where as N-ethylcathinone has a subsequent loss of 28.

Conclusion:A molecular networking mass spectrometry based approach may be used to identify structural analogs of chemicals. Future directions will involve the analysis of compounds with both similar and diverse structures as compared with those used to develop the molecular network to determine if this approach will be useful for classifying new chemical compounds.

A-247

Screen and Identification of Four Biomarkers for Discriminating Non-small Cell Lung Carcinomas and Pulmonary Nodules by MALDI-TOF MS

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Background:

Non-small cell lung carcinomas (NSCLC) is one of the most common Lung cancer. Early detection of NSCLC renders an opportunity to dramatically reduce the disease mortality. In our study, serum peptides were analyzed for the early detection or monitoring of NSCLC comparing with pulmonary nodules, so as to provide alternative detective options rather than the computed tomographic scanning.

Methods:

Serum samples from 15 patients with solitary lung nodules and 12 patients diagnosed with NSCLC were colleted in this study. All blood samples were obtained prior to their treatment or surgery. Serum samples were fractionated using weak cation exchange magnetic bead (SPE-C100; Bioyong Tech, Beijing, China) according to the manufacturer's instructions. The resultant supernatant was transferred into a fresh tube, and the peptides were analyzed directly on a ClinTOF Mass Spectrometer (Bioyong Tech, Beijing, China). A three-peptide mixture (MW of 1532.8582 Da, 2464.1989 Da, and 5729.6087 Da, Cat Nos. P2613, A8346, and I6279, Sigma-Alderich) was used as internal standard. Then detected peptides were identified by linear ion trap-Orbitrap-Mass Spectrometry (LTQ-Orbitrap-MS).

Results:

An average of 105 peptide mass peaks were detected in both the NSCLC and control group. There were 16 peaks having peak frequencies above 50%, and six of them were significant different between the NSCLC and control groups (p-value < 0.05) when considering their relative peak intensity. Four peptides (MW of 1064.6 Da, 1077.0 Da, 1274.5 Da, and 1524.4 Da) were successfully identified by LC-MS/MS. Three different diagnostic models (Fishert, Linear SVM, and RBF) were established within these four peptides, to make a better combination. When two peptides were selected, the RBF model provides the best specificity and sensitivity of 95.04% and 95.72%. When three peptides were selected, the RBF model provides the best specificity and sensitivity of 98.04% and 97.72%. When all the four peptides were selected, the RBF model provides the best specificity and sensitivity of 99.94% and 99.72%, which means a diagnostic panel for identifying NSCLC and pulmonary nodule was successfully established. Further identification of these four peptides provided indicated that peptides of 1064.6 Da and 1077.0 Da were identified as fragment of FGA1, isoform1 of fibrinogen alpha chain precursor. Peptides of 1274.5 Da, and 1524.4 Da were identified as fragment of APOA1, apolipoprotein a-I precursor.

Conclusion:

Four serum peptides were identified as new biomarkers for NSCLC. A diagnostic panel of four serum peptides with 99.94% specificity and 99.72% of sensitivity was established for identifying NSCLC and pulmonary nodules by using the RBF model.

A-248

High Resolution Accurate-mass Mass Spectrometry (HRAMS) Offers Superior Accuracy For Quantitation of Steroids and Proteins

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Background: We demonstrate the clinical laboratory application of high resolution accurate-mass mass spectrometry (HRAMS) for quantitation of steroids (17-hydoxyprogesterone (17OHPG) and androstenedione (ANST)) and a protein biomarker, insulin like growth factor-1 (IGF1). We show how HRAMS resolves interferences that are unresolved on a triple quadrupole instrument in ~5% of patient samples from our daily clinical volume for 17OHPG and ANST. Additionally we show how HRAMS identifies IGF1 variants (V-IGF1) not distinguished from wild-type IGF1 by immunoassays.

Methods:

(a)HRAMS for 17OHPG and ANST

Sample extraction involved equilibration with internal standard, protein precipitation followed by online extraction of the supernatant and chromatographic separation on a reversed-phase column. Analysis was performed on either an API 5000 in MRM mode or a Q Exactive in SIM mode (70,000 resolution). API 5000 and Q

Exactive were compared. Transitions on the API 5000 were: ANST=287.2/97.1 and OHPG=331.3/109.1. On the Q Exactive the ions 287.2006 (ANST) and 331.2268 (OHPG) were added to the inclusion list. Samples with known chromatographic interferences on the API 5000 were analyzed on the Q Exactive.

(b)HRAMS for IGF1

100 µL sample is equilibrated with N15 labeled internal standard and treated with 400 µL acidified ethanol (1N HCl: Ethanol) and incubated at room temperature for 30 minutes followed by addition of 90 µL of neutralizing buffer (1.5 M trizma). The samples were then centrifuged at 3000 rpm for 10 minutes and cooled for 30 mins at -20oC. The samples are centrifuged again and analyzed by online extraction of the supernatant and chromatographic separation on a reversed-phase column. The m/z used to quantitate the wild-type IGF1 was 1093.5298. The Q-Exactive mass spectrometer was operated at a resolution of 70,000 and mass accuracy of 10 ppm was used for quantitation.

Results:

(a)A good correlation between the API 5000 (MRM) and the Q Exactive (SIM) with a slope of 0.9716 (n=162, R^2=0.9944) for ANST and a slope of 0.9567 (n=154, R^2=0.9955) for OHPG was obtained. Interassay imprecision was found to be acceptable using the Q Exactive and was 6-10% for ANST (range = 61-1932 ng/dL) and 11-12% for OHPG (range = 56-1831 ng/dL). The limit of quantitation was confirmed on the Q Exactive to be 15 ng/dL for ANST, and 40 ng/dL for OHPG. Most importantly, patient samples that showed interferences with either ANST or OHPG on the API 5000 showed no interference at the analyte retention time on the Q Exactive.

(b)HRAMS IGF1 assay compared well with the immunoassay slope of 0.9449 (n=1720, R^2=0.9573. In our validation studies for the HRAMS assay for IGF1 15 out of 2465 (0.6%) patients ~50% of the calculated IGF1-IA was V-IGF1. The V-IGF1 was found to have the same retention time as the native IGF1 and m/z difference (Δm/z) of ~4.5Da resulting in an m/z of 1097.9483. While immunoassay is unable to distinguish between the two variants, HRAMS enables identification potential novel IGF1 variants.

Conclusion:

HRAMS offers superior resolution and mass accuracy, which is useful for improving accuracy in quantitation of steroids and proteins in a clinical laboratory.

A-250

Determination of Plasma Catecholamines by LC/MS/MS for Clinical Research

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Background:

Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited for the rapid analysis of multiple analytes. A highly sensitive and specific LC/MS/MS analytical method has been developed for the quantitation of catecholamines (dopamine, epinephrine and norepinephrine) in plasma. This method uses a procedure by filtration on Agilent Captiva ND^{Lipids} tubes and an offline solid phase extraction (SPE) procedure for efficient sample preparation.

Methods:

An efficient solid phase extraction (SPE) sample preparation procedure was developed for the simultaneous extraction of dopamine, epinephrine and norepinephrine in plasma. Calibrators were created by spiking clean plasma with various concentrations of each analyte. The chromatographic system consists of an Agilent Pursuit pentafluorophenyl (PFP) column and a mobile phase comprised of methanol and water containing 1 mM ammonium fluoride. Deuterated internal standards were included for each analyte to ensure accurate and reproducible quantitation.

Results:

Chromatographic separation of all analytes is achieved through the use of a pentafluorophenyl column. The separation of epinephrine/normetanephrine and metanephrine/3-methoxytyramine are especially critical since these compounds share common fragments. Without proper separation by retention time, fragmentation of these compounds can cause interferences with one another and lead to inaccurate quantitation. The described analytical method achieves the required functional sensitivity and is capable of quantitating analytes over a sufficiently wide dynamic range with a single injection. All analytes displayed excellent linearity from 5 to 2500 pg/mL (0.03-16 nmol/L). All calibration curves displayed an $R^2 > 0.999$. Back-calculated accuracies for all calibrators ranged from 95% to 109% and showed inter-day CVs below 5%. Commercially-available quality control material was used to test the reproducibility of this method. Measurements were repeated in triplicate to assess intra-day reproducibility and on three separate days to assess inter-day reproducibility, and CVs were found to be below 4%.

Conclusion:

A robust method for quantifying dopamine, epinephrine and norepinephrine in plasma with excellent reproducibility and accuracy has been developed.

A-252

Pre-column double derivatization method to quantify > 35 clinically relevant amino acids by HPLC-TripleTOFTM 5600

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Background: Clinically relevant amino acids are measured in physiological fluids to diagnose a number of inborn errors of metabolism. Widely used methods are HPLC with various detectors such as UV and mass spectrometry. The gold standard method for amino acid analysis is ion exchange chromatography with ninhydrin post-column derivatization. However, this method is laborious and time consuming. Many reverse phase chromatography methods have difficulty in separating isobars in one run and require extra sample preparation steps and multiple injections. Also, since many amino acids are polar, in reverse phase chromatography ion pair reagents are frequently used but cause problems in cleaning mass spectrometer. To avoid ion-pairing, amino acids are commonly derivatized to make them non-polar for reverse phase separation. Butylation is the most commonly used technique but many amino acids are refractory to butylation. To solve this problem, we developed a double derivatization method. Butanol and dansyl chloride were selected to derivatize amino acid carboxyl group and amino group, separately. TripleTOF™ 5600 (Time of Flight) in a high sensitivity and TOF MS scan positive mode were used for amino acids quantitation with high mass resolution.

Method: 50 µl patients' biological fluids (heparinized plasma, urine and CSF) were used. Isotopically labeled amino acids were used as internal standards. The general sample preparation steps were: 1) protein precipitation; 2) transfer and split of supernatant; 3) drying of supernatant and derivatization of residue with butanol and dansyl chloride; 4) reconstitution of residue and injection of the mixture.

Waters Acuity UPLC coupled to TripleTOF™ 5600 (AB Sciex) with a phenomenex column (Kinetex, C18, 2.6 µm, 100 x 3 mm) was used. Mobile phase A was 2mM ammonium formate / 0.1 % formic acid in water; Mobile phase B was acetonitrile. Flow rate was 0.5 mL/min. The column temperature was 50°C. The gradient was 2-40% B for 15 min, then 100 %B for 3.5 min and back to 2% B with total run time of 22 min.

Results: This method was validated and was able to quantify > 35 clinical relevant amino acids including glutamine, glycine, alanine, citrulline, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine and arginine. In general, the recovery ranged from 90 to 110 % and total imprecision was less than 15%. The quantitation of amino acids with isotopically labeled internal standards showed the best results. Most amino acids were doubly derivatized at carboxyl - and amino - groups except phosphoethanlomine and taurine. The isobaric compounds were well separated. The results from butylation and dansylation were generally in good agreement. The method compared well with ninhydrin derivatization HPLC method.

Conclusion: This double derivatization method is accurate, precise, sensitive and fast for the measurement of > 35 physiologically relevant amino acids.

A-253

Measurement of aldosterone in clinical research; the quest for accuracy

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Background: Liquid chromatography tandem mass spectrometry (LC-MS/MS) is increasingly becoming the method of choice for steroid hormone measurements for clinical research, but thorough method validation is essential to ensure the quality of the method. Through the use of sample preparation, chromatographic separation and mass spectrometric detection, quantification of aldosterone by LC-MS/MS has been shown to provide greater selectivity than more traditional analytical techniques. Chromatographic separation of aldosterone from isobaric steroid species is paramount to obtaining accurate data. Since no higher order reference material is available for aldosterone in matrix, we have assessed external quality assurance samples from the UK National External Quality Assessment Service (UK NEQAS). Here we present a study of the effects of chromatographic selectivity on the analytical bias for

quantification of aldosterone, followed by comparison to an established LC-MS/MS method for aldosterone analysis.

Methods: Aldosterone certified reference material in acetonitrile was purchased from Cerilliant (Round Rock, TX) to create calibrators in stripped serum purchased from Golden West Biologicals (Temecula, CA). QC material was prepared in plasma purchased from SeraLab (Haywards Heath, UK). Samples (n=35) were obtained from UK NEQAS (Birmingham, UK) to compare the analytical bias of the developed LC-MS/MS methods. Plasma samples (n=59) were analyzed using the finalized method and the results were compared to an independent LC-MS/MS method. Samples were precipitated prior to Solid Phase Extraction (SPE) on the Waters® Oasis® MAX µElution plate. Using an ACQUITY UPLC® I-Class system, samples were injected onto either a 2.1 x 50mm Waters AQCUITY UPLC BEH Phenyl column (Method 1) or a 2.1 x 100mm Waters CORTECS UPLC C_{18} column (Method 2) using a water/methanol gradient elution and quantified with a Waters Xevo® TQ-S mass spectrometer.

Results: Method 1 and 2 were shown to provide imprecision of <10%CV across the range 36-720pg/mL. Method 2 improved chromatographic resolution of aldosterone from interfering steroids in comparison to Method 1, which included baseline resolution of the 18-hydroxycorticosterone interference from aldosterone and its internal standard.Method accuracy assessment by the analysis of UK NEQAS samples through comparison to the All Laboratory Trimmed Mean (ALTM) results was performed using Method 1 and 2. The aldosterone scheme has on average 30 participating laboratories (range 26-33), with the largest group using a competitive fixed-time solid-phase radioimmunoassay (average 23 laboratories, mean %CV=9.4%), while the LC-MS/MS group comprised of only 2-3 laboratories (no %CV available). Method 2 demonstrated a greater bias (-23.1%) than Method 1 (-5.3%) when compared to the ALTM. Using Method 2, comparison with samples (n=59) previously analyzed by an independent LC-MS/MS method demonstrated good agreement, showing no significant bias ($p > 0.05$).

Conclusion: Chromatographic selectivity has been investigated for the quantification of aldosterone in plasma for clinical research. LC column selection has been found to be a critical parameter that can significantly change analytical bias for the analysis of aldosterone. This further highlights the need for a reference measurement system for aldosterone, enabling harmonization of methods across different laboratories.

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

A-254

Rapid, Comprehensive and Simultaneous Determination of Inborn Errors of Metabolism using an Untargeted Metabolomics Methodology

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Background

The objective of this study is to validate the use of an untargeted metabolomics methodology to detect metabolic disturbances in fasted plasma samples for the detection of inborn errors of metabolism. Inborn Errors of Metabolisms (IEMs) are caused by defects in the enzymes that help process nutrients, causing an accumulation of toxic substances or a deficiency of substances needed for normal body function. Making a swift, accurate diagnosis of an IEM is critical in preventing developmental disorders. While there are several hundred recognized IEMs, state public health programs only screen for 40 disorders or fewer at birth and do so with an array of targeted assays. The approach described here allows for the detection of metabolic disturbances caused by IEM's beyond what is currently available with targeted assays and at a lower cost.

Methods

All samples were run over three separate chromatographic methods. Proteins were first precipitated from plasma samples via MeOH. The supernatant from the crash was divided into aliquots for each method, dried and then reconstituted in method appropriate solvents. All three methods were run on ThermoFisher Q-Exactives coupled to Waters Acquity UPLCs and included a positive ion reverse phase (RP) method, a negative ion RP method and a negative ion HILIC method. One hundred, twenty-six (126) compounds across three platforms were validated to CLSI standards including assessing precision, linearity, carryover, LOD, interference, stability and others. The Q-Exactives alternated between MS scans from 70-1000 m/z and MS^n scans and were operated at 35K resolution. Compounds were identified by match to authentic standard library based on retention index, accurate mass and fragmentation.

Results

The intra-assay precision (within run), as measured by %CV for all 126 compounds in triplicate plasma samples, was <20% except in 8 instances and the total, inter-assay precision during a multi-day trial was <20% except in 6 instances. When the signal intensities are scaled against a set of six plasma matrix controls that are run on a daily basis, the precision of the assay improves significantly. All 126 compounds showed linearity of >0.95 R^2. In addition to carryover, stability, and interference studies, an accuracy study was performed on a set of 200 pediatric plasma samples. The cohort included 130 samples from patients with 21 known IEMs and 70 samples from healthy individuals. The method correctly identified 20 of the 21 disorders.

Conclusion

Results of this proof-of-concept study demonstrate that metabolomic profiling has the potential to detect a wide range of IEMs and could represent an attractive initial screening option for other diseases with a suspected biochemical, genetic origin. Importantly, this methodology accurately identified the affected individual patients when compared to a healthy population, demonstrating the power of this methodology for personalized medicine. In this presentation, we highlight the testing carried out to validate our untargeted metabolomics methodology and present a case study of a patient that was identified with citrullinemia through biochemical signatures in multiple pathways by screening a single draw plasma sample on this metabolomics platform.

A-255

Development of a Reference Measurement System for Urine Albumin

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Background: Urine albumin and urine albumin/creatinine ratios are used in the detection, treatment, and monitoring of chronic kidney disease. Although inter-method differences and analyte heterogeneity have been reported for urine albumin measurements, accuracy assessments of the available methods have been hindered by the lack of a reference measurement system, including reference measurement procedures and reference materials, for this clinical analyte. To address the need for a reference measurement system for urine albumin, we have developed a candidate reference measurement procedure (RMP) for the value-assignment of urine albumin in a matrix-based standard reference material.

Methods: The candidate RMP incorporates an isotopically-labeled (15N) full-length recombinant human serum albumin (15N-rHSA) material as the internal standard, which permits the absolute quantitation of albumin in human urine. A total of 11 tryptic HSA peptides with 2 transitions per peptide were selected on the basis of retention time reproducibility, peak intensity, and the degree of HSA sequence coverage. The calibrators were generated by spiking charcoal-stripped human urine with the 15N-rHSA internal standard at approximately 22.0 mg/L and the unlabeled NIST SRM 2925: Human Serum Albumin Solution (0.8443 g/L ±0.0320 g/L) with HSA concentrations ranging from 5 to 300 mg/L. The samples were digested with trypsin via a conventional digest method and the products were analyzed using LC-MS/MS (Agilent 6460 mass spectrometer coupled to the Agilent 1290 Series LC system) in the positive ion mode under multiple reaction monitoring (MRM) MS conditions.

Results: The multiplexed urine albumin assay displayed good linearity over a concentration range of 5 to 300 mg/L with r2 values >0.99 over the 23 MRM measurements. Minimal matrix effects were observed from the analysis of the HSA peptides in the buffer system (50 mM ammonium bicarbonate in water) compared to the urine (NIST SRM 3667) system, with CV values ranging from 0.1-2.3%. The inter-peptide and intra-peptide precision for 5 QC levels showed CV values <15%. To investigate the accuracy of the method, a total of 23 calibration curves were generated from the MRM measurements and used to determine the urine albumin content of 15 non-pooled patient urine samples. The combined (all 23 MRM measurements) CV values for each of the 15 patient samples ranged from 5.9-12.7%.

Conclusion: MS-based quantification of urine albumin provides both accurate and repeatable measurements at both micro- and normoalbuminuria levels, which can facilitate early diagnosis of kidney dysfunction. In addition to the quantitative advantages, we are also able to qualitatively evaluate molecular heterogeneity of endogenous urine albumin via the incorporation of multiple peptides that span the HSA sequence in the multiplexed assay. The high degree of selectivity and sensitivity of the MS-based urine albumin assay coupled with the highly purified SRM 2925 calibrator (primary reference standard) will support the value-assignment efforts of the matrix-based urine albumin secondary reference material.

A-256

LC-MS/MS Method Optimization for the Analysis of 25-Hydroxyvitamin D and 24,25-Dihydroxyvitamin D Metabolites in Human Serum and Plasma

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Background: The quantitative analysis of 25-hydroxyvitamin D levels in serum and plasma provides an essential assessment of patient Vitamin D status and an evaluation of potential Vitamin D deficiency. We have optimized our LC-MS/MS method for the quantitation of 25-hydroxyvitamin D2/D3 and 24,25-dihydroxyvitamin D3 levels in patient serum and plasma samples. As part of this method optimization, we have incorporated the ongoing between-run analysis of internal and external quality control samples, including the testing of the NIST Standard Reference Materials SRM 972, SRM 972a, SRM 968e, and SRM 1950 human serum and plasma samples for additional accuracy and precision assessments of the 25-hydroxyvitamin D metabolites levels.

Methods: 25-hydroxyvitamin D2/D3 and 24,25-dihydroxyvitamin D3 analytical calibration curve standards and controls were prepared in normal human plasma depleted of Vitamin D metabolites. Isotopically labeled hexadeuterated 25-hydroxyvitamin D2/D3 internal standards were added to 200 uL of samples, and extracted using liquid/liquid extraction. Extracts were then dried under a stream of nitrogen, reconstituted in methanol, and transferred to autosampler vials for injection to the LC-MS/MS. Analysis was performed using an AB SCIEX API4000 LC-MS/MS system coupled with an Agilent 1100 LC and operated using APCI in the positive ion mode, with Multiple Reaction Monitoring (MRM) for quantitation of the Vitamin D metabolites.

Results: Linearity was demonstrated across the range of 1.25 -180 ng/mL for the 25-hydroxyvitamin D2/D3 metabolites, with calibration curve R2 >0.999, and across the range of 2-100 ng/mL for the 24,25-dihydroxyvitamin D3 analyte, also with calibration curve R2 >0.999. Six levels of 25-hydroxyvitamin D2/D3 controls prepared in depleted normal human plasma, and tested by two different analysts, demonstrated between-run LC-MS/MS results (n = 52) within 2.0% of target concentrations with precision (%CV) within 3.7%. LC-MS/MS testing of 24,25-dihydroxyvitamin D3 normal human plasma controls showed between-run results (n =24) within 2.5% of target concentration levels, with %CVs within 2.0%. LC-MS/MS between-run analysis of the NIST SRM materials demonstrated 25-hydroxyvitamin D metabolites results within 2.3% of target concentrations. LC-MS/MS testing of 94 patient serum and plasma samples was completed for the assessment of comparative Vitamin D metabolite concentration levels.

Conclusion: This LC-MS/MS method has been demonstrated to be an accurate and precise method for the analysis of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D3 levels in human serum and plasma samples. As an additional ongoing measure of method accuracy, precision, and specificity, we continue to analyze standard reference material and external quality assessment samples as part of our routine testing using this LC-MS/MS method.

A-257

Development And Validation Of A High Sensitivity MicroLC-MS/MS Underivatized Method For Estradiol In Human Serum

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Background: There are considerable demands to accurately measure estradiol (E2) at very low concentrations (<5 pg/mL) in postmenopausal women, men, pediatric patients, and patients receiving breast cancer treatment. Historically, high sensitivity E2 assays used in clinical laboratories are radioimmunoassays, which may lack specificity and accuracy for measuring E2 over a wide concentration range. Most current high sensitivity LC-MS E2 methods can overcome these analytical limitations, but requires large sample volume and involves complex sample preparation with dansyl chloride derivatization. Our study aims to develop a high sensitivity, underivatized method using MicroLC-MS/MS to reliably measure E2 concentrations below 5 pg/mL using low sample volume.

Method: Estradiol-d4 (C/D/N isotope, Quebec) was used as the internal standard (IS) and calibrators were made by spiking E2 standards (Sigma, MO) into the E2-depleted serum (Golden West Biologicals, CA). 290µL of each calibrator and samples were mixed with 10µL of 2.5 ng/mL IS, and extracted with 3mL extraction buffer consisting of 90% hexane (Sigma, MO) and 10% ethyl acetate (Sigma, MO). The organic phase was evaporated under nitrogen at 37°C, and the dried residue was dissolved in 60µL of 1:1 methanol/water (v/v) prior to an injection of 5µL into the MicroLC-MS/MS. Analytes were separated on a micro-C18 column (0.5x50mm, 3µm, YMC America, PA) with a flow rate of 35µL/min and a run time of 3.5min using the Eksigent micro-LC 200 system (SCIEX, MA). The gradient started with 10% mobile phase A (0.01% ammonium hydroxide in water) and 90% mobile phase B (0.01% ammonium hydroxide in acetonitrile). The mobile phase B was increased to 98% over 1.5min and then held steady for 1 min. E2 and IS are detected by the SCIEX QTrap 6500 mass spectrometer in negative mode using the following transitions: E2: 271/145 (quantifier), 271/143 (qualifier); IS: 275/147.

Results: In this method, it is crucial to use HPLC columns with stability at the pH of 10. Our method demonstrated good linearity over a concentration range of 3– 820 pg/mL with r2 > 0.999. Total precision displayed CVs< 15% for all three QC levels (4.4 pg/mL, 49 pg/mL and 555 pg/mL) and the limit of quantitation was 3 pg/mL (CV< 20%). Method comparison studies for samples with E2 < 100 pg/mL showed: [RIA E2] = 0.92[LC-MS E2] +1.18, mean bias = -1.0 pg/mL (n= 29); [Cobas e602 E2] = 1.1[LC-MS E2]+ 3.60, mean bias = +6.0 pg/mL (n=40). Hemoglobin and bilirubin was observed to negatively interfered with this method at H-index of 370 and I-index of >9, respectively. There is no significant interference of triglycerides up to 800 mg/dL.

Conclusion: Our MicroLC-MS/MS method without derivatization, shows good analytical performance and can reliably measure E2 in blood samples with a LOQ of 3 pg/mL.

A-258

Evaluation and Comparison of Nonderivatization and Derivatization Tandem Mass Spectrometry Methods for Analysis of Amino Acids and Acylcarnitines in Dried Blood Spot

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Introduction

Flow injection tandem mass spectrometry (FIA-MS/MS) has been frequently used to analyze amino acids (AA), acylcarnitines (AC), and succinylacetone (SUAC) in dried blood spots (DBS) for inborn errors of metabolism research. Original sample preparation techniques detect butyl esterification (i.e., derivatized) of AAs, ACs, and SUAC. However, with improved sensitivity of MS instruments, it is possible to detect AAs, ACs, and SUAC as their native free acids (i.e., nonderivatized). This simplifies analytical operation and minimizes the use of corrosive chemicals. Using quality control (QC) DBS samples enriched with different levels of multianalytes, we conducted a comprehensive study to evaluate and compare nonderivatization and derivatization tandem MS methods on a triple quadrupole mass spectrometer.

Methods

We developed a method in which AAs, ACs and SUAC were extracted in a single extraction resulting in significant reduction in labor and time. The 3.2 mm DBS punches were extracted by incubating with acetonitrile-water-formic acid mixture containing hydrazine and stable-isotope labeled internal standards. The extracts were derivatized with n-butanolic-HCl followed by evaporation and reconstitution with mobile phase. For the nonderivatization method, the extracts were simply dried and reconstituted with mobile phase. In both cases, the extracts were analyzed by Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer. Quantification of 12 AAs, 18 ACs, and SUAC was achieved using a meta-calculation software, iRC PRO™. The off-line automated tool streamlined data processing of peak area, concentration and user-defined formulas.

Results

We utilized DBS QC samples to evaluate and compare the two tandem MS methods. The QC samples contained enriched multianalytes at three concentrations: low, intermediate and high. For each method, we evaluated within-run precision by means of ten successive, independent measurement of DBS samples (n=10) and run-to-run precision by means of ten independent measurement in seven different test series (n=70). We also compared quantitative results of multianalytes from nonderivatization and derivatization methods.

Within-run Precision

For the derivatization method, the within-run precisions (n=10) for AAs, ACs, and SUAC at three concentrations were less than 8.9% (low), 8.3% (intermediate), and 9.0% (high).

For the nonderivatization method, the within-run precisions for AAs, ACs, and SUAC were less than 7.6% (low), 7.2% (intermediate), and 9.8% (high).

Run-to-run Precision

For the derivatization method, the run-to-run precisions (n=70) for AAs, ACs, and SUAC were less than 15.0% (low), 15.6% (intermediate), and 16.1% (high).

For the nonderivatization method, the run-to-run precisions for AAs, ACs, and SUAC were less than 12.8% (low), 12.8% (intermediate), and 12.6% (high).

Method Comparison

The method differences of 12 AAs and SUAC between quantitative values resulting from butyl esters and free acid techniques at three concentrations were less than 3.8% (low), 4.8% (intermediate), and 3.2% (high). The method differences of 18 ACs were less than 14.2% (low), 11.4% (intermediate), and 10.5% (high). Therefore the two methods were highly correlated. Our data are consistent with the reported results from a comprehensive empirical analysis.

A-259

Mass-spectrometric relative quantitation of glycated hemoglobin S

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Background: Glycated hemoglobin (reported as HbA1c) is an indicator of the average glucose level in the blood over the previous several months. The presence of hemoglobin genetic variants can affect the accuracy of some widely used HbA1c tests if the operator is unaware of the presence of genetic disorder. Hemoglobin S (HbS) is the most common hemoglobin mutant and consists of a β-chain substitution of Glu with Val at the seventh position. The aim of the study was to measure the extent of the glycation of the β chains in order to compare glycation rates for normal and mutated (β*) β-chains of heterozygous hemoglobin S (HbAS).

Methods: We analyzed 20 hemolysate samples from HbAS subjects both with and without diabetes. The diluted and desalted lysate samples were introduced into a triple quadrupole mass spectrometer (AB Sciex API-4000) at a flow rate of 50 µl/min. The instrument was operated in ES+ mode, six 10-sec scans were summed over 600-1400 m/z range. The relative quantification was performed by comparison of ion intensities of structurally similar intact globin chains in the deconvoluted spectra (β vs β*, α^g vs α, β^g vs β, β^{*g} vs β*, where g - glycated). For samples with low HbA1c, boronate affinity concentration of the glycated fraction was perfomed prior to analysis.

Results and conclusion: The ratio β*/β: 40/60 was found to be relatively stable for all samples analyzed. We found the glycation rate to be slightly higher for the β* compared to the normal β. The mass-spectrometric approach developed easily allows for relative quantitation of glycated forms of HbS and could be used to examine glycation rates for other hemoglobin variants. Further studies are needed to determine the extent to which the different glycation rates may impact clinical interpretation of HbA1c results obtained by various routine assay methods.

A-260

Vedolizumab Quantitation in Serum using SRM And microLC-ESI-Q-TOF Mass Spectrometry

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Background: Vedolizumab is a humanized $IgG_1\kappa$ therapeutic monoclonal antibody (mAb) that inhibits T-cell migration to the gut mucosa by selectively targeting $\alpha_4\beta_7$ integrin and inhibiting its ability to bind to mucosal-addressin-cell-adhesion-molecule-1 (MAdCAM-1). Vedolizumab was recently FDA approved for the treatment of patients with moderate-to-severe ulcerative colitis and Crohn's disease who have no response or have lost response to TNF-α inhibitors. Currently there are no clinically available methods to measure vedolizumab. There is demand from the gastroenterology practice to guide therapy and improve outcomes for these patients refractory to multiple therapies. **Objective:** To develop and compare mass spectrometry (MS) methods to quantitate vedolizumab using: (1) tryptic peptides unique to the mAb by LC-MS/MS and (2) its intact light chain (iLC) accurate mass by microLC-ESI-Q-TOF/MS as a surrogate for drug concentrations. Furthermore, determine if vedolizumab concentrations correlate with C-reactive protein (CRP) concentrations. **Methods:** Vedolizumab was spiked into pooled human serum to generate a 7-point standard curve (5, 10, 25, 50, 100, 150, 250µg/mL) and control material. Proteotypic peptides unique to the heavy (HC) and light (LC) chains of vedolizumab were identified for quantification by selective reaction monitoring (SRM). Horse IgG was added to samples as an internal standard, followed by reduction, alkylation and trypsin digestion. The mixture was analyzed by LC-MS/MS (AbSciex API 5000). For iLC analysis monoclonal immunoglobulin Rapid Accurate Mass Measurement (miRAMM) was used. Briefly, infliximab was spiked into serum as internal standard and immunoglobulins were purified using Melon Gel. Light chains were released using DTT and injected on an Eksigent microLC system. Spectra were collected on an ABSciex-Triple-TOF-5600-MS. Vedolizumab iLC were quantified by summing multiple charge states corresponding to vedolizumab based on retention time and accurate mass measurements. Assay characteristics were defined by measuring precision and linearity. Subsequently, vedolizumab was measured in residual sera from patients undergoing therapy (n=110 samples from 28 unique patients) using both methods. Chart-review was conducted to correlate infusion date with vedolizumab concentration. Clinically-ordered CRP (immunoturbidimetry, reference interval (RI) ≤8mg/L) was also compared to vedolizumab concentrations. **Results:** Lower limit of quantitation (LOQ) for both HC and LC peptides was 5.0µg/mL; while LOQ for vedolizumab iLC quantitation was 10µg/mL. Both methods were linear up to 250µg/mL, n=10, R^2>0.99. Inter- (n=10 days) and intra-assay (n=10 runs) precision ranged from 3-19% for both methods using three levels of controls (10, 50, 150µg/mL). Method comparison using Passing-Bablok regression fit (x=proteotypic method) was y=0.79x + 4.78 (r=0.90). Vedolizumab concentrations, measured in patient sera by both methods in different time points of treatment went from <5–160µg/mL. Serial measurements (varying from 2-13) were available for 19 patients and trended inversely with infusion dates (R^2=0.86). Vedolizumab was also inversely correlated with CRP. When dichotomized into within (n=13) or above RI (n=24), vedolizumab measured 23±21 vs. 37±23 µg/mL (p=0.03). **Conclusions:** This study not only demonstrated that vedolizumab can be quantitated in patients by both proteotypic peptides and intact light chain MS, but more importantly, showed that those measurements may correlate with inflammation and aid in therapeutic monitoring of response to treatment.

A-261

Quantitation of Ubiquinone (Coenzyme Q10) and Retinyl Palmitate in Serum/Plasma using Liquid Chromatography Electrospray Tandem Mass Spectrometry

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Introduction: The monitoring of vitamin levels in patients utilizing supplementation therapy is important for the prevention of potential toxicities and measurement of treatment outcomes. Vitamins play an essential role in human health and wellness. While essential, many vitamins cannot be synthesized within the body and must be obtained externally, most commonly from dietary intake. Retinyl Palmitate and Ubiquinone (Coenzyme Q10) are powerful antioxidants that are responsible for maintaining a variety of essential functions throughout the body. Coenzyme Q10 is considered an essential co-factor in the mitochondrial respiratory chain responsible for oxidative phosphorylation and plays a unique role in the electron transport chain. Retinyl Palmitate (RP) is a synthetic alternate form of vitamin A (retinol) and is widely used in nutritional supplements in its place. Vitamin A serves in multiple roles: it is important for growth and development, maintenance of the immune system and good vision.

Mindful of the numerous unique features and the idiosyncratic nature of these molecules we set out to improve their detection utilizing positive electrospray ionization tandem mass spectrometry spectroscopy (ESI- LC-MS/MS). Our aim was to improve the sensitivity and to simplify the extraction process and create a robust method suitable for the routine analysis from serum or plasma samples.

Method: Aliquots of 200 µl of standard, control and patient serum/plasma samples were spiked with Coenzyme Q10-D9 serving as an internal standard. The extraneous proteins were precipitated from the samples with ethanol and followed by the addition of hexane. Samples were analyzed on an API 3200 triple quadrupole mass spectrometertandem mass spectrophotometer (Applied Biosystems) equipped with a TurboIon Spray® source and a Shimadzu Prominence 20A HPLC system. Chromatographic separation was achieved using a LUNA 3µ PFP (2) 50 x 2.0 mm, 100Å column (Phenomenex) at a flow rate of 0.85ml/min, for a total run time of 5 minutes. The MS/MS was operated in positive electrospray and multiple reaction monitoring (MRM). Two transitions were monitored for each analyte: 880.7 -> 197.3 and 880.7 ->237.3 for Coenzyme Q10 (CoQ10), 269.4 ->93.0 and 269.4->107.0 for Retinyl Palmitate and 889.7->206.3 and 889.7 -> 189.1 for Coenzyme Q10-D9. **Results:** Validation was performed on 127 previously run samples, adhering to CLSI-derived protocols. Data analysis was performed using EP Evaluator® for the following parameters: precision (inter-, intra-, LOD/LOD), linearity, recovery, correlation, carry-over, interference and stability. CoQ10 linearity testing showed the assay was linear from 0.22 - 12.0 mg/L with a slope of 0.954 and an observed error of 6.2%. RP demonstrated linearity from 0.03 - 2.0 mg/L and a slope of 0.994 with an observed error of 8.8%. Both analytes recovered between 93.0-106% and the correlation coefficient between CoQ10 and RP comparing our method and the reference method was r=0.9205 and 0.9958 respectively. Correlation of our assay

demonstrated no significant bias and all precision studies were CV% = ≤20.0%. No significant interfering substances were observed.

Conclusion: We have successfully developed and validated a robust, simple and effective method for extracting and monitoring Coenzyme Q10 and Retinyl Palmitate levels in patient samples.

A-263

Evaluation of the effects of 25OHD2 and 3-epi 25OHD3 on five automated 25OHD Immunoassays with comparison to a liquid chromatography tandem mass spectrometry method

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Background: Vitamin D testing is becoming increasingly important with the recognition that it is correlated with autoimmune diseases, cardiovascular diseases and cancer, and vitamin D deficiency is common in the worldwide. Recently, automated immunoassays are becoming popular to cope with the increasingly workload. However, the efficiency to separate and measure $25OHD_3$, $25OHD_2$ as well as 3-epi $25OHD_3$ was different, which may cause variation between methods.

Methods: Our objective was to evaluate the effects of $25OHD_2$ and 3-epi $25OHD_3$ on Roche, Siemens, Abbott, IDS and DiaSorin immunoassay systems with comparison to a liquid chromatography-tandem mass spectrometry (LC-MS/MS). We selected 332 serum samples from routine vitamin D assay requests, including 166 serum samples contained only $25OHD_3$, 111 serum samples contained both $25OHD_3$ and $25OHD_2$, 32 serum samples contained $25OHD_3$ and 3-epi $25OHD_3$, and analyzed with these methods.

Results: In the samples with no $25OHD_2$ or 3-epi $25OHD_3$, all the immunoassays correlated well with LC-MS/MS (r≥0.872), DiaSorin and Roche showed negative bias less than 2.5 ng/mL while Abbott and IDS showed positive bias less than 2.0 ng/mL. However, Siemens showed the biggest positive bias 12.5 (11.0-14.0) ng/mL and the poorest Kappa (0.468) while other four immunoassays showed excellent agreement with LC-MS/MS-1(Kappa>0.8)(Using 20 ng/mL as the cut-off for vitamin D deficiency). When there is $25OHD_2$ or 3-epi $25OHD_3$ in the samples, the correlation and clinical agreement decreased between all the immunoassays and LC-MS/MS. After using the regression models to transfer the cut-offs for immunoassay methods, Siemens had the biggest improvement of Kappa values (0.637) and all the immunoassays showed an acceptable agreement with LC-MS/MS.

Conclusions: Most automated immunoassays showed acceptable correlation and agreement with LC-MS/MS when there is no detectable $25OHD_2$ and 3-epi $25OHD_3$. Both $25OHD_2$ and 3-epi $25OHD_3$ affects the immunoassay methods a lot and bias existed between methods. Using one general cut-off for all the methods may be not appropriate.

A-264

Determination of Plasma Renin Activity by LC/MS/MS for Clinical Research

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Background:

Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited for the rapid analysis of analytes in complex matrices. A highly sensitive and specific LC/MS/MS analytical method has been developed for the determination of plasma renin activity (PRA) for clinical research. Plasma samples are incubated for 3 hours at 37°C. A sample preparation procedure by solid phase extraction (SPE) allows efficient extraction of Angiotensin I in plasma. Plasma renin activity is calculated by subtracting Angiotensin I concentration in a blank plate.

Methods:

For Generation sample, the plasma sample is incubated under controlled pH conditions for 3 hours at 37°C. The reaction is quenched and internal standard is added. The mixture is subjected to SPE extraction and analyzed by LC/MS/MS. For Zero sample, a duplicate plasma sample is immediately subjected to SPE extraction and analyzed by LC/MS/MS. Plasma Renin Activity is calculated by subtracting Angiotensin I concentration in the Zero sample from the Generation sample. The result is divided by the duration of incubation (3 hours = 10800 seconds) for a final value in ng/L/s. Angiotensin I (Proteochem) calibrators are prepared in 1% bovine serum albumin buffer pH 6 solution. Quantifier and qualifier transitions were monitored. Isotopically labelled Internal Standard (AnaSpec) was used. Bio-Rad Lyphochek controls were used. The chromatographic system consists of an Agilent Poroshell 120 SB-C18 column coupled with a guard column and a mobile phase comprised of methanol and water containing 0.2% formic acid.

Results:

The described analytical method achieves the required functional sensitivity and is capable of quantitating Angiotensin I over a sufficiently wide dynamic range. Angiotensin I displayed an excellent linearity from 0.1688 to 100 ng/mL. The calibration curve displayed an $R^2 > 0.9996$. Back-calculated accuracies ranged from 93% to 114%. Commercially-available quality control material was used to test the reproducibility of this method. Measurements were repeated in triplicate to assess intra-day reproducibility, and on three separate days to assess inter-day reproducibility, and CVs were found to be below 6%.

Conclusion:

A robust analytical method for quantifying angiotensin I in plasma by LC/MS/MS which is applied for the determination of Plasma Renin Activity has been developed. Typical method performance results are well within acceptable criteria.

A-265

Targeted Screening of Drugs of Abuse using Laser Diode Thermal Desorption and mass spectrometry

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Background:

Toxicology laboratories generally use screening methods to obtain a semi-quantitative response for samples suspected of containing drugs of abuse. Some screening techniques are fast but less specific and generate by far too many false positive results. Using mass spectrometry combined with ultra-fast high-throughput LDTD ion source enhances specificity while maintaining and even surpassing the speed and throughput of traditional screening methods.

The Laser Diode Thermal Desorption (LDTD) ion source uses an infrared laser diode to indirectly thermally desorb neutral species of drugs of abuse molecules from urine sample extracts. These neutral species are carried into a corona discharge region, where they undergo efficient protonation and are introduced directly into the mass spectrometer. The total analysis time is under 9 seconds with no carry-over.

The objective of this experiment is to validate a simple urine extraction, the analysis method and to test different real samples using the LDTD-MS/MS. A cross validation study against the LC-MS/MS approach for the analysis of targeted drugs of abuse (PCP, ecstasy metabolite, amphetamine and methamphetamine) was done in order to evaluate the performance of the proposed alternative LDTD-MS/MS method

Methods:

A calibration curve and quality control samples were prepared in blank urine. 100 µL of calibrators, QC and patient specimens were transferred to an Eppendorf tube. 20 µL of internal standard (Amphetamine-d5, Methamphetamine-d9, PCP-d5 and MDMA-d5 at 750 ng/mL) in a mixture of Water:Methanol (1:1)) and 250 µL of sodium hydroxide (1N) were added. The mixture was vortexed. A liquid-liquid extraction was then performed by adding 250 µL of 1-Chlorobutane. Following the mixing and centrifugation at 5000 rpm for 3 min, 100 µL of the organic layer was transferred in an Eppendorf tube and 10 µL of HCl (0.01N) in methanol solution was added and mixed. 6 µL was deposited in the LazWell Plate and evaporated to dryness. The LDTD laser power was ramped to 45% in 3 seconds, and shut down after 2 seconds. Positive ionization mode was used and AB Sciex-5500 QTrap system was operated in MRM mode.

Results:

The calibration curves show excellent linearity with r higher than 0.995 between the quantification range of 4 to 800 ng/mL for Amphetamine, Methamphetamine, PCP, MDA, MDEA and MDMA. Inter-run accuracy and precision are between 98.4 to 107.7% and 2.6 to 9.3%, respectively, for every drug. No matrix effect or carryover was observed. This method was cross validated with results from a traditional LC-MS/MS method with real patient specimens. All negative samples correlated accordingly. A correlation higher then 0.99 between LC-MS/MS and LDTD-MS/MS methods for positive samples was calculated. All negative samples are detected as negative in both methods.

Conclusion:

A fast liquid-liquid extraction combined to LDTD-MS/MS technique provides a unique ultra-fast and specific mass spectrometry high-throughput method for screening. This method has demonstrated accurate, precise and specific results with a run time of 9 seconds per sample.

A-266

Development of a LC-MS/MS assay for the detection of sulfonylurea drugs, and application of the assay in emergent hypoglycemia cases.

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Sulfonylurea agents are commonly used in the treatment of type 2 diabetes mellitus. The medications decrease blood glucose by increasing the release of endogenous insulin from the beta cells. Sulfonylureas are most common drugs that causes hypoglycemia with overdose or when ingested by nondiabetic patients. Since the consequences of hypoglycemia can be devastating, a rapid differential diagnosis and treatment is essential for patients. Therefore, detection of the presence of sulfonylurea allows for the correct identification of the underlying cause of hypoglycemia and rule out the other causes, such as insulinoma, sepsis, and hepatic disease. **Objective:** This study was designed to develop and validate a qualitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for the detection of sulfonylurea compounds in serum, and utilized the assay to screen for sulfonylurea exposure in emergent hypoglycemia cases. Analytes include tolbutamide, chlorpropamide, tolazamide, acetohexamide, gliclazide, glipizide, glyburide, and glimepiride. **Methods:** Serum samples spiked with deuterated internal standard were processed by protein precipitation using acetonitrile, followed by centrifuge and separation of the supernatant from the protein pellet. The sample was dried under nitrogen in a 37°C water bath. The drugs were reconstituted in 80% mobile phase A and 20% mobile phase B. LC-MS/MS was performed using an Agilent HPLC with an ABSciex 3200 LC-MS/MS in positive ESI mode. Compounds were identified by a combination of retention time, two MRM transitions and the product ion spectrum. Separations were performed using a Phenomenex Luna 3u C8(2) 100Å, 50 × 2.0 mm, with a gradient from 30% to 95% organic over 5.5 min (Mobile phase A: 20 mM ammonium acetate in 95:5 HPLC Honeywell Water : methanol; mobile phase B: 100% methanol). Validation of the final method included determining the lower limit of detection (LOD), linearity, within-run imprecision, between-run imprecision, matrix effect, carry over, and recovery for each analyte. **Results:** The calibration curves for each analyte exhibited consistent linearity and reproducibility in a range of 5 - 1000 ng/ml. Within run coefficient of variation ranged from 4.3% to 15% at 50 ng/ml. Between-run coefficient of variation ranged from 10% to 21% at 50 ng/ml. No significant ion suppression was detected for any of the analyte (88 - 97%). Recovery ranged from 95 to 120% with an average of 103%. No carryover was observed at toxic levels to each analyte. **Conclusion:** We have developed and validated a sensitive and specific LC-MS/MS assay for sulfonylurea detection. Since April 2014, the method has been used in thirteen emergent hypoglycemia cases, in which four cases were positive for glipizide. The results were achieved with a rapid turnaround time of 2-3 hours, and provided real-time information for the differential diagnosis by the treating team.

A-268

Use of an Animal-free Synthetic Surrogate Serum Matrix for Preparation of Calibrators and Controls in Immuno- and LC-MS Based Clinical Assays

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Background:

The objective is to determine the utility of a well-defined, stable, animal-free matrix for use as a serum (or stripped serum) substitute in clinical assays. The potential clinical benefits would include the complete absence of both clinically relevant endogenous analytes as well as any potentially harmful blood-borne pathogens. This, along with the ability to manufacture with precise lot-to-lot consistency could prove clinically useful as a blank, calibrator and control matrix as well as a sample diluent. The matrix under evaluation was prepared with 2% recombinant human serum albumin (rHSA) expressed from an animal-free rice expression system. Such a synthetic surrogate serum matrix could provide more consistently reliable clinical performance compared to animal-derived matrices for both ELISA and LC-MS based clinical assays.

Methods:

The 'synthetic surrogate serum' was tested in commercially available IVD ELISA kits to different analytes including beta-2 microglobulin and thyroglobulin. Blank and mock samples were prepared by spiking respective analyte reference materials at varying concentrations into the synthetic surrogate serum and comparing results to spiked pooled human serum, spiked stripped serum and spiked matrix provided by the kit manufacturers. The synthetic surrogate serum was also tested in direct comparison to pooled human serum in an LC-MS based clinical assay for measuring blood levels of methotrexate (MTX). Several comparisons were performed including measuring MTX spiked human serum samples and patient samples using either pooled human serum or synthetic surrogate serum calibrators.

Results:

For the ELISA kits, several concentrations of analyte were tested, spanning the range of each of the respective kits. For all analytes, measurements showed interferences when pooled human serum was utilized indicating endogenous levels of target analyte. This was also observed when stripped serum was utilized with the thyroglobulin ELISA kit. As expected, no interferences were observed in any of the kits when the synthetic surrogate serum was used. Further, synthetic surrogate serum matched the performance of the matrix provided by the kit manufacturers indicating a suitable matrix for these ELISA kits. In the LC-MS based MTX assay, comparison of pooled human serum calibrators used to calculate synthetic serum substitute calibrator concentrations was described by the Deming equation $y=1.048x - 6.14$ with a correlation coefficient of $R^2 = 0.9995$. Comparison of the values assigned to patient samples when using calibrators from pooled human serum or synthetic surrogate serum was described by the Deming equation $y = 0.963x + 3.52$ with a correlation coefficient of $R^2 >0.999$.

Conclusion:

We have formulated a simple, animal-free matrix which has been shown to be suitable as a calibrator/blank matrix as well as a patient sample diluent. This has been shown in commercially available IVD ELISA kits as well as in an LC-MS based clinical assay. Unlike typical animal based matrices, this synthetic surrogate serum offers a reproducibly defined formulation and is completely void of any analytes of interest or potentially harmful blood-borne risk factors.

A-269

A Simple and Sensitive Method for Quantification of 5HIAA and VMA by Liquid Chromatography-Tandem Mass Spectrometry

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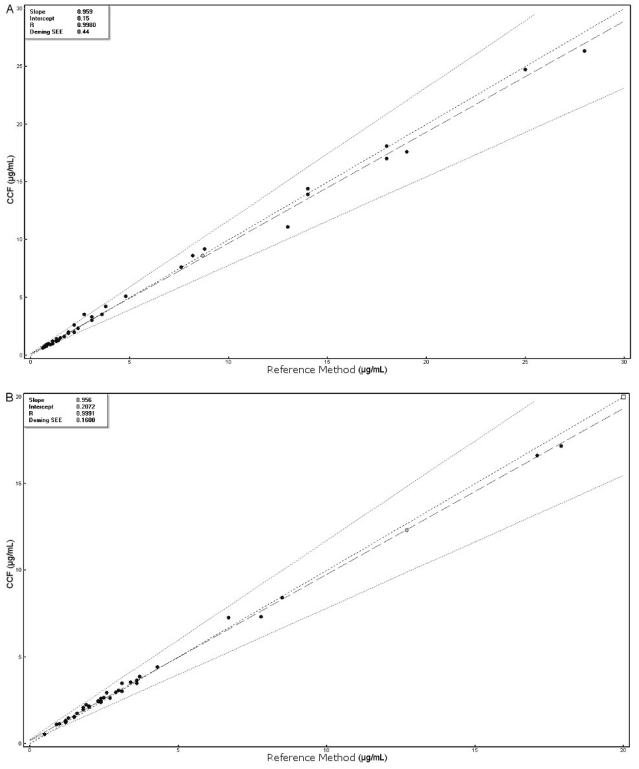
Background: 5-Hydroxyindole-3-acetic acid (5HIAA) and Vanillylmandelic acid (VMA) are important in the detection and diagnosis of carcinoid and neurogenic tumors, respectively. 5HIAA, the major metabolite of serotonin, plays a major role in the study of neurologic disorders and monitoring the progression of carcinoid disease. Measurement of urinary VMA has been beneficial in the diagnosis and follow-up of pheochromocytoma and other catecholamine secreting tumors, as VMA is present in higher concentrations than other catecholamines in urine.

Objective: To develop a simple and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for measurement of 5HIAA and VMA in urine. Methods: Patient urine sample (50µl) and 100µl of internal standard (IS) solution (0.5µg/mL 5HIAA-d5, and 0.5µg/mL VMA-d3 in acetonitrile) were combined and vortex mixed. Samples were centrifuged at 15500 g for 3 minutes and 50µL supernatant was mixed with 200µL water. The resulting solution (15µL) was analyzed on an LC-MS/MS system by multiple reaction monitoring (MRM) in the negative ESI mode using a Scherzo SM-C18 analytical column. Total chromatographic time was 11 minutes. Results: A mixing study determined that matrix effects were compensated for by the deuterated internal standards. No carryover was observed up to 99.0µg/mL and 97.5µg/mL for 5HIAA and VMA, respectively. Precision of the assay for both 5HIAA and VMA was shown to be within 10% CV at three concentration levels over 10 days. Linearity of the assay was determined to be 0.2 to 50µg/mL for both 5HIAA and VMA. Results from patient sample comparisons (n=40) with an independent clinical lab is shown in Figures 1A (5HIAA) and 1B (VMA). Conclusion: This LC-MS/MS method offers simple sample preparation and sensitive quantification of 5HIAA and VMA in urine and has been validated for use in our clinical lab.



A-270

Online Solid Phase Extraction and LC/MS/MS Detection of Thyroid Hormones in Biological Fluids

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Thyroid hormones play critical roles in the regulation of biological processes such as growth, metabolism, protein synthesis, and brain development. Specifically, thyroid hormones, 3,3',5,5'-tetraiodo-L-thyronine (thyroxine or T4) and 3,3',5-triiodo-L-thyronine (T3), are essential for development and maintenance of normal physiological functions. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3), are important for the diagnosis and monitoring of thyroid diseases. Most clinical laboratories measure thyroid hormones using immunoassays. The immunoassay-based methods offer a relatively rapid, high patient sample throughput that lends itself to automation but are significantly compromised by problems with assay interference and are perturbed by changes in protein levels that alter the free hormone availability.

Liquid chromatography mass spectrometry (LC/MS) has been reported to offer superior specificity and speed over the immunoassays for determination of thyroid hormones in biological matrices such as serum and tissues. Nevertheless, the reported sample preparation procedures, typically by liquid-liquid extraction followed by solid phase extraction (SPE), involve multiple time consuming steps, and are less compatible with automation.

In the present work, an online SPE-LC/MS method has been developed for the determination of thyroid hormones in biological fluids. The method exploits RP-Amide or C8 as the trapping column and Phenyl phase as the separation column, respectively. The preliminary experiments demonstrated that, under the optimized conditions, both RP-Amide and C8 effectively trapped the thyroid hormones extracted from spiked rabbit plasma sample which had been protein precipitated. And with both traps, sharp peak shapes were observed. However, the RP-Amide traps are advantageous over the commonly used C8 traps with higher signals and recoveries. Additionally, RP-Amide traps are more flexible with washing solvent as it is compatible with 100% aqueous mobile phases and can be used with up to 20% methanol with minimal sample loss.

A-272

The PhoTorrentTM Atmospheric Pressure Photoionization (APPI) Source Utilized for High Efficiency Photoionization of Testosterone and 25-OH Vitamin D3

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Background:

Atmospheric Pressure Photoionization (APPI) is commonly used to ionize compounds of interest for environmental research and is becoming popular in clinical research. It is an effective ionization method for many compounds which are not conducive to electrospray ionization (ESI) or atmospheric chemical ionization (APCI), leading to enhanced sensitivity and lower background for a wide range of compounds. The IONICS PhoTorrentTM APPI source is a modular assembly, easily interchangeable with the ESI or APCI hardware on a high performance dual probe ion source. This study demonstrates the characteristics of the IONICS modular PhoTorrent APPI source for two clinical compounds (Testosterone and 25-OH Vitamin D3). Performance under a range of conditions, including various total flow rates and dopant levels, was determined.

Methods:

The IONICS PhoTorrent APPI source module achieves high efficiency, broad range photoionization with the aid of a 10.6eV lamp from Syagen Technologies. The PhoTorrent APPI module was mounted onto the dual probe source of an IONICS 3Q 320 LC-MS/MS, in place of the ESI/APCI probes. Standards for Testosterone and 25-OH Vitamin D3 were purchased from Sigma-Aldrich and prepared in concentrations ranging from 0.0001 -10 ng/mL (Testosterone) and 0.1-100 ng/mL (25-OH Vitamin D3). Standards were dissolved in 20/80 water/methanol with 0.1% formic acid. The injection volumes were 10 µL. Duplicate injections were made for each concentration. The samples were eluted using a Shimadzu Nexera UHPLC with a C18 column using a mobile phase of 20/80 water/methanol, 0.1% formic acid, 5mM ammonium acetate.

Results:

The IONICS PhoTorrent APPI source demonstrated sensitivity to as low as 100 ag/µL for Testosterone while maintaining excellent linearity (R2 = 0.9997) over the range of 0.0001-10 ng/mL. This was achieved using a total solvent flowrate for Testosterone of 300 µL/min. A dopant of toluene to 10% of the total flow rate was added to enhance photoionization.

The 25-OH Vitamin D3 achieved a sensitivity as low as 100 fg/µL while maintaining linearity (R2 = 0.9999) over the range of 0.1-100 ng/mL. The 25-OH Vitamin D3 was run with a total flowrate of 500 µL/min, again with a 10% dopant of Toluene. In general, it was observed that increasing flow rate provided sharper LC peaks, resulting in the expected increase in signal to noise ratio. Minimal variation in peak area was observed across a total flow range of 100-500 µL/min.

Conclusion:

The PhoTorrent APPI source therefore demonstrates high linearity and excellent LLOQ's, and is well suited to flow rates of 100-500 µL/min. Development of the modular PhoTorrent APPI Source assembly facilitates switching between modes, from APPI to ESI and APCI, increasing the number and range of compounds that can be analyzed effectively with minimal hardware changes.

A-273

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

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Objective

Developing a LC/MS/MS method to quantitatively monitor the plasma level of monosialogangliosides in patients with GM3 Synthase Deficiency (GSD), an inherited neurological disorder characterized by seizure and profound developmental stagnation, for clinical diagnosis and therapeutic evaluation during 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 attached to a ceramide portion with variable length on the fatty acid chains. Physiologically, they are believed to play critical roles in the regulation of various receptor-mediated

cell signaling pathways and cellular events. Disruption in their metabolic pathways pathologically leads to the pathogenesis of numerous neurodegenerative disorders, such as Parkinson disease, Alzheimer disease, and ganglioside GM3 synthase deficiency (GSD). Therefore, 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 three monosialoganglioside species, GM1, GM2, and GM3, in human plasma has been developed and validated. This assay employed DMTMM & PAEA chemical derivatization for signal enhancement and D3-labeled monosialogangliosides as internal standards (IS). The analytes and ISs were extracted from plasma using protein precipitation procedure, cleaned up with a mixture of water/methanol/chloroform, dried under nitrogen purging, and derivatized with DMTMM & PAEA. Thereafter, the samples were injected into a Shimadzu Nexera UHPLC system interfaced to an AB Scix Qtrap 5500 mass spectrometer that operating in ESI positive and Multiple Reaction Monitoring (MRM) mode to achieve highly sensitive and specific detection.

Validation

Considering 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 chemical derivatization with DMTMM & PAEA to increase the abundance of their doubly charged molecular ions in positive ESI. The sensitivity of monosialoganglioside species in positive ESI was observed to be enhanced for 15-20 times 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 measurements of monosialogangliosides GM1, GM2, and GM3, respectively. Thereafter, we validated this quantitative assay based on the FDA guideline for extraction recovery, precision, accuracy, stability, and matrix effect. The extraction recovery was found to be above 80% for each monosialoganglioside species using our sample preparation strategy. The relative percent error and coefficient of variation from measurements were below 11 and 11% for each monosialoganglioside species. The loss from derivatized analytes during storage was revealed to be insignificant (<10%) under studied conditions. Matrix effect from plasma was observed to be minor (<20%) on analytes during the analysis.

Conclusion

In summary, we developed and validated a novel quantitative assay for determination of monosialoganglioside species in human plasma using LC/MS and chemical derivatization.

A-274

A Novel Quantitative LC-MS/MS Method for Salivary Cortisol

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BACKGROUND Cortisol is a steroid enzymatically derived from cholesterol mainly in the adrenal gland and is important in glucose metabolism and stress response. The cortisol concentrations undergo a cyclic diurnal variation with the lowest concentrations at midnight and the highest in the morning. However, in Cushing's syndrome the diurnal variation is lost and midnight concentrations are similar to morning concentrations. As such, midnight salivary cortisol is a good diagnostic biomarker of Cushing's syndrome. The preferred method for measuring salivary steroids is LC-MS/MS due to its high specificity and sensitivity. The objective of this work was to establish a quick and sensitive method for measuring salivary cortisol. **METHOD** Saliva (200 µL) collected using a Salivette device (Sarstedt, Newton, NC) was mixed with internal standard (50 µL; d_4-cortisol 5 ng/mL in 1:1 methanol:water) and vortex mixed, followed by addition of 1 mL of methyl-*tert* butyl ether. The mixture stood at room temperature for an hour then was vortex mixed for 30 sec, and centrifuged at 2,000 g for 3 minutes. The organic layer (900 µL) was transferred into a glass tube and dried under a flow of nitrogen at 35ºC. The residue was reconstituted with 1:1 methanol:water (200 µL) and injected (25 µL) onto an Accucore C18 analytical column (3.0 x 50 mm, 2.6 micron; Thermo Fisher Scientific, Waltham, MA) in a Prelude LC system coupled with a TSQ Quantiva mass spectrometer (Thermo Fisher Scientific). The mass spectrometer with heated electrospray ionization was set in the positive ionization and multiple reaction monitoring mode. The transitions were 363.3->121.2 and 363.3->267.1 for cortisol and 367.4->121.1 and 367.4->273.1 for d_4-cortisol. Quantification was based on peak area ratio of cortisol to the internal standard. **RESULTS** The analytical cycle time was 4.25 minutes per injection. This method was free from matrix effect or interference. It was linear from 0.3 to 15.6 ng/mL with analytical recovery of 95-118%. No significant carryover was observed from samples with concentrations up to 47.5 ng/mL. The total CV was 14.8%, 7.3%, and 6.6% at 0.7 ng/mL, 10.7 ng/mL, and 21.6 ng/mL respectively. Comparison (n=33) with another LC-MS/MS method offered by an independent clinical laboratory gave a Deming regression correlation coefficient (R) of 0.925, slope of 1.04, an intercept of -0.002, and a standard error of estimate of 0.86. The mean bias between these two methods was 3.6%. **CONCLUSIONS** This fast LC-MS/MS method is sensitive and accurate making it suitable for measuring salivary cortisol in a clinical lab.

A-275

Workflow Study of a New HPLC, a New Tandem MS and a New Data Processing Software for General Clinical Use

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Background: There is a growing presence of LC-tandem MS in clinical laboratories because this analytical technique can provide definitive identification and quantitation of target compounds using chromatographic separations and specific transitions of tandem MS from precursor ions to product ions. Users can use LC-tandem MS to develop their own tests, use a smaller amount of samples, and measure several analytes in a single run. In addition to choosing proper MS ionizations and LC chromatographic separations for analysis of target compounds (analytical phase), clinical laboratories often encounter challenges with sample clean-up (pre-analytical phase) and data processing (post-analytical phase) when facing the demand to provide test results in a timely manner with increasing numbers of incoming samples. This study is to focus on the workflows of the pre-analytical phase (sample clean-up and delivery to MS detection), analytical phase (LC separation and MS analysis) and post-analytical phase (data processing) of three Class I medical devices for general clinical use.

Methods: The HPLC instrument consists of two separate channels, both of which include an online sample cleanup Turboflow column for removing sample matrix, and an analytical column for chromatographic separation. This HPLC is capable of cross-channel sequencing by only introducing the portion of the chromatogram of target compounds into the mass spectrometer therefore doubling the sample throughput. This HPLC can operate in two different workflows: (1) TX mode for sample matrices that require on-line sample cleanup and (2) LX mode for less complex sample matrices that can use dilute-and-shoot technique bypassing Turboflow Column. The Tandem MS has a selected reaction monitoring scan. The workflows of Tandem MS include HESI and APCI ionization modes with polarity switching. The data processing software provides three levels of user permissions for technicians, supervisors, and lab directors. This software has built-in flexibility in assigning roles and responsibilities, and audit trail function is provided for streamlining record keeping.

Results and Conclusion: The ability to run tests overnight, unattended was conducted using the example compound Alprazolam in synthetic serum using TX workflow with HESI positive ion mode. A total of 2000 crashed synthetic serum samples spiked with Alprazolam and isotopically-labeled internal standard were analyzed continuously for 100 hours, with an additional 44 QC samples inserted at intervals during the same 100 hours. Cross-channel RSD's (n=2000) of retention time, concentration and ion ratio (m/z 274 to m/z 281) of Alprazolam were observed at <2%. The precision studies of between-instruments (n=120, 5 replicates, 4 runs, 2 channels, 3 units) of different ionization modes on HESI probe and APCI probe were conducted using four example compounds in polarity switching mode (+/-) in either HPLC TX workflow, or HPLC LX workflow . HESI (LX workflow with synthetic urine sample matrix): Reserpine(+); Chloramphenicol (-); APCI (TX workflow with synthetic serum sample matrix): Testosterone (+); Estradiol (-). The corresponding between-instruments RSD's of concentrations were < 4% for Reserpine and Testosterone, and <10% for Chloramphenicol and Estradiol. The software streamlined the post-analytical phase of processing data and helped ensure data integrity.

For in vitro diagnostic use.

A-276

Development and application of a rapid and simple multiplexed UPLC-MS/MS method to measure triazole antifungals and metabolites

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Background: Triazole antifungals have become central to the treatment of invasive fungal infections, most notably in immunosuppressed populations such as those receiving bone marrow transplants. However, interactions with other drugs as well as environmental, genetic and disease-specific factors have led to wide variability in circulating drug levels leading to both decreased efficacy, or conversely, increased toxicity. As a result, there is growing clinical demand for therapeutic drug monitoring of these antifungals along with their metabolites. Here, we present a simple and rapid stable isotope dilution liquid chromatography tandem mass spectrometry method to measure fluconazole, voriconazole, voriconazole-N-oxide, posaconazole, itraconazole, and hydroxyitraconazole in serum.

Methods: Briefly, analytes were extracted from 10 µL of serum by simple protein precipitation with acetonitrile in the presence of deuterated stable isotope internal standards. Chromatographic separation was achieved using a two solvent gradient elution UPLC method with a reversed phase C18 column. Mass spectrometric analysis was performed using electrospray ionization (ESI) and collision-induced dissociation tandem mass spectrometry.

Results: Total run time was 3 minutes, with each analyte showing a high degree of linearity ($r^2 > 0.99$) from 0.01 µg/mL to 10 µg/mL (0.1 µg/mL to 100 µg/mL for fluconazole), accuracy and precision from sub- to supra-therapeutic levels (DEV and CV < 15%), as well as a high degree of correlation between our measurements and those obtained from a national reference laboratory. The validated method was then applied to clinical serum samples and showed a substantial variability in the relative serum concentrations of voriconazole to voriconazole-N-oxide and between itraconazole and hydroxyitraconazole, suggesting significant inter-patient variability in drug metabolism. Additionally, the method was also developed and applied to clinical cerebrospinal fluid specimens.

Conclusion: A simple and rapid LC-MS method to monitor multiple triazole antifungals and metabolites from serum and cerebrospinal fluid will provide a common platform for therapeutic drug monitoring. This will allow for a better understanding of antifungal metabolism and distribution, and subsequently better clinical management for a growing and vulnerable population of immunosuppressed patients.

A-277

Serum methylmalonic acid quantitation by LC-MS/MS for monitoring methylmalonic acidemia

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Background: Methylmalonic acidemia is an inherited metabolic disorder that is monitored and managed with serum methylmalonic acid (MMA) concentration and acylcarnitine and urine organic acid profile analysis. Due to the heterogeneous etiology for methylmalonic acidemia, an analytical assay for serum MMA levels requires a large clinical reportable range (0.1 - 1000 µM). Current literature describes multiple mass spectrometry method using MMA quantification to monitor Vitamin B12 deficiency, with a smaller emphasis on the challenges of quantifying MMA for methylmalonic acidemia. Here, we aim to develop a simple liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify serum MMA across a wide dynamic range using small sample volumes for monitoring pediatric patients with MMA.

Methods: Extraction procedure was optimized to reduce extraction time and sample volume. 50 uL of patient serum was mixed with d3-MMA and 4.6 M formic acid. After 5 minutes pre-treatment, sample was loaded onto Biotage 96-well ISOLUTE SLE+ 200 supported liquid extraction plate. MMA and d3-MMA were then eluted using methyl tert-butyl ether (MTBE). Eluted MTBE was evaporated under nitrogen at 50 ºC, reconstituted in 100 µL of water, and then 10 µL was injected into the Waters Alliance 2795 HPLC coupled with triple quadruple tandem mass spectrometer (Waters QuattroMicro). Separation of MMA from endogenous isobaric succinic acid was achieved on Phenomenex Gemini C18: 100 x 3.00 mm x 3 µm by gradient method at 0.6 mL/min started with 85% mobile phase A (water) and 15% mobile phase B (methanol with 0.1% formic acid and 2 mM ammonium acetate) and ended with 95% mobile phase B. Electrospray ionization was set at negative mode and multiple reaction monitoring was used to monitor MMA (117 to 73 and 117 to 55) and d3-MMA (120.1 to 76 and 120.1 to 58). The sample-to-sample run time was 7 minutes. Controls and calibrators were made in-house by spiking Seracon II stripped serum with MMA.

Results: To accommodate serum MMA measurement across several orders of magnitude from pediatric patients, assay development was focused on carryover, linearity, and sample volume. Carryover was minimized through needle wash studies, and no significant carryover was observed up to 500 µM after maximizing the number of needle wash cycles attainable for a 7 minute run using 40% methanol. The assay was shown to be linear between 0.1 - 500 µM (R^2 = 0.999). Extracted patient samples diluted 8 fold with water had acceptable agreement with straight samples (N=18, 0.15 - 500 µM), suggesting that sample volume can be reduced to 20 µL. Intra-assay imprecision was determined to be 1.9 % coefficient of variation (N = 3, 1.3 µM). LC-MS/MS method comparison with a reference lab yielded a Deming regression slope of 1.088, intercept of -1.388, and R^2 of 0.999 (N = 18, range 0.1-500 µM).

Conclusion: With fast turnaround time, small sample volume, excellent chromatographic separation, improved selectivity through ion ratio monitoring, and a wide dynamics range, we have shown a promising new method for measuring serum MMA levels by LC-MS/MS to monitor and manage pediatric populations with methylmalonic acidemia.

A-278

Method development for the determination of 17α-hydroxyprogesterone in human serum by isotope dilution liquid chromatography tandem mass spectrometry

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Background: Hormones are an important class of clinical diagnostic markers. Accurate measurement of hormone levels is necessary for proper diagnosis and treatment of many diseases. 17α-Hydroxyprogesterone (17OHP) is a metabolic precursor of cortisol; elevated levels of 17OHP are indicative of congenital adrenal hyperplasia. Quantification of 17OHP in serum has traditionally employed immunoassay methods, which suffer from poor antibody specificity. These methods are subject to interferences by other similar steroids, which result in falsely elevated levels. Normal level of 17OHP : Female: 0.1-12 ng/mL; Male: 0.3 to 2.5 ng/mL. We developed ID LC/MS/MS as another method for the determination of 17OHP in serum

Methods: Human serum samples were obtained from male and female human serum. 17OHP-d_8 was used as an internal standard. 0.5 mL of serum sample was taken into an amber vial. An appropriate amount of isotope standard solution was spiked into the sample vial to make a 1:1 weight ratio. After equilibration for 1 hour, 5 mL of CH_2Cl_2 was added to sample. Sample was shaken for 5 min and centrifuged at 3,000 rpm for 5 min. CH_2Cl_2 phase was separated and dried under nitrogen stream. Then sample was dissolved with 100 µL of 95% ethanol. Aliquot of the ethanol solution was filtered and then analyzed by LC/MS/MS. The LC column was Biphenyl and kept at 40°C during the chromatographic run. The mobile phase was gradient of A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water), and the flow rate was 0.25 mL/min

Results: Two levels of 17OHP in male and female serum were determined. The optimum solvent for the extraction of 17OHP in serum was CH_2Cl_2 among many other solvents. The optimum LC conditions were gradient of A (0.1% formic acid in acetonitrile) and B (0.1% formic acid in water); 0 (30% A)-21 min (50% A)-31 min (70% A)-32 min (100% A)-37 min (100% A)-38 min (30 % A)-43 min (30% A). 17OHP and 17OHP-d_8 were monitored at mass transfer m/z 331.3/97.3 and 339.3/100.3 respectively. The level of 17OHP in male serum showed nearly 0.7 ng/mL and that of female showed about 1.8 ng/mL. These results showed some difference compared to commercial immunoassay methods.

Conclusion:

17OHP is an indicative of congenital adrenal hyperplasia and very trace level in human serum. An optimized ID LC/MS/MS method was proposed as another method for the determination of 17OHP in serum. Extraction of 17OHP from serum matrix and HPLC separation from interferences were successfully established by this method. This method can be proposed as an accurate method for the development of certified reference material.

A-279

Determination of Urine Caffeine and Caffeine Metabolites by use of Polarity-Switching LC-MS/MS

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Background: Caffeine is a psychoactive stimulant commonly found in beverages such as coffee, tea, soft drinks, and energy drinks. It is a dietary compound of considerable health interest and has been studied as a risk factor for many diseases and conditions. We used an LC-MS/MS method for measuring urine caffeine and its metabolites in a representative sample of the US population (National Health and Nutrition Examination Survey NHANES] 2009-2010) in part to explore the potential use of these biomarkers in assessing dietary caffeine exposure. While LC-MS/MS was well suited for this application, it had one intrinsic limitation. Caffeine and its xanthine metabolites were best detected with positive mode ionization, whereas uric acids typically performed best with negative mode ionization. In the absence of the ability to perform analyses in both ionization modes in the same injection, we had to analyze each sample twice to obtain optimal results. Employing a new generation tandem mass spectrometer we explored the possibility to use polarity switching to measure these compounds in the same chromatographic injection.

Methods: Urine samples were diluted, amended with stable isotope labeled internal standards, alkalinized to convert specific metabolites to stable forms, and acidified and filtered prior to analysis. A UHPLC system with a solid core C_{18} column and a binary water/methanol gradient (0.05% formic acid) was used to chromatographically separate the analytes. Detection was performed on a tandem quadrupole MS/MS. Quantitation, confirmation, and internal standard MS/MS transitions were identified and optimized for all analytes. Polarity switching was performed at 20 ms. Analytes were quantified by interpolation against an 11-point calibration curve covering the concentration range expected in a population setting (0.005-600 µmol/L depending on the analyte).

Results: The performance of our polarity switching LC-MS/MS method was equivalent to or exceeded that of its predecessor. Limits of detection were 0.003-0.1 µM depending on the analyte. Between run imprecision (CV) determined over 30 days at 3 QC pool concentrations was 5-10% for 14 of 15 analytes. Spike recoveries were typically ±10% of being quantitative (100%). A comparison of our new and existing methods (180-sample convenience set) showed that results were highly correlated (Pearson $r \geq 0.9985$, $P < 0.0001$) and bias was low (Bland-Altman bias -2.3-3.6%; weighted Deming regression slope 0.95-1.04). The ability to measure all compounds in a single run resulted in a 45% reduction in analysis time, reduced solvent consumption, increased column lifetime, and simplified data review, thus improving overall sample throughput while reducing analysis costs.

Conclusion: Polarity switching permitted the analysis of all analytes in a single chromatographic injection while simultaneously using both ionization modes. This is an improvement upon existing LC-MS/MS methods in which separate chromatographic runs for each ionization mode were necessary because polarity switching was either not possible or could not be performed sufficiently fast because of instrumental limitations. Our new method is being used for the NHANES as well as other biomonitoring studies.

A-280

Measurement of 24-h urine free cortisol using ID-LC/MS

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Background:

The urinary free cortisol (UFC) has generally been determined by immunoassay. However, immunoassay is known to be nonspecific with cross reaction and inaccuracy. Therefore we developed candidate reference method for urinary free cortisol using isotope dilution-liquid chromatography mass spectrometry (ID-LC/MS) and evaluated the method.

Methods:

After solid-phase extraction, the cortisol and internal standard (cortisol d4) detected in the multiple-reaction monitoring mode using an positive electrospray ionization; cortisol m/z 363->121, cortisol d4 m/z 367->121. Intra-assay imprecision and inter-assay imprecision were evaluated with three different concentrations of QC samples. Matrix effect, stability, lower limit of detection and lower limit of quantification were also evaluated. In addition, we compared UFC values of forty-five 24-h urine specimens measured by our ID-LC/MS with those measured by ARUP lab using ID-LC/MS and those measured by DxI chemiluminescent immunoassay in our laboratory. Cross-reactivity of prednisolone was also evaluated.

Results:

Intra-assay CVs (n = 8) were 1.49-3.88% and inter-assay CVs (n = 20) were 3.15-5.90%. The limit of detection and limit of quantification of UFC were 0.5 ng/mL and 1.0 ng/mL, respectively. Significant matrix effect was not observed. The immunoassay showed large positive proportional and constant bias compared with our ID-LC/MS (y = 4.5455x + 11.898, r = 0.665). In addition, there was positive proportional bias of UFC by our method comparing with those by ID-LC/MS of ARUP lab but showed good correlation (y = 1.37x + 0.19, r = 0.999). Cross-reactivity of prednisolone with cortisol by our ID-LC/MS method was 0.9%, which was significantly lower than 19.3% by chemiluminescent immunoassay (Table 1).

Conclusion:

UFC determination by ID-LC/MS showed good precision, sensitivity, and low cross-reactivity with prednisolone, indicating use of ID-LC/MS is necessary for patients taking synthetic steroid hormones. However, there was also some bias between different LC/MS methods, which should be standardized by use of common standard material for calibration.

A-281

Towards a "Random Access" Model for Clinical LC-MS/MS

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Background: Advances in analytical performance and high-throughput capability have vastly improved the scope of clinical LC-MS/MS. One application where LC-MS/MS continues to lag behind many alternative platforms is that of "random-access" functionality. This largely limits LC-MS/MS to batch analyses. Here, we describe a working LC-MS/MS model incorporating routine batch methods for vitamin D and immunosuppressant drugs (ISDs) operating in parallel with a third method, busulfan, requiring a "random-access" type approach.

Methods: LC-MS/MS was performed using a TLX-2 online sample preparation liquid chromatography (SPLC) system (Thermo-Scientific, Franklin, MA) coupled to an ABSciex 5500 Q-Trap mass spectrometer (ABSciex, Concord, Ontario, Canada). Batch methods for vitamin D

and ISDs were performed on separate SPLC multiplexing channels, 1 and 2 respectively. Each channel operates with a separate sample clean-up (TurboFlow) column and analytical LC column combination in series multiplexed into a single MS/MS. Channel 1 uses a solvent system composed of 0.1% formic acid in water and methanol. Channel 2 uses 15 mM ammonium acetate/0.1% formic acid in water and methanol. The third method within this model is for the anti-neoplastic drug busulfan. Therapeutic drug monitoring for busulfan requires pre-dose blood draw (trough level) and re-draw at 6 hour intervals thereafter over a period of

up to 96 h. Busulfan analysis cannot be carried out in batch mode; it is

imperative that analysis is performed on sample receipt as plasma levels are required to guide acute changes in dosing. Plasma samples for busulfan were cleaned up by methanolic protein crash with isotopic dilution (busulfan-d8) followed by online SPLC on either channel.

Results: MS/MS for busulfan on channel 1 was monitored using the molecular ion transition *m/z* 247.3>151.1. MS/MS for busulfan on

channel 2 was monitored using the ammonium adduct precursor ion transition (*m/z* 264.2>151.1). Total analysis time on either channel was less than 15 min per sample. Comparison between the two busulfan channels demonstrated that they are interchangeable. Deming

regression (n = 41) for channel 1 versus channel 2 was Channel 1 = 1.04 * Channel 2 - 0.33; r = 0.998. Deming regression between our bi-channel busulfan data (LC-MS/MS) and reference method (GC-MS) data was GC-MS = 1.03 * LC-MS/MS - 0.24; r = 0.998; n = 36. Cross-center (n = 22) verification of our method demonstrated a mean bias of 0.0% (bias range: -10.2% to 12.4%) against the all-method mean across the concentration range 0.88 - 12.14 µmol/L (n = 6).

Conclusion: This model successfully combines batch and "random-access" type methods on a single multiplexing LC-MS/MS platform. Increased speed and throughput is achieved by running busulfan on both channels interchangeably. It also offers robustness allowing continued analysis in the event of a hardware problem on one SPLC channel. This approach offers simplicity, with fixed solvents on each channel and no column switching required. Since all three methods operate on APCI positive mode, a single probe is used throughout. We have demonstrated a working LC-MS/MS model for successfully combining batch and "random-access" type methods for use in the routine clinical laboratory.

A-282

Analysis of Bile Acid Profiles by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Objective: Primary bile acids are synthesized in the liver and conjugated to either glycine or taurine prior to excretion in the bile, where they serve many important physiological functions such as cholesterol homeostasis and lipid digestion. Within the intestine, bacterial enzymes metabolize conjugated, primary bile acids into numerous forms. In this work we focus on bile acid profiling by LC-MS/MS, utilizing multiple reaction monitoring (MRM) mode, and in patients with necrotizing enterocolitis (NEC).

Method: Bile acid extraction from serum or plasma was performed by methanol precipitation, 20 minute room temperature incubation and centrifugation. After centrifugation, the supernatant was dried under nitrogen. The residue was dissolved in methanol and 10 µl were injected for LC-MS/MS analysis. Bile acid extraction from stool samples was achieved by homogenization, lyophilization, and addition of 0.1M NaOH. Samples were then incubated for one hour at 60°C. Water was added and samples were homogenized. Following centrifugation, bile acids were isolated using solid phase extraction (C18 cartridges, Waters). Resultant sample was dried under nitrogen, dissolved in methanol, and injected (10 µl) for LC-MS/MS analysis.

LC-MS/MS analysis was performed on a Shimadzu HPLC system connected to a 4000 Qtrap (ABSCIEX) mass spectrometer in MRM and negative ion mode. Separation was performed on a Kinetex C18 column (150×4.60 mm, 2.6 µm ID, Phenomenex). The mobile phase A consisted of 5 mM ammonium acetate in water and mobile phase B consisted of 5 mM ammonium acetate in methanol. Bile acids were eluted over a 12 minute gradient. The gradient started at 80% B, increased to 93% at 7.5 minutes, then 98% at 7.6 minutes. After a 2.4 minute hold, the gradient was decreased to 80% for the final 2.2 minutes.

Results: The linearity was up to 12.5 µM for 14 metabolites, and 25 µM for 5 additional metabolites with correlation coefficient from 0.9933-0.9996. The limit of quantitation was 0.05 µM for all 19 bile acids. The recoveries were 99.4-105% for Tauro Ursodeoxycholic acid (TUDCA), 95.3-110% for Taurocholic acid (TCA), 99.7-105% for Hyodeoxycholic acid (HDCA), 93.4-105% for Taurochenodeoxycholic acid (TCDCA), and 96-105% for Lithocholic acid (LCA). The intra-assay precision CV was 2.8-14.4% for 0.2 µM and 4.1-13.4% for 7 µM. The total precision was evaluated during a 22 week period (one run/week). The CV was 9.7% -19.7% at a concentration of 7.0 µM, 11% -21% at 14 µM. This method was compared with a commercial LC-MS/MS method for three major bile acids. The correlation coefficients were 0.9984 for Cholic acid (CA), 0.9994 for Chenodeoxycholic acid (CDCA), and 0.9989 for Deoxycholic acid (DCA), respectively. Differences in the bile acids profiles were observed in patient's samples collected after surgery compared to samples collected after disease treatment.

Conclusion: We have implemented an LC-MS/MS method for the separation and detection of nineteen bile acids in plasma/serum and stool samples within a twelve minute analysis time. Absolute quantification of bile aids in both normal pediatric population and patient samples is currently underway to determine normal pediatric reference intervals and changes in the bile acid levels associated with NEC.

A-283

A combined method for Liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurement of aldosterone/plasma renin activity ratio

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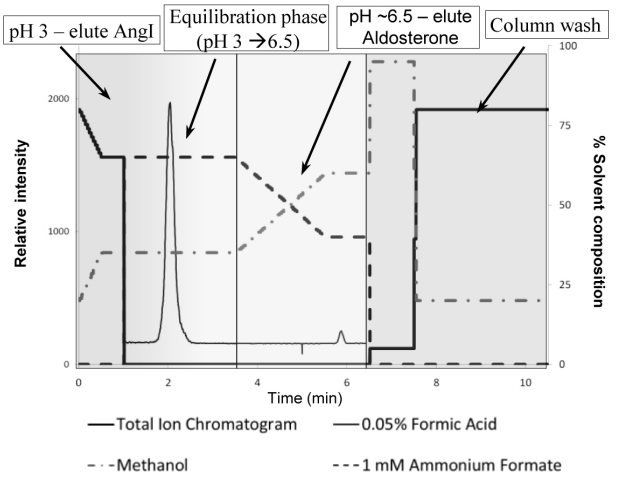
Background: The ratio of aldosterone to plasma renin activity (PRA) is used to help diagnose and evaluate hyperaldosteronism. Aldosterone/PRA methods by LC-MS/MS provide benefits in sensitivity and specificity; however, differences in the chemical behavior and ionization efficiency between the renin product angiotensin I (angI), and aldosterone have until now prevented simultaneous determination of these analytes. This study therefore presents a novel combined LC-MS/MS method for determination of the aldosterone/PRA ratio.

Methods: AngI and aldosterone were quantified from EDTA plasma or stripped serum spiked with isotopically-coded internal standards (IS) and subjected to solid-phase extraction with Waters HLB extraction cartridges. AngI was generated for 1 hour at 37°C in 27.5 mmol/L maleic acid buffer with 0.5 mmol/L AEBSF and chromatographic separation of angI and aldosterone employed an Agilent 1260 quaternary pump with a Phenomenex EVO C18 column (150 mm × 3.0 mm, 5 µm) at 800 µL/min. Analysis was on an Agilent 6460 triple quadrupole mass spectrometer operating in dynamic multiple reaction monitoring mode. The solvent gradient employed 0.05% formic acid, methanol, and 1 mM ammonium formate. Figure 1 displays an example chromatogram with the gradient conditions, and total run time was 10.5 minutes.

Results: Modulation of the solvent gradient as well as pH allowed detection of angI and aldosterone by LC-MS. AngI is detected in positive mode by b5 and y8 ions, while aldosterone was detected in negative mode using transitions 359.4 ◊ 331.4/189.1. AngI is eluted at approximately 2 minutes, while aldosterone is eluted at 5.8 minutes. Detection limits are comparable to immunoassays for each analyte (1 - 50 ng/mL angI, 50 - 2000 pmol/L aldosterone).

Conclusion: By employing a quaternary pump and a three solvent system, we demonstrate a novel LC-MS/MS method for combined analysis of aldosterone and PRA, and for improved workflow in the clinical laboratory.



A-284

A Simple and Robust Nano Liquid Chromatography-Mass Spectrometry Method for the Analysis of Hemoglobin Variants

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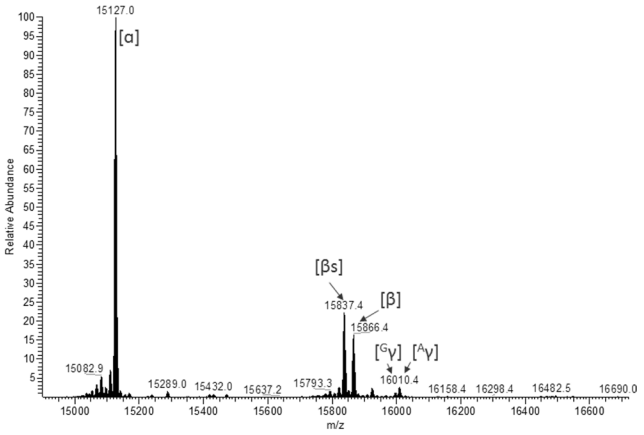
Background: Hemoglobinopathies are a group of disorders affecting red blood cells with abnormal hemoglobin and are the most common inherited disorders. Gel electrophoresis and chromatography assays are the most common techniques used for hemoglobin analysis. In some instances the results from these methods can be ambiguous due to the co-migration of bands in gel and co-elution of different variants on chromatography. The objective of this work was to develop a simple and robust nano liquid chromatography-mass spectrometry (nLC-MS) method for the analysis of intact hemoglobin chains to identify abnormal hemoglobin variants. **Method:** Whole blood (10 µl) and 50 µL of extraction buffer (1:1 mix of 0.2% formic acid in deionized water and 0.2% formic acid in acetonitrile) were vortex mixed and centrifuged. Supernatant (25 µL) was mixed with 75 µL of 0.2% formic acid in deionized water and extracted using a ZipTip (0.2 µL of C18 resin). The ZipTip procedure is as follows: wash with 0.2% formic acid in acetonitrile, condition with 1:1 (0.2% formic acid in deionized water:0.2% formic acid in acetonitrile), then 0.2% formic acid in deionized water. Load sample, wash with 0.2% formic acid in deionized water. Elute with 0.2% formic acid in acetonitrile. The extracted hemoglobin solution was analyzed on an nLC-MS coupled with a Q-Exactive high resolution MS. The raw spectra were deconvoluted in Xcalibur to identify hemoglobin variants (Figure 1). **Results:** This nLC-MS method was successful in identifying abnormal hemoglobin variants in patient samples (n=29) previously identified using alternate methods. **Conclusion:** This simple and robust nLC-MS method for the identification of hemoglobin variants provides accurate results.



A-285

Sensitive Measurement of Free T3 and Free T4 in Serum by LC-MS/MS, using a Simple Ultrafiltration Sample Preparation Procedure

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Background:

The measurement of free thyroxine (FT4) and free 3,3′,5-triiodothyronine (FT3) in serum are important because these values can be used to help researchers assess various states of thyroid function in men, women, and children. Currently most measurements of FT3 and FT4 are performed by equilibrium dialysis immunoassay methods, which may suffer from a lack of specificity. Measurement by LC/MS/MS has the potential to increase the accuracy of these measurements; however sensitivity has posed a challenge due to the very low levels of circulating FT3 and FT4 in serum.

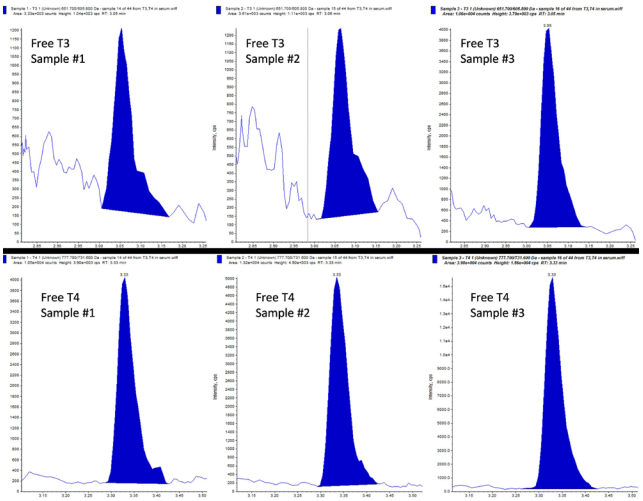
Methods:

Here we present a sensitive method using the AB SCIEX QTRAP® 6500 system, employing a simple and rapid ultrafiltration sample preparation to isolate the free T3 and free T4 prior to analysis by LC/MS/MS. Liquid chromatography separation was accomplished using a Phenomenex Kinetex C18 column (2.6um, 3.0 x 50mm) at a flow rate of 500uL/minute, and mass spectrometric detection was performed in positive MRM mode, using electrospray ionization.

Results:

Unlike earlier attempts to analyze FT3 and FT4 by LC/MS/MS, this sensitive method requires a relatively small injection volume of only 50uL of the final sample, derived from an original sample volume of 400uL serum. We have performed a comparison study using a cohort of samples that have been previously analyzed by immunoassay, and an excellent correlation has been observed. The method LOQ was <0.5 pg/mL for both FT3 and FT4. Replicate injections of the calibration standards demonstrated excellent linearity, accuracy and precision over the concentration range from 0.5 pg/mL to 100 pg/mL of FT3 and FT4. As this method requires an injection volume of only 50uL of the prepared sample, we believe this represents the most sensitive method reported in the scientific literature.

Representative chromatograms are shown in Figure 1, demonstrating both low and high concentrations of FT3 and FT4 in serum samples.



A-286

Development of a serum based exosomal biomarker signature to distinguish indolent and aggressive prostate cancer

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Background: Current tumor markers for prostate cancer lack specificity and are not able to accurately identify those with aggressive disease who require immediate treatment. Exosomes are cell membrane-derived vesicles found in numerous noninvasive bodily fluids such as serum, saliva, urine, and amniotic fluids. They have been demonstrated to carry specific proteins, lipids, mRNA, and microRNAs. Thus, exosomes can potentially harbor cancer biomarkers. Despite the vast information that exosomes can provide, the isolation of pure exosomes is still a challenge in the field. The current methods of isolating exosomes are time-consuming, laborious, and non-specific with most procedures isolating a heterogeneous population of microvesicles.

Objectives: The goal of this study was to develop a more targeted and reproducible method for exosome isolation. We used a combination of centrifugation, and affinity enrichment to devise a quick and robust method for this process. Using such procedure, we wanted to delineate a biomarker signature to distinguish prostate cancer patients with aggressive disease and those from benign controls.

Method: We used affinity reagents to exosomal membrane proteins (CD63/CD9/hsp70) coupled with ultracentrifugation to isolate exosomes from serum of prostate cancer patients with aggressive disease and those from benign disease controls. The isolated exosomes were subject to downstream proteomics analysis using quadrupole and ion-trap mass spectrometers. The identified peptides were further analyzed using gene ontology algorithms, allowing us to map the identified peptides onto cellular signal transduction processes.

Results: In total, over 800 proteins were identified from our serum samples. Of these, a protein signature of ~40 proteins was able to distinguish aggressive disease from benign controls. Gene ontology studies demonstrate that these proteins function in pathways related to growth & proliferation (mTOR), cytoskeleton (alpha-actinin), extracellular matrix (alpha sarcoglycan), and altered signaling (centrasomal protein of 152kD).

Conclusion: Using our optimized serum exosome isolation procedure coupled to downstream proteomics analysis, we were successful in delineating a protein biomarker signature to separate aggressive prostate cancer from benign disease. This signature, or its components, may be useful as an adjunct to prostate biopsy, in appropriate triage of patients with elevated PSA.

A-287

Quantitative Determination of D/L-Methamphetamine in Urine by Liquid Chromatography-Tandem Mass Spectrometry

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Background

The D-methamphetamine isomer possesses the well-known psychostimulant effects of the drug, while the L-methamphetamine has no effect on the central nervous system. Long-term use of D-methamphetamine is associated with depression and suicide as well as serious heart disease and violent behaviors. Consumption of over-the-counter and prescription medications such as Vick's inhaler and Desoxyn respectively may yield positive methamphetamine results. Chiral separation of D and L stereoisomers can help determine if the source was putatively licit or illicit. Cyclodextrins are commonly utilized as chiral selectors in capillary electrophoresis methods but these methods are not common methodologies implemented in forensic laboratories. In this study, a LC-MS/MS method for separation and quantification of methamphetamine stereoisomers derivatized with Nα-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide (Marfey's reagent) is presented. The validated method was applied to the identification of methamphetamine stereoisomers in human urine samples.

Methods

Urine samples were prepared by solid phase extraction using a Strata-X™ cartridge in a 96 deep-well SPE plate. Analytes were eluted from the cartridge with methanol followed by evaporation to dryness under stream of nitrogen at 45 ºC. Samples were reconstituted in a solution of sodium bicarbonate and the Marfey's reagent (Sigma-Aldrich) was added. The derivatization reaction was performed at 56 ºC for 1 h. After quenching the reaction with HCl and centrifuging the mixture, 5 µL of sample were injected into the LC-MS/MS system. Chromatographic separation was performed on a Kinetex C18, 100x2.1 mm, 5 um column under isocratic conditions with 45% water (A) and 55% methanol (B) for 5 min. Prior to the isocratic conditions a short gradient of 10% to 55% B over 1 min was run. Mass spectral data were obtained in positive electrospray mode using an Agilent 6400 Series Triple Quadrupole LC/MS Systems. Derivatized methamphetamine MRM transitions monitored were 402.1 → 284.0, 118.9 and methamphetamine MRM transitions monitored were 150.1 → 119.1, 91.1. Six levels of the calibration curve were prepared with the measurement range from 50-5000 ng/mL.

Results

The incubation time and Marfey's reagent concentration added for derivatization were optimized. Preliminary data showed a good separation of the derivatized methamphetamine stereoisomers in less than 5 min. The method has a limit of quantitation in all matrices of 50 ng/mL. Imprecision and accuracy for the 3 quality controls tested met all acceptable criteria. The average precision and accuracy values were 8% and 90% respectively. The average recovery of the derivatized methamphetamine versus non-derivatized methamphetamine was 92%. The validated method was applied to the identification and quantification of methamphetamine stereoisomers in human urine samples. Although the derivatization procedure in the present study may increase the sample preparation duration, the conventional reverse-phase separation on a C18 column was achieved in less than 5 min. Additionally, a higher sensitivity was achieved in all matrices tested compared to other chiral separation methods.

A-288

A Fast and Simple LC-MS/MS Quantitative Method for Benzodiazepines in Urine

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BACKGROUND Benzodiazepines (BZD) were introduced in the early 1960s and are an important class of psychotropic drugs that are used often in clinical practice as tranquilizers, sleep inducers, antiepileptics, hypnotics, anticonvulsants, anxiolytics, and muscle relaxants. Due to the tranquilizing effects of BZD, they are often used for suicide attempts, abused recreationally, and employed for drug facilitated sexual assault. Therefore, they are frequently encountered during emergency toxicological screenings, drug abuse testing, and forensic medical examinations. This method was developed for rapid and accurate measurement of BZD compounds in urine for pain management services. **METHODS** BZDs and/or metabolites (Lorazepam, Temazepam, 7-Amino-Clonazepam, Hydroxyalprazolam, Hydroxytriazolam, Nordiazepam, Oxazepam) were included in this method. Urine sample (50 µL) was mixed with an enzyme solution (50 µL; 10,000 U/mL *H. rufescens* β-glucuronidase in 1M sodium acetate, pH 4.5) and an internal standard mix (50 µL). The resulting mixture was vortex mixed for 15 seconds, followed by incubation at 60°C for 30 minutes. The sample was then centrifuged at 17000 g for 3 minutes. The supernatant was mixed with 250 µL methanol, vortex mixed for 15 seconds, and centrifuged for 10 minutes at 17000 g. The supernatant (250 µL) was mixed with 250 µL water and 25 µL of the mixture was injected onto an Accucore C18 column (3.0 x 50 mm, 2.6 micron; Thermo Fisher Scientific, Waltham, MA) in a TLX2 system coupled with a TSQ Ultra mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was set at positive heated electrospray ionization and multiple reaction monitoring mode. Quantification was based on

peak area ratio of the individual analyte to the corresponding internal standard. Two transitions were monitored for all the compounds while one for each internal standard. **RESULTS** The chromatography was 4.35 minutes per injection. This method was free from matrix effect or interference. It was linear from 60-9500 ng/mL with analytical recovery ranging from 80-120% for all compounds. No significant carryover was observed from samples with concentrations up to 10,000 ng/mL. The total coefficients of variation (CV) ranged from 2.9%-9.4% for the tested concentrations of 100 ng/mL, 2550 ng/mL, and 5000 ng/mL. **CONCLUSION** This LC-MS/MS method requires simple sample preparation and offers precise and accurate measurement of the BDZ. It was validated to test urine samples for pain management service.

A-289

Recently Postmenopausal Women Have Elevated Urinary 8-iso-Prostaglandin F2α (8-iso-PGF2α) Assayed by LC-MS/MS after Solid Phase Extraction

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Background: the adverse effects of menopause are reported to include alterations in lipid profile, body mass index, insulin levels and also increased risk of hypertension, cardiovascular diseases, osteoporosis, diabetes mellitus, cancer and other degenerative changes in postmenopausal females. It has been correlated with the increased production of free radicals after menopause. Oxidative stress is reported to increase after menopause, suggesting that the decrease in sex hormones occurring at the time of menopause could predispose women to higher levels of reactive oxygen species (ROS). Recently, F2-isoprostanes are shown to be reliably measured and used as the most reliable markers of oxidative stress status. Plasma or tissue 8-iso-PGF2α concentrations provide a "snap-shot" assessment of oxidative stress. The potential for interferences arising from serum or plasma during sample preparation makes the measurement of urinary concentrations that show no diurnal variations and less subject to interferences provide a better assessment of oxidative stress.

Objective: the objective of the study was to determine the association between the status of oxidative stress and menopause in healthy recently postmenopausal women using urinary 8-iso-PGF2α as a marker for identifying oxidative stress. Hypothesis of an important antioxidant role for estrogens would be reflected in strong inverse correlations with urinary 8-iso-PGF2α concentration.

Methods: urinary 8-isoprostaglandin F2α (8-iso-PGF2α) and estradiol were measured in 21 young women and 23 recently postmenopausal women. The subjects were healthy, were not smokers, and were not taking any medications or vitamins. Blood pressure, serum cholesterol, and glucose did not differ between the groups. Solid phase extraction columns (C18) were used for the extraction of 8-iso-PGF2α using a single solid phase extraction (SPE) cartridge. Chromatographic separation was achieved using C18 analytical column with 0.1% formic acid in water and a mixture of methanol: acetonitrile under isocratic conditions, and detection by negative mode selected reaction monitoring (SRM) mass spectrometry (MS), using an 8-iso-PGF2α standard to identify specific chromatographic peaks. The quadrupoles were set to detect the negatively charged molecular ions [M-H]− and a high mass fragment of 8-iso-PGF2α at m/z 353→193 amu.

Results: The limits of detection and quantitation for 8-iso-PGF2α were 12.3 and 31.6 pg/mL, respectively. The intra- and interassay imprecisions were 4.1% and 6.7%, respectively. 8-iso-PGF2α (238±41 [postmenopausal] versus 154±36 [non menopausal women] pg/mg creatinine, $P<0.05$) were higher in postmenopausal. Levels of 8-iso-PGF2α showed significant negative correlation with estradiol levels in postmenopausal women ($r = 0.57$, $P<0.01$). No such correlation was found in non menopausal women ($r= 0.29$, $P> 0.05$). Associations were adjusted for age, body mass index (BMI), physical activity.

Conclusion: This study supports an association between the status of oxidative stress and menopause in healthy recently postmenopausal and that endogenous E2 play a role to modify oxidative stress by decreasing urinary 8-iso-PGF2α concentrations as assayed by LC-MS/MS.

A-290

Development and Validation of Metanephrines and Catecholamines in urine by LC-MS/MS

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BACKGROUND: Urinary determination of biogenic amines excretion is used to diagnose phaeochromocytoma. Liquid chromatography-tandem mass spectrometry has high analytical specificity and can allows rapid testing for identification of these disorders.

OBJECTIVE: Development a simple, rapid and sensitive LC-MS/MS method to determination of biogenic amines metanephrines and catecholamines in urine using deuterated internal standard (IS).

RESULTS: 1000 µL of urine samples containing the internal standard were treated by liquid-liquid extraction using ethyl acetate. Chromatographic separation was performed on a BDS HYPERSIL C18 column (125 mm x 3 mm, 3 µm) and mobile phase water:methanol (98:2, v/v) with 0.25% of formic acid at 200 µL/min. Detection was performed on a triple quadrupole mass spectrometer with electrospray ionization (ESI+). The method has a chromatographic running time of approximately 8 min. The precision value was less than 7.0% for all analytes. The average of recovery was 92-108% for all analytes.

CONCLUSIONS: The LC-MS/MS method has been developed successfully for the quantitative analysis of these biogenic aminas and has applied for clinical analysis

A-291

A Rapid and Sensitive LC-MS/MS Method for the Analysis of Free Thyroid Hormones

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

Accurate and sensitive measurement of low levels of free hormones is necessary to assess thyroid function for both veterinary and human clinicians. Reverse tri-iodothyronine (rT3) is an inactive form resulting from T4 biotransformation. Since rT3 functions as the feedback inhibitor of thyroid hormone production, the measurement of rT3 can be an important diagnostic marker in clinical implication. The intent of this application was to develop an LC-MS/MS method for the analysis of thyroid hormones (T3, rT3, and T4) at the free form levels using the highly efficient and selective Raptor™ Biphenyl column. The clinical applicability of the method was demonstrated by analyzing the fortified thyroid hormone in phosphate buffer saline (PBS) containing 4% human albumin.

Human albumin dissolved in PBS solution was used to prepare calibration standards ranging from 2 to 400 pg/mL. Standard solutions were spiked with internal standard, T4-13C6, and mixed with acetonitrile in a glass vial. An aliquot of ethyl acetate was added, stirred, and then centrifuged. The organic phase was removed and placed into a glass vial, and evaporated to dryness under a stream of nitrogen. The dried extract was reconstituted in a 30:70 water:methanol solution and injected on to the Raptor™ Biphenyl column (100x2.1mm, 2.7µm) for the analysis on a Waters ACQUITY UPLC® I-Class System coupled with a Waters Xevo® TQ-S mass spectrometer using electrospray ionization in positive ion mode.

Good linearity (1/x weighted) was obtained for all three forms of thyroid hormones with coefficients of variation (r2) > 0.990 from 2 to 400 pg/mL (for T3) or 5 to 400 pg/mL (for T4 and rT3). The %deviation was < 15%. Simultaneous analysis of all 3 analytes was performed with a fast 3.5 minute total run time for each injection with complete separation of T3 and rT3 isobars. It was demonstrated that the Raptor™ Biphenyl column is excellent for rapid and sensitive analysis of thyroid hormones. With the established method, as low as 2 pg/mL (T3) or 5 pg/mL (T4 and rT3) of thyroid hormones can be accurately determined with less than 3.5 minutes of analysis time. The analytical method is thus applicable to the clinical analysis of free thyroid hormone at low pg/mL levels.

A-292

The Analysis of Vitamin D and Metabolites in Plasma by LC-MS/MS

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

Vitamin D deficiency has been linked to an increased risk for many chronic diseases including diabetes, heart disease, autoimmune diseases, and some cancers. Vitamin D exists in two forms, vitamin D2 and vitamin D3. Each undergoes metabolism to form 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3. For accurate determination of vitamin D levels in the blood, it is important to distinguish between these metabolites and to separate them from major matrix interferences. In this method, the Raptor™ ARC-18 column combines the speed of superficially porous particles (SPP) with the resolution of highly selective USLC® technology to produce a simple and rugged method for the determination of vitamin D metabolites in serum and plasma.

The suitability of ARC-18 column for the analysis of vitamin D metabolites was first demonstrated for the analysis of neat standard solution containing fat-soluble vitamin D and metabolites. The ARC-18 was then used to analyze the 25-hydroxyvitamin D metabolites of fortified and plasma (Rat). The quantitation was performed with calibrated standards prepared in 4% human albumin PBS solution. Three levels of fortified metabolite concentration were measured with acceptable accuracy and precision.

The metabolites were fortified into Rat plasma and extracted with a liquid-liquid extraction method. Plasma (150mL) was mixed with 0.2M ZnSO4 (150mL) in a 2-mL glass HPLC vial. Added 300mL of methanol containing 25ng/mL of d6-25(OH)D3 (internal standard) and vortex mixed (10secs). Added 750mL of hexane, mixed for 30secs, and then centrifuged for 10mins at 4300rpm. The hexane layer (~650mL) was removed and placed into the micro-vial and evaporated to dryness under nitrogen at 55°C. The dried extract was reconstituted with 75mL of 15:85 water:methanol solution and injected (5 mL) for analysis.

The calibration standards were prepared in 4% human albumin in PBS solution and subjected to the same extraction procedure for the fortified plasma samples. A good linearity was obtained for both 25(OH)D2 and 25(OH)D3 from 1 to 150ng/mL (with 1/x weighting). The r was ~0.999 and the %deviation was ≤10%. The quantitation results of 3 levels of fortified plasma samples showed method accuracy from the %recovery of within 8% of the nominal concentration for all QC levels. The %RSD was from 0.3-10.7 indicating good method precision.

Separating fat-soluble vitamins by LC can be time consuming. The Raptor™ ARC-18 column, however, can analyze these difficult compounds using reversed-phased chromatography (RPC) in less time than traditional columns to increase productivity. Plus, in the bioanalytical arena, the ARC-18 can quantitate the metabolites of vitamin D using the same column and mobile phases.

A-293

Comparison of Tacrolimus Quantification using the Waters MassTrak LC-MS/MS assay with the Abbott Architect Immunoassay

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* Imir Metushi and Alec Seitman have contributed equally to this abstract.

Background: Tacrolimus is a widely prescribed immunosuppressant for organ transplantation. However, due to its narrow therapeutic window it requires periodic therapeutic drug monitoring. Immunoassays which use monoclonal antibodies are widely used to monitor tacrolimus concentrations. Tacrolimus is metabolized by the CYP3A subfamily of enzymes giving rise to metabolites which are structurally similar to the parent drug that can cross react with the immunoassay. Methods: We verified performance claims of the Waters MassTrak assay and performed a patient comparison relative to the Abbott tacrolimus immunoassay on the Architect platform. We performed linearity studies by using pooled blood from patient samples measured in quadruplicates at 9 concentrations: 0.95, 4.9, 8.8, 12.7, 16.7, 20.6, 24.5, 28.5, and 32.4 ng/mL. Precision studies were performed by using three concentrations of pooled patient blood samples; low (0.5 ng/mL), medium (15 ng/mL) and high (30 ng/mL). A total of 40 patient blood specimens were run on both the MassTrak and the architect immunoassay over 20 days. In order to cover the dynamic range of the assay, we choose to divide the samples into four groups: 10 samples from each of the four ranges (0-6 ng/mL; > 6-13 ng/mL; > 13-21 ng/mL and > 21 ng/mL). Results: The Waters MassTrak assay was linear from 1 to 32.4 ng/mL with an R2 = 0.997. Precision studies of the MassTrack assay at three different concentrations over the measured analytical range were (mean ± CV): 0.6 ng/mL ± 20%, 16.0 ng/mL ± 5.4%, 31.2 ng/mL ± 5.8%. Matrix effect studies were conducted to determine whether blood could suppress ionization of tacrolimus; comparison of signal intensity between pretreated blood samples and solvent samples revealed a difference of less than 10% for both tacrolimus and the internal standard (ascomycin). Patient comparison data between the MasTrak LC-MS/MS and Abbott immunoassay showed excellent correlation with an R2 = 0.97. However, the MassTrak method produced concentrations for tacrolimus that were consistently lower than the immunoassay with a negative bias ranging from 0 to 37% ($p < 0.0001$; via two-tailed paired t-test test). Conclusion: For the first time we report a direct comparison of the Waters MassTrak assay with the Abbott immunoassay for quantifying tacrolimus. Overall, the MassTrak LC-MS/

MS assay was linear over the clinically relevant range of 1 to 32.4 ng/mL. Tacrolimus measurements in patient blood samples correlated well with the immunoassay (R2 = 0.97), however, the immunoassay values were consistently higher than those measured using the MassTrak method. We suspect that the higher measured concentrations in the immunoassay method are likely due to cross reactivity with tacrolimus metabolites by the antibody used in this assays.

A-295

Stability of 5-Hydroxyindole-3-acetic Acid and Vanillylmandelic Acid in Urine with Unadjusted pH at Various Laboratory Storage Conditions

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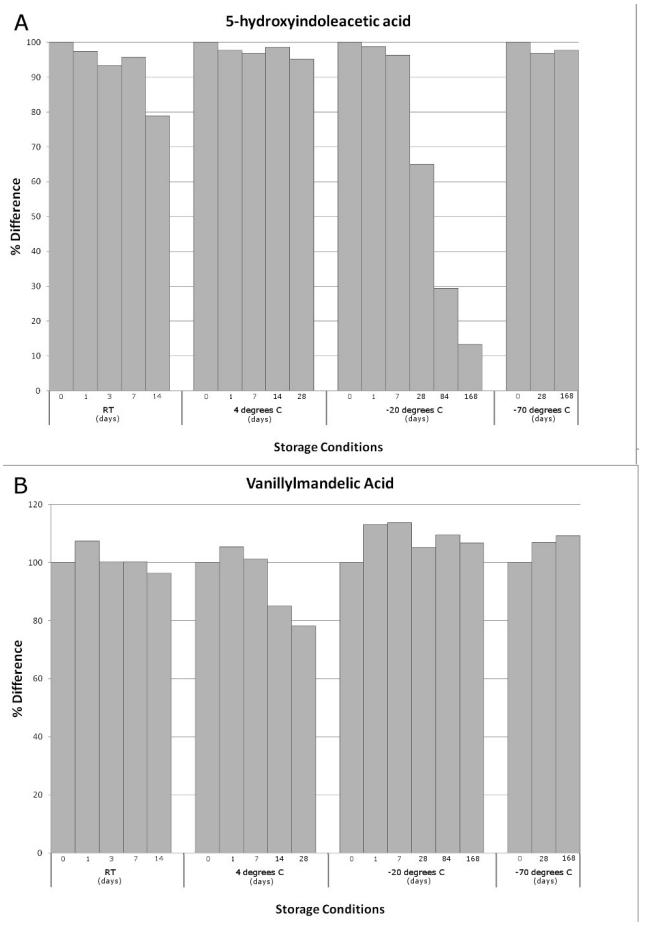
Background: Quantification of 5-hydroxyindole-3-acetic acid (5HIAA) and vanillylmandelic acid (VMA) in urine is used for the diagnosis and treatment follow-up of carcinoid and neuroblastic tumors, respectively. Several major reference laboratories provide acceptable storage conditions for 5HIAA and VMA in urine on their online directories. Many labs require acidification to improve stability. However, the stability of each analyte at various laboratory storage conditions is under represented in the literature. The objective of this study was to examine the short and long-term stability of 5HIAA and VMA in pH-unadjusted urine at room temperature (RT), 4°C, -20°C and -70°C.

Methods: Pooled patient urine was spiked with 5HIAA and VMA at 3.5 mg/L and 4.0 mg/L, respectively. The measured pH of the unadjusted urine pool was 6.2. Aliquots of the spiked urine were made for each storage condition and baseline samples were analyzed immediately. At defined time intervals for RT (1, 3, 7 and 14 days), 4°C (1, 7, 14, and 28 days), -20°C (1, 7, 28, 84 and 168 days) and -70°C (28 and 168 days) aliquots were removed and stored frozen at -70°C. By the end of this study all samples were thawed and analyzed in a single batch by an LC-MS/MS method.

Results: The stability of 5HIAA and VMA for RT was 7 and 14 days, respectively. While 5HIAA was stable for 28 days VMA was stable for only 14 days at 4°C. Interestingly, when samples were frozen at -20°C, the stability of 5HIAA regressed to 7 days, whereas VMA remained stable for 168 days. Both analytes were found to be stable for the entire study period (168 days) at -70°C.

Conclusion: 5HIAA and VMA in urine have adequate stability for routine clinical laboratories without adjusting the pH.



A-298

Ultra-Fast quantitative analysis of Immunosuppressants in Dried Blood Spots using Laser Diode Thermal Desorption coupled to tandem mass spectrometry

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Background:

Immunosuppressant drugs are used as a proliferation signal inhibitor in organ transplants. Dried blood spots (DBS) on paper become a desirable method of sample collection. This technique reduces the steps for sample collection and shipping. To optimize the dosing regimen, concentration results from a sample are rapidly needed. The ultra-fast Laser Diode Thermal Desorption (LDTD) technology combined to a tandem mass spectrometer system is used for rapid turnaround time of sample results.

The Laser Diode Thermal Desorption (LDTD) ion source uses an infrared laser diode to indirectly thermally desorb neutral species of Immunosuppressant molecules from a dried sample extract. These neutral species are carried into a corona discharge region, where they undergo efficient protonation and are introduced directly into the mass spectrometer. Total analysis time is under 9 seconds with no carry-over.

The objective of this experiment is to validate the DBS extraction conditions, the analysis method and test different real patient samples using the LDTD-MS/MS. A cross validation study against the LC-MS/MS approach for the analysis of Immunosuppressants (Cyclosporin A, Tacrolimus, Sirolimus and Everolimus) was performed in order to evaluate the performance of the proposed alternative LDTD-MS/MS method.

Methods:

Lyophilized calibrators for Cyclosporin A, Tacrolimus, Sirolimus and Everolimus as well as Quality Control material were obtained from Chromsystem and UTAK. 25 µL of calibrators, QC and patient specimens are spotted on a Whatman 903 card and dried at room temperature (protected from light) for at least 2 hours. Six DBS punches of 3 mm were transferred in a glass tube. 100 µL of water was added and the tube was transferred to an ultrasonic bath for 10 minutes. 100 µL of internal standard (Ascomycin (5 ng/mL), Cyclosporin A-d4 (250 ng/mL), Ramycin-d3 (3 ng/mL) and Everolimus-d4 (5 ng/mL) in a mixture of ZnSO4 (1M):Methanol (20:80)) was added. The mixture was vortex-mixed. A liquid-liquid extraction was then performed by adding 200 µL methyl-tert-butyl ether (MTBE). After vortexing and centrifugation, 45 µL of the organic layer was transferred in a tube and 5 µL of desorption solution was added and mixed. 4 µL was deposited in the LazWell Plate and evaporated to dryness. The LDTD laser power was ramped to 80% in 6 seconds, and shut down after 2 seconds. Positive ionization mode was used, and the AB Sciex 5500 QTrap system was operated in MRM mode.

Results:

The calibration curves show excellent linearity with r > 0.995 between the quantification ranges of the Chromsystem standard. Intra-run accuracy and precision between 86.4 % and 107.5 % and 0.7 to 18.3%, respectively, were calculated. All QC values meet acceptance criteria of 15%. No matrix effect or carryover was observed. This method was cross validated with results from a traditional LC-MS/MS method with real patient specimens. All negative samples correlated accordingly.

Conclusion:

DBS provides an easier way for sample transport management and is an ideal match to the unique advantages provided by the LDTD technology ultra-fast analysis of Immunosuppressant drugs. This method has demonstrated accurate, precise and stable results with a run time of 9 seconds per sample.

A-299

Reference Intervals for Midnight and Morning Cortisol in Saliva Measured by an LC-MS/MS Method

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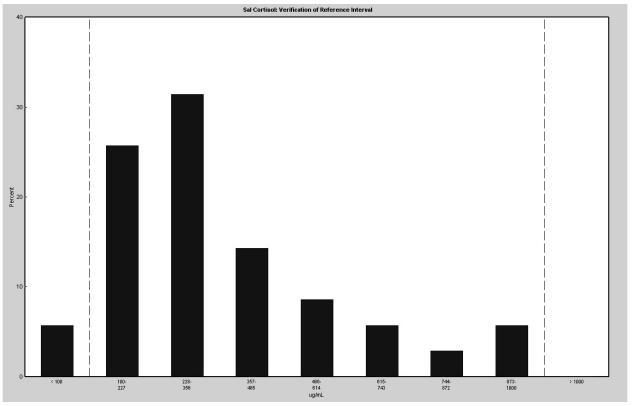
BACKGROUND Cortisol is a steroid enzymatically derived from cholesterol mainly in the adrenal gland. The cortisol concentrations undergo a cyclic diurnal variation with the lowest concentrations at midnight and the highest in the morning. Cushing's syndrome is the result of excessive secretion of glucocorticoids, primarily cortisol, and presents as a complex metabolic disorder. In Cushing's syndrome, the diurnal variation of cortisol is lost and midnight concentrations are similar to the morning concentrations. Therefore, measuring midnight levels of cortisol is important for the diagnosis of Cushing's syndrome. Salivary concentrations (typically <1000 µg/mL) correlate well with free serum concentrations (<1600 µg/mL) and do not require blood draws. There are different published reference intervals for both morning and midnight cortisol levels in saliva. In this work, we aimed to verify the reference intervals for morning and midnight salivary cortisol measured by an LC-MS/MS method. **METHODS** Collection of saliva samples was approved by the Institutional Review Board. In brief, saliva was collected in Salivette (n = 41) from healthy adults (22 females), aged 19-71 y (45±14). The exclusion criteria were: working night shift, taking any systemic steroid medications, or using any topical steroid cream. AM samples were collected between 6-8AM and midnight samples were collected between 11PM and midnight. The Salivettes were centrifuged and the samples were frozen at -70ºC until analysis. Statistics were performed using EP Evaluator Release 9 (Data Innovations, South Burlington, VT). **RESULTS** The morning cortisol results displayed a slight positive skew in the distribution for this reference population (Figure 1). The reference interval for (n=35) AM salivary

cortisol using a transformed parametric method provided an overall range of 100 to 1000 µg/mL, while the midnight range (n=41) was <100 µg/mL by nonparametric method (central 95%). **CONCLUSION** The reference range determined in this healthy adult population was not gender dependent and showed known diurnal variation.



A-300

Determination of Chromium, Manganese and Nickel in Human Serum by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

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In this study, a method to determine the trace elements Chromium, Manganese and Nickel in serum by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was developed and validated. These trace elements are very important for physiological function but, in high concentrations they may cause toxic effects to humans and in low concentrations, can cause developmental impairment.

The Chromium's clinical importance is due to its compounds in Glucose Tolerance Factor (GTF) as insulin cofactor, increasing the membrane's fluidity to promote the insulin binding toits receptor. It may be found in oil, asparagus, beer, mushroom, plum, whole grains, meat, offal and vegetables.

Manganese may cause toxic effects at high concentrations, such as respiratory diseases and nervous system disorders. On the other hand, its lack may result in growth disorders by bone frailty, osteoporosis, acromegaly, multiple sclerosis, and ovarian or testicular degeneration. It may be found in grains, nuts, peppers and fruits. It is also present in igneous rocks, fertilizer industries, metallurgical industries, ceramics, glass, batteries and gasoline.

Dermal exposure to Nickel may result in dermatitis and exposures by inhalation causes respiratory diseases. Nickel is considered carcinogenic (composite) and possibly carcinogenic (metal) by IARC. It may be found in beans, cabbage, lettuce, spinach and some fruits such as figs, pineapple, plum andraspberry. It is present in the steel, battery, liquid effluents steel, oil refinery and fertilizer plants, paper and pulp.

Therefore, a rapid, sensitive and accurate ICP-MS method was developed and validated for Simultaneous determination of 61Ni (HEHe), 52Cr (HEHe) e 55Mn (No Gas) in human serum. 500 µL of serum samples and calibrators standards were prepared by dilution (1:10, v/v) in an acidic solution consisting of nitric acid (0.1%) and triton-X 100 (0.05%). Germanium (72Ge) and Scandium (45Sc) were added as internal standard (IS).

The method was validated in terms of lower limit of quantification (LLOQ), linearity, precision, accuracy and comparability with Graphite Furnace Atomic Absorption Spectrometry (GFAAS). Instrumental linearity was verified in the range of 0.01-100.0 µgL-1 for Chromium, 0.1-100.0 µgL-1 for Manganese and 0.5-100.0 µgL-1 for Nickel. The medium range of recovery over an interval of 0.5-100.0µgL-1 for the Chromium was 99.9-106.4%, for Manganese it was 95.7-102.3% and for Nickelit was 100.2-103.9%.

The intra and inter-day precision were less than 3.5% for Chromium, 7.2% for Manganese and 11.7% for Nickel.

In conclusion, the ICP-MS method has been developed successfully for the quantitative analysis and biomonitoring of these trace elements.

A-301

A quantitative LC-MS/MS determination of serum 17-hydroxyprogesterone utilized for the diagnosis of congential adrenal hyperplasia

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Background: Inherited genetic errors in steroid biosynthesis lead to hormone imbalances within affected individuals, which can propagate into life-threatening diseases. Cogential adrenal hyperplasia (CAH) has severe manifestations in 1 in 14 000 persons in the United States of America, resulting in salt wasting crisis in neonates as well as incorrect gender assignment in virtualized females, while mild forms of CAH are observed in less than 1 in 100 individuals in the USA, leading to sinus and pulmonary infections, orthostatic syncope and shortened stature. Ninety percent o f diagnosis cases are caused by a mutation in the gene that expresses the cytochrome P450 enzyme 21-hydroxylase. This mutation leads to elevated levels of 17-hydroxyprogesterone and the manifestation of CAH. Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) allows for the rapid quantification of those steroids involved in the diagnosis of CAH, while using a lower volume of blood than radioimmunassays; important for the neonatal patient population targeted for testing. The developed liquid chromatography tandem mass spectrometry method for the quantification of 17-hydroxyprogesterone will provide physicians with information to assist their diagnosis and treatment of CAH.

Methods: A low volume and sensitive LC-MS/MS method has been developed for the quantification of 17-hydroxyprogesterone in 100 µL of serum. Sample preparation for both analytes relied upon a methyl *tert*-butyl ether liquid-liquid extraction, while chromatographic separation of analytes was carried out using an Agilent 1290 liquid chromatographic separations module (Santa Clara, CA, USA) and Agilent Poroshell C18 column (2.1 x 100 mm, 2.7 µ) with a Methanol and 5 mM ammonium formate gradient over 6 minutes. Identification of both analytes relied upon an Agilent 6460 triple quadrupole mass spectrometer using multiple reaction monitoring for quantification; 17-hydroxyprogesterone (331.0 ◊ 97.1/109.1 [H-085, Cerilliant, Round Rock, Texas, USA]) and 17-hydroxyprogesterone-d8 (339.2 ◊ 100.0/112.1 [H-096, Cerilliant]).

Results: The separation of steroid molecules by LC ensures the minimization of any interferences, which is critical in achieving accurate quantitative results. In separating these analytes, a lower limit of quantification (LLOQ) of 100 ng/L was achieved, as well as an upper linear range of 250000 ng/L. Across this linear range all calibration curves displayed excellent linearity of R2 > 0.999 and satisfied accuracy and precision requirements of ±10% for calibrators and quality control samples. In achieving this linear range, the diagnosis of CAH in patients will be enabled without the need for sample dilution or reanalysis.

Conclusion: The achieved linear range of 100-250000 ng/L and specificity provided by LC-MS/MS will ensure the proper diagnosis of patients afflicted with CAH, while reducing the need for sample dilution or reanalysis.

A-304

Combining High Resolution Mass Spectrometry and In Silico Structure Searches to Identify Compounds Contributing to False Positive Immunoassays

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Objective: Capture antibody-based assays are susceptible to off-target binding thus increasing false positives. We employed two techniques, high-resolution mass spectrometry (HRMS) and an *in silico* structure search, to identify compounds likely to cause false positive results in urine specimens that were positive by amphetamine (AMP) or MDMA immunoassays but failed to confirm by tandem mass spectrometry.

Methods: 100 false positive (FP) immunoassay specimens for AMP and/or MDMA were diluted five-fold and analyzed by a 1260 liquid chromatograph coupled to an Agilent 6230 time of flight mass spectrometer (TOF-MS). Full scan data from 100-1000 m/z were collected and signals with $>10^6$ counts were analyzed using MassHunter software and the Personal Compound Database Library (Agilent Technologies) to establish a library of chemical formulas from the obtained high resolution masses. Criteria for a positive identification were retention time ±0.15 minutes, mass error ±10ppm, and match score greater than 70. Separately, Scifinder (Chemical Abstracts) was used as an *in silico* structure search to generate a library of compounds that are structurally similar to amphetamine, MDMA, or compounds known to cross react with AMP and MDMA immunoassays. Initial 'hits' included thousands of organic compounds that were filtered based on frequency of citation in the literature as well as medicinal or biochemical characteristics. Chemical formulas and exact masses of 145 structures were then compared against masses identified by TOF-MS. To assess if compounds identified in the TOF-MS data or structure-based searches had cross-reactivity with the AMP or MDMA immunoassays, available verified standards (32 compounds total) were purchased and prepared in synthetic urine at half-log concentrations between 0.1 and 100ug/mL and analyzed by a Beckman AU5810 instrument with Siemens Emit II Plus reagent packs.

Results: Compounds known to have cross-reactivity with the immunoassays were identified in the structure-based search, validating the approach. In comparing chemical formulas and exact masses of 145 structures (of 20 chemical formulas) against masses identified by TOF-MS (area counts >1 x10^6), 10 mass matches correlating to 38 compounds represented in 67 specimens were identified. Of the 32 compounds tested by immunoassay, cross-reactivity was observed with 13 compounds by MDMA and 8 compounds by amphetamine immunoassays.

Discussion: Urine analysis by HRMS correlates accurate mass to chemical formulae but provides little information regarding compound structure. Utilizing structural data of target antigens and known-cross reacting compounds, one can search compound databases to correlate HRMS-derived chemical formulas with structural analogues. While this study demonstrates the utility of this approach, urine contains numerous metabolites that can also contribute to immunoassay FPs that may not be identified based on available compound libraries.

Conclusion: Because antibody specificity correlates with antigen structure, *in silico* structure based searches in combination with HRMS methods, facilitate identification of potential compounds that contribute to antibody cross-reactivity.

A-305

Analytical Quantitation of Tenofovir and Emtricitabine in Human Hair using LC-MS/MS for the Exploration of Hair as a Long-Term Adherence Marker

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Background:

There are a number of limitations associated

with self-reporting in assessing adherence to HIV antiretrovirals. Plasma and peripheral blood mononuclear measurements are reflective of short-term drug exposure. Hair and dried-blood spots (DBS) provide a more long-term exposure assessment. The objective of this study is to develop and validate a quantitation method for tenofovir/emtricitabine in hair using

LC-MS/MS and to access the utility of hair as a marker of long-term adherence, compared to DBS, in HIV-negative men enrolled in pre-exposure prophylaxis studies (iPrEx).

Methods:

Human hair was analyzed for tenofovir/emtricitabine using a Shimadzu LC-20AD HPLC system coupled to a Micromass Quattro Ultima triple quadrupole mass spectrometer using electrospray positive ionization. Multiple reaction monitoring analyses was performed on a reverse phase column (Synergi POLAR-RP, 4 µm, 4.6 x 150 mm). Flow rate was1 ml/min

utilizing a gradient system of aqueous to 45% methanol both containing ammonium acetate (5 mM), 0.06% trifluoroacetic acid, and 3 mg/L ammonium phosphate for 6 minutes. Sample preparation consisted of

the incubation of hair (5 mg) in a 50% aqueous methanol solution containing 1% trifluoroacetic acid, 0.5% hydrazine, and tenofovir-d6 and emtricitabine-^{12}C-$^{15}N_2$ (internal standards) at 37°C overnight in a shaking water bath. Following centrifugation, the supernatant was then evaporated to dryness, and reconstituted with mobile phase for analysis by LC-MS/MS. The linear concentration ranged from 0.00200 to 0.400 and 0.0200 to 4.00 ng/mg of hair for tenofovir and emtricitabine, respectively and the lower limit of quantitation were equivalent to the lowest point of the standard curve. Precision and accuracy were determined through the analysis of quality control samples at 3 concentrations (0.320, 0.0400, and 0.00600 ng/ml hair (tenofovir) and 3.20, 0.400, and 0.0600 ng/ml hair (emtricitabine)). Precision, defined by the coefficient of variation (CV), ranged from 6.40% to 13.5% (tenofovir) and 2.45% to 5.16% (emtricitabine). Accuracy, defined by relative error (RE), ranged from -2.25% to +1.00% (tenofovir) and -0.250% to +4.06% (emtricitabine). Extraction efficiency was determined to be 91.3% (tenofovir) and 99.1% (emtricitabine). No

carry over peaks were observed and no matrix effect (CV for matrix factor (MF) of tenofovir, emtricitabine, internal standards, and IS-normalized MF does not exceed 15%) was observed in six different lots of blank hair. **:** iPrEx Open Label Extension (OLE) enrollment consisted of 1603 HIV-negative men. Visits were performed at baseline and weeks 4, 8, 12, 24, 36, 48, 60, and 72. Plasma and DBS were collected at 4 and 8 weeks and every subsequent 12 weeks, while hair samples were collected every 12 weeks.

Results:

Tenofovir hair concentrations and tenofovir-diphosphate in DBS were strongly correlated (Spearman's correlation coefficient r 0.734, $p<0.001$), as were tenofovir hair levels and emtricitabine-triphosphate concentrations in DBS (r 0.781, $p<0.001$). However, a stronger correlation between emtricitabine hair levels with tenofovir-diphosphate in DBS exists compared to that with emtricitabine-triphosphate in DBS (r 0.587, $p<0.001$).

Conclusion:

Previous studies have shown that DBS are predictive of efficacy and the strong correlation between hair and DBS suggest that hair concentrations could be predictive as well.

A-306

Development and Validation of Dilute and Shoot- Flow injection Tandem Mass Spectrometric method for the Detection and Quantification of Phenobarbital in Human Urine

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Background: A new, sensitive and specific Dilute and Shoot- Flow injection Tandem Mass Spectrometric method for the identification and quantification of Phenobarbital in urine was developed and validated according to the US FDA and SAMHSA guidelines for the first time. Although, a variety of approaches have been employed to identify both licit and illicit drugs for urine drug testing analysis such as immunoassays, gas chromatography- mass spectrometry and high performance liquid chromatography-mass spectrometry, but also inherent several limitations such as (i) Immunoassays lacks specificity, susceptible to cross reactivity and can only be used for screening, (ii) GC-MS analysis necessitates complex derivatization of the sample. LC-MS/MS is now recognized as a gold standard technique for quantitative screening of multiple drugs, it requires an expensive HPLC column for separation, long run time, excess solvent loss, and elaborated sample preparation procedures like solid phase extraction or liquid- liquid extraction, resulting low throughput workflows which is the current rate limiting step and increases the turn-around time of the analysis. Faster methods are highly preferred in clinical settings, since majority of the patients will be tested positive for pain drugs, indicating adherence to agree upon treatment plan.

Method: Our devised method involved simple dilution of Phenobarbital spiked urine, fortified with Phenobarbital-d_5 (Internal Standard) followed by flow injection using 5mM Ammonium acetate / 70% Acetonitrile as a carrier solvent, bypassing the column compartment and detected by mass spectrometer employing negative ESI-MRM mode. Quantification and confirmation of Phenobarbital at LLOQ level (5ng/ml) was possible in this method by using MRM channels (signal/noise: 29 and 10.5) respectively.

Results: The validated method was found to be linear with the dynamic range of 5- 200ng/ml and the $r^2 > 0.9998$ was achieved. The precision (%CV) and accuracy (%RE) results for intra and inter assay at 3 QC levels (12.5, 45, 160ng/ml) and LLOQ (5ng/ml) were > 3.0% and 5.0% respectively. It is worth mentioning that, this method is devoid of significant matrix (absolute and relative) effects, where % CV's were found to be 7 and 6.2 respectively, at three QC levels and LLOQ. Phenobarbital in urine at two QC levels (12.5, and 160) was subjected to stability studies conditions: Bench top (8hr) at room temperature, freeze thaw (3 cycles), and 2 months at -20^{o}C, excellent stability of Phenobarbital was found with % recovery of 98-100.5%.

Conclusion: The key components of this method are: Simple dilution and FI-MS/MS quantification of phenobarbital in urine with 2 minutes run time (analyte peak obtained at 0.7 minutes), enabling higher throughput analysis for urine drug testing, without compromising the data integrity when compared to traditional LC/MS/MS protocols. Nevertheless, no change in the signal intensity or sensitivity was found even after 1200 injections proving that this method was robust. Hence, the acquired results proved that this novel method is simple and scalable and has the capacity to process ~1500 samples/day, and can lead to the transfer of existing methodologies to the newer robust platforms, permitting the development of more sensitive and rapid techniques recommended for clinical and toxicological studies.