

**POSTER SESSION ABSTRACTS:  
TUESDAY, OCTOBER 18**

**TP-1: The Use of HILIC HPLC/MS/MS for the Quantitative Bioanalysis of the Polar Drug Carboplatin from Plasma**

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Carboplatin is a drug that is used as a chemotherapy treatment for cancer. The determination of the in-vivo pharmacokinetics of carboplatin is important especially when novel combination treatments are used. However, the bioanalytical measurement of carboplatin by HPLC/UV methods is insensitive due to the lack of significant UV absorption by carboplatin. Although derivitization of carboplatin has been used, these methods only produced limits of quantitation near 0.13  $\mu$ M and required run times of >25 minutes per sample. Other HPLC/MS methods required the extensive clean-up of the biological samples. In addition, the HPLC/MS methods suffered from assay interferences due to the lack of retention of the polar carboplatin on the reversed phase column. Here we report on the development of a simple precipitation method with a polar HPLC column (HILIC) for the sensitive HPLC/MS/MS quantitative analysis of carboplatin from plasma. The assay gave precision and accuracy of better than  $\pm 25\%$  across the entire range of 25-10,000 nM concentrations of carboplatin in plasma. This HPLC/MS/MS assay is useful for the routine low nM measurement of carboplatin from plasma to support PK studies.

**TP-2: Development of an On-Line Extraction Turbulent Flow Chromatography Tandem Mass Spectrometry Method for Cassette Analysis of Caco-2 Cell Based Bi-Directional Assay Samples**

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Caco-2 cells are frequently used for screening compounds for their permeability characteristics and P-glycoprotein interaction potential. Bi-directional permeability studies performed on Caco-2 cells followed by analysis by HPLC-UV or LC-MS method constitutes the "method of choice" for the functional assessment of efflux characteristics of a test compound. A high throughput LC-MS/MS method has been developed using on-line extraction turbulent flow chromatography coupled to tandem mass spectrometric detection to analyze multiple compounds present in Hanks balanced salt solution in a single analytical run. All standard curves (P-gp substrates: quinidine, etoposide, rhodamine 123, dexamethasone, and verapamil and non-substrates: metoprolol, sulfasalazine, propranolol, nadolol, and furosemide) were prepared in a cassette mode (ten-in-one) while Caco-2 cell incubations were performed both in discrete mode and in cassette mode. The standard curve range for most compounds was 10 nM to 2500 nM with regression coefficients (R<sup>2</sup>) greater than 0.99 for all compounds. The applicability and reliability of the analysis method was evaluated by successful

demonstration of efflux ratio greater than 1 for the P-gp substrates studied in the Caco-2 cell model. The use of cassette mode analysis through selected reaction monitoring mass spectrometry presents an attractive option to increase the throughput, sensitivity, selectivity, and efficiency of the model over discrete mode UV detection.

**TP-3: EpiTags™ - A Standardized, Scaleable Approach to Multiplex Protein Analysis**

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We describe a new method for quantitative proteomics utilizing antibodies developed against unique continuous linear peptide sequences found within every protein. Starting with the genomic database, in silico techniques are used to identify continuous linear sequences for any protein that are unique relative to the entire proteome. Antibodies are then raised against synthetic peptides that comprise these unique sequences and antigen affinity purified to yield mono-specific type reagents. Antibody recognition of the selected epitopes is assured by fragmenting proteins in the sample prior to analysis. Protein concentration is determined based on interpolation against synthetic peptide standards and need for a protein standard is obviated. This presentation demonstrates proof of concept of this peptide-based immunoassay approach starting with generation of low nM affinity and high specificity antibodies to synthetic peptides, through to quantitative analysis of proteins in a multiplex platform. The EpiTag system provides a quantitative measurement solution for any protein in any sample. The system is platform independent and works equally well on beads (e.g. Luminex) or planar arrays.

**TP-4: Fast Gradient LC Separations Using Your Existing LC**

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The quest to pursue "ultra" fast LC separations is gaining momentum as the result of recent product introductions to address this need. These new products claim to deliver the same separations while using 5-10 fold less time. What may be lesser known is that routine performance of "ultra" fast separations can be achieved using many of the ordinary main stream HPLCs that one may already have available. Achieving this level of performance is simply a matter of knowledge of the separation principles involved and their practical implementation. The key principle involved is balancing velocity with diffusion in the mobile phase. The implementation of the LC set up involves volume control in the system and its eluted peaks. The implementation of the LC analyses involves making good choices in stationary and mobile phases as well as full proper control of critical system parameters such as mobile phase temperature (entering column). Application of the above concepts allows routine

analyses of >500 samples per day per LC (or LC/MS) operating at 7 mm/s (5 mL/min, 5x “normal”), clearing 10 column volumes per min (10x “normal”) and delivering peaks <1 s wide. One result of this added capacity is the straightforward and scalable positive impact on turnaround times of existing analyses. In addition, the added capacity allows many new applications (previously not feasible) to be pursued that may benefit from greater molecular specificity and precision that can result from adding a high efficiency separation. Extensive example data demonstrating the principles, set up, analyses, and applications will be provided.

**TP-5: Micro Parallel Liquid Chromatography (μPLC) for Confirmation Screening and Secondary Assays**

Tom Onofrey

Nanostream, Inc. 580 Sierra Madre Villa, Pasadena, CA 91107

Researchers seek tools to confirm hits and eliminate false positives from screening studies. In addition, assay development can be both costly and time-consuming. This talk presents data for separation-based assays from a micro parallel liquid chromatography (μPLC) system, a high-throughput variant of HPLC. The system—which includes an autosampler, UV absorbance and fluorescence detectors, software, and 24-column microfluidic cartridges—facilitates assay development, offers quantitative information about hits, and provides additional information on compound purity and solubility. This talk will present data from assays used in lead identification and for phase 2 metabolism studies to demonstrate the advantages of reduced interference and a ratio-metric readout. The system will be compared to plate-reader approaches as well as to other analytical solutions.

**TP-6: A New Hydrophobic Interaction Chromatography (HIC) Column with Improved Hydrolytic Stability for Protein**

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HIC is a popular technique for protein purification. It does not denature proteins during the purification process as compared to reversed phase HPLC and preserves biological activity. We have developed a new silica based HIC stationary phase with multifunctional amide group attachments. The surface chemistry of this phase was optimized by inclusion of hydrophobic and hydrophilic ligands which were designed to improve the hydrolytic stability of silica support in the aqueous media. These modifications resulted in superior resolution and peak efficiencies of proteins and provided a versatile column with sufficient longevity. We have demonstrated the use of this silica based HIC column for separation of proteins and peptides. These include 1) Mixture of proteins 2) Mixture of peptides 3) Trypsin digests of proteins 4) Human skeletal muscle cell extract 5) Snake venom proteins 6) Serum proteins 7) Pancreatin 8) Thrombin 9)

-Amylase 10) membrane proteins and others. We have compared the new silica column with other HIC columns for efficiency, peak shape, resolution, capacity and resolution. The new HIC column displayed high capacity and superior overall performance. The high capacity of this analytical HIC phase makes the column an excellent choice as first dimension of a multidimensional chromatography analysis.

**TP-7: High Resolution IMAC: Applications Using Immobilized Copper and Iron**

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Immobilized metal-affinity chromatography (IMAC) is used as a tool for protein/peptide purifications in a wide variety of applications. Because fractions collected from low resolution IMAC cartridges usually contain impurities, the demand exists for the IMAC phase in an analytical column format. To achieve this, we used living/controlled radical polymerization and polymer chain collapse to engineer the surface of 10 μm, nonporous, polymeric beads with isolated, metal-containing nano-particles that act as IMAC interaction sites. Surface-bound nanoscale particles were visible in transmission electron microscope (TEM) images of bead cross-sections. TEM was also used to visualize individual ferritin molecules interacting with nano-particles on the substrate surface. In the copper mode (Cu-histidine interaction), the resolving power of the column was tested by injecting a mixture from a library of prion-related peptides. As expected, the column was capable of separating prion peptides differing in number of octapeptide repeat units (PHGGGWGQ), (PHGGGWGQ)2, and (PHGGGWGQ)4. Immobilized copper mode has also been applied to 1) monoclonal antibodies from impurities, Fc and Fab MAb papain digest fragments, and MAb fragments from forced reduction experiments, 2) mixtures from a library of prion-related peptides, and 3) histidine tagged protein aggregation variants. In the immobilized iron mode (Fe-phosphate), we enriched phosphopeptides from standard protein digests before a secondary separation of the fractions by reversed phase. During extended use, the analytical IMAC column has demonstrated performance and robustness equal to high resolution, ion exchange protein columns.

**TP-8: Automated Nanospray Chip System for Medium Throughput Mass Spectrometry**

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Nanospray chip technology enables automated high sensitivity mass spectrometry in applications where sample consumption must be kept to a minimum and reliability of the spray is critical, i.e., clog-resistant, no sample cross-contamination, and accurate with consistent flow and spray characteristics. In demanding applications such as ligand-native protein binding and in clinical diagnostics, the number of samples to be analyzed per day may be in the hundreds. A chip for just 16 samples each and is compatible with existing robotic sample dispensing equipment may be sufficient to dramatically boost productivity. This report presents preliminary results obtained with such a system.

**TP-9: Characterization of Protein Expression Changes in Liver Microsomes in Response to Drug Treatment using iTRAQ™ reagents and 2-D LC/MS/MS**

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The goal of quantitative proteomics research is to characterize protein expression levels and their modified forms in an effort to understand biological states at the molecular level. The ability to obtain high-confidence protein identification and simultaneous quantification of proteins makes LC/MS a valuable tool for both biomarker discovery and validation. As the liver is the primary site for metabolism of drugs, it is important to understand the changes in protein expression in response to drug treatment, specifically the cytochrome P450 proteins. Proteins from mouse liver microsome samples (control and phenobarbital treated) were digested and labeled with iTRAQ™ reagents. The peptides were fractionated by strong cation exchange and analyzed by reverse phase LC/MS/MS on a hybrid triple quadrupole linear ion trap mass spectrometer. Each fraction was analyzed twice, where the second analysis excluded MS/MS of all confidently identified peptides. The ratios for all the identified peptides were determined and Pro Group Viewer was used to calculate the protein expression ratios. MS/MS data was used for peptide identification and quantification, with over 200 different proteins identified, quantified and the results compiled with Pro Group Viewer. Pro Group determines the minimal set of proteins (including distinct isoforms) based on all putative peptides and the confidence in those peptides. A protein-level confidence is reported for each protein. The challenging aspect of the P450 family is the high degree of sequence similarity between some isoforms in some P450 subfamilies. Using the isoform resolving functionality in Pro Group, we have been able

to unambiguously identify and quantify 21 of these P450 proteins. The expression changes correlate very well with the previous targeted MRM quantification work on these proteins. The Panther Gene ontology tool can then be used to further understand the changes in the drug treated liver samples. The combined use of iTRAQ reagent, Pro Group Viewer and the mass spectrometer's ion trap MS/MS capability enabled the robust identification and relative quantitation of proteins needed for biomarker discovery. The cumulative work on this project demonstrates a workflow using stable-isotope chemistries and a MS platform for non-targeted biomarker discovery followed by targeted validation of putative biomarkers.

**TP-10: Use of iTRAQ™ Technology for Prevalidation of Protein Biomarkers Identified by 2-Dimensional Gel Electrophoresis and MALDI-MS**

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Today, toxicoproteomics still relies mainly on 2-dimensional gel electrophoresis (2-DE) followed by mass spectrometry (MS) for detection and identification of proteins, which might characterize a certain state of disease, predict carcinogenicity or indicate toxicity. We utilized the classical 2-DE approach for the evaluation of protein biomarkers that are predictive for chemically induced hepatocarcinogenesis in rats (Fella et al., Proteomics, 2005). We were able to identify statistically significantly deregulated proteins in N-Nitrosomorpholine (NNM) exposed rat liver tissue. Biological relevance of these potential biomarkers has been proven. However, in order to ensure reliable results, these protein expression patterns need to be prevalidated using independent technology platforms. We used iTRAQ reagent technology, a MS-based protein quantitation method, which was recently developed by Applied Biosystems (Framingham, USA). The iTRAQ reagents enable isobaric peptide tagging for quantitative proteomic analysis by using amine-specific, stable isotope reagents in up to four different samples simultaneously. The labelled peptides are identical in mass and, therefore, also identical in single MS mode. In MS/MS mode low-mass reporter ion signals allow quantitation, while peptide fragment ion signals allow protein identification. We were able to identify and quantify hundreds of proteins in four rat liver samples simultaneously. Many of the potential hepatocarcinogenic biomarkers from classical 2-DE/MS showed the same expression level when using the iTRAQ technology (annexin A5, Rho GDP dissociation inhibitor, major urinary protein precursor, adenosine kinase...). Therefore, our results show the usefulness of this new quantitation strategy for verification and prevalidation of biomarker proteins identified using classical proteomic approaches. In comparison with 2-DE/MS, the iTRAQ

technology allows a significant decrease in working and instrumental time for protein identification and quantitation by about one order of magnitude. In addition, the advantage of multiplexing up to four different samples increases comparability of results.

**TP-11: Simultaneous Collection of Online LC-MS/MS and LC Fractions With Post-Column Splitting: Maximizing MS Information Of Complex Samples With Nanoelectrospray Ionization**

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LC-MS continues to be the gold standard for acquiring qualitative and quantitative information for complex mixtures. Applications such as metabolomics, biomarkers and proteomics involve the analysis of known and unknown species with a desire to quantify and characterize each component. Sample complexity limits the amount of time for MS/MS and MS<sub>n</sub> during an LC run. This work will describe the development of a system that enables coupling of LC columns with flow rates up to 1.0 mL/min to the ESI Chip with use of a post-column splitter. The system enables collection of LC-MS and LC fractions simultaneously. Software links LC-MS retention times to specific LC fractions collected in 96 or 384-well sample plates. Infusion of LC fractions enables optimization of MS/MS parameters such as collision energy for complex, multi-component fractions demonstrating greater than 10 times improvement in S/N compared to online LC-MS/MS. Ibuprofen metabolites in human urine were investigated by both online LC/MS and chip-based infusion nanoESI of LC fractions. This work will highlight the improved data obtained by extended analysis of LC fractions for obtaining MS/MS data on low level metabolites.

**TP-12: Analytical and Preparative Supercritical Fluid Chromatography (SFC) Using Monolithic Columns**

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As monolithic columns have become commercially available, their use in HPLC has become more widespread because of their markedly decreased backpressure and longer lifetime over traditional particulate columns. However, their use in SFC has been reported very rarely. Are they advantageous for SFC as well? While use of a highly compressed gas, typically CO<sub>2</sub>, provides for lower backpressure in SFC than HPLC, backpressure still limits analytical and preparative throughput. Increased backpressure is also associated with a need for more frequent instrument maintenance. Preparative instruments are particularly susceptible to failure due to precipitation of relatively concentrated mixtures in CO<sub>2</sub> and concomitant pressure increases. Applicability of monolithic columns to high-throughput

analytical SFC/MS and preparative SFC of small drug-like molecules is investigated. We compare the performance of 1) monolithic columns in SFC vs HPLC and 2) monolithic columns vs particulate columns in SFC.

**TP-13: Eksigent's Microfluidic Flow Control Applied to Fast LC/MS Analysis of Pharmaceutical Small Molecules**

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Traditional approaches to faster chromatography have involved higher column pressures, shorter columns and faster flow rates. This alternative, micro flow approach uses far less solvent at traditional HPLC pressures, resulting in less waste generation. Here, the micro flow LC system has been combined with mass spectrometry, and the benefits and limitations of capillary LC/MS with fast gradients are explored. Finally, the system is demonstrated in an open-access pharmaceutical research mode using Masslynx® software from Micromass.

**TP-14: Cell Metabolomics using 1H-NMR -- Progress on Data Analysis for Genetically Modified Cells**

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Cell cultures are promising, cost effective alternatives to living animals for early phase toxicology and many other studies. Cell-based metabolomics using NMR spectroscopy can report about adverse effects of treatment with potential drug candidates, all kind of environmental effects, and genetic modification, for example. As part of the program to study genetically modified bacteria and stem cells we have conducted metabolomic analysis of such cells using 1H NMR. Both living cells and cell-extracts were subject of analysis. Recently we have made progress with analysis of previously acquired data, which will be presented on the poster. We have found clear clustering in correlation with simple genetic changes both for the living cells and the extracts. The loading plots offer access to potential biomarkers for this condition, as well as insight into the modulated biochemistry of the cells. The technology can be adapted for a variety of applications, which will also be discussed.

**TP-15: Multidimensional PCA Scores and Loadings Plots -- Advantageous Use of ArrayTrack, a Free Software from FDA**

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Multivariate data analysis of metabo\*mic data most often uses PCA scores and loadings plots. Two dimensional correlations along selected principal components are most common, although the advantage of three dimensional scores plots has been demonstrated many times as well. Recently we have started using the free software from FDA, ArrayTrack, for visualization and analysis of our NMR-based metabo\*mic data on living cells, cell extracts, and horse saliva with great success. In this poster we shall demonstrate on real-life examples the usefulness and superiority of three dimensional plots versus two dimensional ones, as well as some user-friendly capabilities of the software itself. Beside 3D scores plots, we shall show and discuss three-dimensional loadings plots as well, an obvious extension of such analysis, which has not been presented in the literature yet to our best knowledge.

**TP-16: Sample Purification for Static Nanospray MS using Wall-Coated Pipette TrapInTips™**

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While highly suited for MALDI-MS, in-pipette sample purification is difficult to implement with static nanospray methodology. Geometric constraints of pipette tips limit sample transfer efficiency into glass nanospray emitters, and conventional loading/extraction requires either pressurized back-loading into a pipette or coupling one pipette tip to a longer pipette. The TrapInTip™ (New Objective), a novel gel-loader pipette tip containing reverse-phase sorbent-coated walls, eliminates high pressure/vacuum requirements for loading a packed bed. TrapInTip geometry permits sample aspiration from the pipette tip and direct loading of static nanospray emitters. TrapInTips demonstrate concentration, desalting, and excellent MS signal for 0.05uM BSA tryptic digest solutions and 0.10uM BSA solutions containing NH<sub>4</sub>HCO<sub>3</sub>. Manual gradients using eluents of successively increasing organic concentrations display excellent peptide-separation ability. An ion trap mass spectrometer (LCQ Deca™, Thermo Electron) was outfitted with a nanospray source (PicoView® 150, New Objective) for offline analysis. Chromatographic ability of TrapInTips was tested by preparing a 0.5uM BSA tryptic digest. Four different eluent solutions containing 10%, 20%, 40% and 60% ACN were prepared with 0.1% formic acid. Loading, washing, and elution onto each coating material produced significant signal enhancement for concentrations as low as 0.05uM without NH<sub>4</sub>HCO<sub>3</sub> and 0.1uM with NH<sub>4</sub>HCO<sub>3</sub>. Two 0.40uM BSA digests, one containing 50uM NH<sub>4</sub>HCO<sub>3</sub>, served as concentrated reference samples; for these references, no MS signal was

observed in the presence of NH<sub>4</sub>HCO<sub>3</sub>, and decreased S/N ratio was observed for the BSA solution containing 2% ACN without NH<sub>4</sub>HCO<sub>3</sub>. In addition, TrapInTips provided excellent chromatographic separation from consecutive aspiration/expulsion cycles with eluent compositions ranging from 10-60%. For three different sorbents, BSA samples eluted from TrapInTips revealed detectable MS signal in the presence of NH<sub>4</sub>HCO<sub>3</sub> and improved S/N without added salt. The proficiency of the TrapInTip to conduct offline LC-MS for proteomic research offers the requisite purification, concentration, and separating facility for identifying complex peptide mixtures.

**TP-17: Enhanced MALDI Tissue Imaging by Precise Matrix Deposition using a Novel Chemical Printer**

Steve Wishnies Shimadzu Biotech,USA

MALDI MS is a powerful tool in imaging molecules from relatively small populations of cells in specific regions of heterogeneous tissue architectures. To generate MALDI imaging matrix solution is deposited onto tissue sections in discreet digital micro-droplets using a piezoelectric delivery device (using a Chemical Printer) and each droplet is analyzed. The spectra from specific tissue map locations are then compiled into image analysis software which can then be linked to database tools to compare between sample types and identify the statistical significance between tissue types and further probe for biomarkers of disease.

**POSTER SESSION ABSTRACTS:  
WEDNESDAY, OCTOBER 19**

**WP-1: Lead Discovery Screening Using Separations and Mass Spectrometry Measurement in Functional Biomolecular Assays**

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There are several compelling reasons to seek more molecularly specific detection techniques for use in high throughput lead discovery screening. One motivation is to reduce attrition for otherwise promising validated targets where no HTS assay can be developed using the conventional tools. Many conventional assays require non-native substrates (e.g. fluorescence labels), which can yield results that are not biologically relevant. So another motivation for more molecularly specific measurement techniques is to allow the use of the native substrate at biologically relevant concentrations. Radioactive labels can yield biologically relevant results, but pose high safety / disposal costs and still require efficiently separating the substrate from the substrate product in the presence of drug candidate compounds. Optical readouts usually require additional derivatization reactions (non-specific) and often don't have sufficient precision or dynamic range to distinguish hits from noise (particularly in activation assays) due to chemical noise. The combination of separations (GC or LC) and mass spectrometry (MS) offers a powerful solution to many of these challenges and is often adaptable to most small molecules thereby permitting use of native substrate at biologically relevant concentrations without the danger or expense of radioactive substrates. In these MS based assays, ultra fast, high-resolution separations prevent interference of the substrate with the substrate product even in the presence of drug candidate compounds. Traditional assays generally cannot readily adapt to the challenges / interferences encountered from the drug candidate compounds. The frequent result is missed hits and a large number of false positives (often >10 fold the number of leads). In contrast, separations/MS based assays can readily incorporate MS/MS or HRMS into the assay to anticipate the challenges / interferences encountered from the drug candidate compounds and virtually eliminate false positives by achieving sufficient molecular specificity. In addition, separations/MS often have enough excess precision (5% RSD) and dynamic range (3 orders of magnitude) to easily distinguish hits from noise (even in difficult activation assays) and yield high Z' factors ( $Z' > 0.7$ ). In this presentation, results from GC/MS and LC/MS based screens are presented. While MS based measurements are not likely to ever become as fast as optical measurements, the observation of this work is that the total screening project time required is very similar. When optical approaches are used, assay development is usually very time consuming (primary bottleneck). Assay development time is significantly decreased using separations/MS (~5 fold vs. optical) and incorporation of MS/MS or HRMS into the assay generally requires only an additional day of method development. Rapid MS assay development compensates

for the extra measurement time required. Furthermore, hits from separations/MS based screening tend to be validated in secondary assays (cell and X-ray crystal structure), presumably due to the lack of false positives. A restricted number of valid hits, free of false positives, will save time and resources as their associated projects progress into and through the lead optimization phase. Saving time through the more specific hit discovery coupled with the ability to perform otherwise infeasible screens make separations/MS a tool of choice for HTS. Patent pending

**WP-2: Accurate Mass Profile Filtering of Ion Chromatograms at Unit Mass Resolution**

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Accelerating LC/MS analysis for metabolite identification critically relies on effective data processing since the rate of data acquisition is much faster than the rate of data-mining. The rapid and accurate identification of metabolite peaks from complex LC/MS data is a key component to speed up the process. Current approaches that routinely use the ion chromatogram can suffer severely from matrix effects. This poster describes a new method to automatically extract and filter metabolite-related information from LC/MS in the presence of complex biological matrices at unit mass resolution. This performance is illustrated by LC/MS analysis of the metabolites of verapamil from rat microsome incubation spiked with biological matrix bile. Profile-mode MS data were acquired on a unit mass resolution triple stage quadrupole instrument, Q TRAP, and externally calibrated with a unique procedure that corrects for both mass axis and mass spectral peak shape to facilitate metabolite identification with high mass accuracy. Through the double-filtering effects of accurate mass and isotope profile, conventional extracted ion chromatograms of parent drug verapamil at m/z of 455, demethylated verapamil at m/z of 441, and dealkylated verapamil at m/z of 291, which had substantial false positive peaks, were simplified into chromatograms that are substantially free from matrix interference. The filtered chromatograms can be combined into a composite ion chromatogram resembling that from radio-labeling the parent drug.

**WP-3: A priori Virtual Screening to Maximize Analytical Throughput in the Quality Control Lab**

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A priori virtual screening is a set of software filters that allow an investigator to assign a suitable analytical method for each constituent in a chemical library in the absence of empirical data. Libraries presented in structure / data files, MS-Excel® files or MS-Access® files are imported into the software and the filters parse compounds based on inherent physico-chemical properties. Constituents of the library are binned into

analytical queues including LC-MS, NMR and GC-MS. The result is increased throughput via correct assignment of methodology and decreased need for re-analysis. Filtering a library populated with 27,684 drug-like and lead-like compounds suggested that 72.5% should be run by LC-MS, 25% would be best suited for NMR and the remaining 2.5% should be run via GC-MS. An empirical study revealed that approximately 94% of the 20,071 compounds run by LC-MS passed the analysis. Proton-NMR of the 6,921 compounds binned into the NMR queue demonstrated a 95% pass rate. The remaining 692 compounds were not analyzed by GC-MS but by a combination of NMR and GC with a 93% pass rate. The a priori process offers the investigator the ability to virtually screen a chemical library and demonstrates a unique advantage over empirical trial and error when assigning system suitability in the quality control laboratory.

#### **WP-4: Metabolism of Resveratrol Analogs**

Tanya N. Gamble<sup>1</sup>, Gary Impey<sup>1</sup>, Kuang Yu Chen<sup>2</sup>, Julie Wingate<sup>1</sup>  
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Resveratrol is a natural substance found in grapes, berries and peanuts that has been shown to have anti-oxidative, anti-inflammatory, and estrogenic effects. It has also been shown to have chemopreventive properties. Resveratrol has a relatively high IC<sub>50</sub>, and effort has been put into finding a more potent analogue. This paper investigates the metabolism of the cis- and trans- isomers of the resveratrol analog, 3, 4, 5, 4'-tetramethoxystilbene (MR-4). MR-4 was incubated with microsomes and various co-factors to produce phase I and phase II biotransformations. The metabolites were analysed using a 4000 Q TRAP® LC/MS/MS system and a Q STAR XL® MS/MS system. Unlike resveratrol, the MR-4 analogues primarily formed phase I metabolites.

#### **WP-5: Parallel Liquid Chromatography to Increase Throughput for ADME and DMPK Studies**

Paran Patel  
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Demand for ADME profiling has increased in early stages of drug discovery (i.e., in lead optimization) as well as in downstream areas closer to the clinic (i.e., drug metabolism). The increase in demand has resulted in the proliferation of instrumentation and techniques aimed at the characterization of various physiochemical properties and the need for software solutions to extract meaningful results from increasing amounts of data and types of data formats. Recent advances in high-throughput liquid chromatography systems and in analysis software have reduced the total amount of time associated with characterization of ADME properties such as log P/log D, solubility, and permeability—all using a familiar and standardized platform. The increase in analytical throughput has also allowed researchers in drug metabolism to overcome LC and sample preparation

constraints associated DMPK assays (e.g., drug drug interaction studies) performed using MS/MS. Examples will be shown to demonstrate how these strategies can be used to accelerate ADME assays conducted at the lead optimization and pre-clinical stages of drug discovery.

#### **WP-6: Strategies and Techniques of Major Metabolite Profiling for Structure Optimization in Drug Discovery**

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Oral bioavailability is an important ADME property. If it is low, it may lead to the failure of a drug candidate. High-throughput in vitro stability assays have been widely used to identify high clearance compounds in drug discovery. This information can be expanded to the identification of major metabolic pathways to address metabolism liabilities. This poster will describe the strategy and techniques for major metabolite profiling. Using this approach, the major metabolic pathways are identified at the physiologically relevant drug concentrations. Metabolism-structure relationships are studied by profiling compound series analogs in parallel. Exact structures of the major metabolites are determined using MS<sup>2</sup> and MS<sup>3</sup> with NMR studies performed with isolated metabolites. These studies provide important information for discovery chemists to block metabolically labile positions on molecules and design more stable compounds.

#### **WP-7: Quantitative LC-MS/MS Analysis of Pregabalin and its Enantiomer in Urine and Perfusate Matrices Using a New Generation of Chiral HPLC Column**

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Pfizer's new drug, Lyrica (pregabalin), is the first and only FDA-approved treatment for both painful diabetic peripheral neuropathy and postherpetic neuralgia, two of the most common neuropathic pain conditions. Lyrica is also FDA-approved for the adjunctive treatment of partial onset epileptic seizures in adults. Pregabalin is a structural derivative of the inhibitory neurotransmitter g-aminobutyric acid (GABA). Pregabalin and its R-enantiomer are difficult to separate by chromatography because of their g-amino acid structure. In this study, we have successfully applied a new generation of chiral HPLC column packed with glycopeptide-based chiral stationary phase (CSP) to separate and quantify them in urine and perfusate matrices to support an isolated perfused kidney (IPK) study for pregabalin. To speed up the screening procedure on the CSPs, a column coupling strategy was used and the Chirobiotic T column was identified to be most effective. The pH and the type of organic solvent used in the mobile phase were found to be the two key factors for achieving baseline chiral resolution. This method has been validated for the

quantitative analysis of pregabalin and its enantiomer in both rat urine and kidney perfusate at a range of 50 - 20000 ng/mL. Urine samples were diluted with internal standard working solution before being injected on to the column. Ion exchange solid phase (SPE) extraction was done to remove the proteins in perfusate sample prior to injection. The method was rugged and has successfully been used to assay more than 1000 samples.

**WP-8: Strategies For Automating Metabolite Profiling and Detailed Structure Elucidation**

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Our approach for metabolite profiling utilizes: 1) An automated system capable of obtaining both metabolic stability and metabolite profiling information on a single sample; and 2) Automated data processing software capable of finding, confirming and identifying non-blank parent-related metabolites using a proprietary algorithm called AutoShift and software package called MetLab Profiler. This combination greatly accelerates the process of providing metabolic "hot-spot" information in a timely manner. Often, more detailed information is required on a potential metabolite, such as, site of attachment and complete structure elucidation. More advanced MS/MS techniques, such as data-dependent MSn analysis can get part of the way towards this goal. However, multi-dimensional NMR analysis is often required to propose complete structures. Traditionally this transition from LC/MS to NMR requires tremendous scale-up of the metabolite to provide milligram levels of material for analysis: prep-scale chromatography and elaborate purification schemes are the norm. With the advent of capillary NMR the scale requirements have been greatly reduced to low microgram levels, but the issue of rapid and efficient isolation still remains. Our approach for detailed elucidation utilizes: 1) Advanced MSn analysis; 2) An automated system capable of analytical scale separation, peak isolation, on-line sample enrichment and NMR-friendly sample preparation; and 3) A high-field NMR equipped with a capillary NMR probe for low-volume, low-level sample analysis. This combination greatly reduces the sample requirements for detailed NMR analysis and goes a long way towards approaching the scale typical of frontline LC/MS analysis.

**WP-9: Improved Sensitivity for the Quantitation of Resolved Epimers of Budesonide Extracted from Rat Plasma Utilizing an API 5000™ LC/MS/MS System**

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Budesonide (BUD) is a potent glucocorticosteroid with anti-inflammatory properties. Its PK characteristics lead to low plasma concentrations following inhalation of therapeutic doses. Requirements for the current rat toxicological investigation include an LLOQ similar to

that reported in the literature for human plasma (<0.5 ng/mL), but with limited sample volume (50 mL). Utilizing an API 5000™ LC/MS/MS system, this LLOQ could be achieved without derivatization. Previous methodology for 22R/22S-BUD in human plasma involved a protein precipitation, followed by SPE. Derivatization was also necessary to increase ionization efficiency by +APCI and to aid in the separation of 22R/22S epimers. Evaporation and reconstitution of the 22R/22S-BUD-21-acetate extract resulted in a tenfold concentration of the analytes. Detection limits of 0.25 ng/mL were achieved from 1.0 mL of plasma, equating to 50pg column. Alternative methods report LLOQs of 0.1 ng/mL, achieved without chromatographic resolution of the 22R/22S epimers. Co-elution of the epimers results in narrower peak widths thereby increasing s/n. However, evidence suggests that a larger volume of distribution and two to three times greater potency for the 22R-epimer. Therefore, in the current study, it was necessary to achieve epimeric resolution. Despite literature claims, ionization of 22R/22S-BUD-21-acetate epimers revealed no gains in sensitivity, and moderate gains in chromatographic resolution. Consequently, derivatization was unnecessary. Given the improved sensitivity of the API-5000™ system extraction could be performed by PPT as opposed to time consuming SPE. In previous validated methodology utilizing the API 4000™ system, an LLOQ of 0.5 ng/mL was achieved. The API 5000™ system furnished an LLOQ of 0.05 ng/mL (2 pg on-column) and pre-validation feasibility data suggests a quantifiable range from 0.05 – 50 ng/mL. This additional sensitivity can be utilized to reduce the plasma volume required for extraction, dilute extracts to minimize ion suppression, reduce injection volume to minimize carry-over and allow repeat analysis, or extract with lower recovery but higher throughput techniques.

**WP-10: An Improved Capillary LC-MS/MS and Its Application to High sensitivity and High Throughput Small Molecule Quantitation**

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High sensitivity quantitation of small molecule species present at very low concentrations is of vital clinical importance and requires a LC/MS system with maximal sensitivity. A low flow LC (nano- or capillary- LC) is often employed for such purposes where the sample volume available for analysis is limited. Common approaches for capillary LC-MS employing a regular interface for conventional flow rates suffer from impaired post-column band broadening and hence reduced separation efficiency and sensitivity. We describe here an improved capillary LC/MS/MS protocol for highly sensitive quantitation of small molecules with comparable throughput and robustness to conventional LC/MS/MS. A binary micro-flow LC system that delivers stable gradient flows at the 1-30 uL/min was coupled to a triple quadrupole mass spectrometer

equipped with a Turbo V ion source via a fully integrated stainless steel electrospray interface. The interface comprises of a large bore stainless steel electrode that is tapered as a micro-ionspray emitter. A monolithic PS-DVB column (200  $\mu\text{m}$  id x 50 mm) was used for steroids separation with gradient flow rates set at 3.0  $\mu\text{L}/\text{min}$ . A 30 micron fused-silica transfer line after the column was inserted directly to the end of the emitter. With the capillary LC/MS/MS system described here, the replicate analysis of most common clinically prescribed steroids with more than 30 MRM transitions indicated that this system provided fast quantitative profiling within 8 min and good reproducibility ( $n=10$ ) of retention time and peak intensity with RSD values of less than 0.5 and 9.0%, respectively. Sensitivity measurements were performed on dilution series of steroids spiked into plasma samples and provided 4 orders of magnitude of linear dynamic range and a detection limit of tens of fg on column. The integration of the precise gradient delivery system, monolithic capillary column and the novel micro-ionspray interface in a compact format minimized dead volumes as reflected by the narrow peak width (0.1 min, FWHM) and asymmetry (less than 1.2 at 10% peak height) calculations. Long-term endurance test of this system is being conducted and results will be presented.

**WP-11: Analysis of Amide Cyclized Peptides From Rat Brain Homogenate Using LC/MS**

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Quantitation of peptides in biological matrices, such as blood or tissue, remains technically challenging, mainly due to the difficulties in sample preparation and detection. The majority of methods reported for peptide quantitation in the literature use indirect measurements such as Western blotting, immunohistochemical analysis or radioimmunoassay. In recent years, there has been an increased interest in utilization of LC/MS techniques for peptide quantitation. In this work, a rapid and sensitive method for the determination of amide cyclized peptides in rat brain homogenate using LC/ESI/MS has been developed. In this study, the peptides were spiked into the brain homogenate. Extracting peptides from brain homogenates was difficult, because brain homogenate contains high amounts of lipids, endogenous peptides and proteins. Precipitation of proteins with acetonitrile resulted in low recovery of the analytes. Recovery of the peptides from brain homogenate reached 85-100% after adding 0.1 % TFA to the acetonitrile. Adding acid may have disrupted protein binding in the brain homogenate and improved the recovery. The detection limit of the peptides was evaluated on a ThermoFinnigan LCQ Deca ion trap mass spectrometer and a Waters Quattro Ultima triple quadrupole mass spectrometer. Since these peptides only generate minor fragment ions, the SIM scan mode was used instead the MRM mode on the triple quadrupole instrument. The detection limit of the peptide on the LCQ Deca was 10 fold lower than that of Ultima. A linear calibration curve from 0.5  $\mu\text{g}/\text{ml}$  – 20  $\mu\text{g}/\text{ml}$  was

achieved. The developed method was applied to study the stability of L- and D- peptides in rat brain homogenate. The results indicated that D- peptide was stable up to 6 hr of incubation at 37°C, but L- peptide was very unstable.

**WP-12: Using a Multimode Ion Source on an Accurate Mass Instrument to Improve Sample Throughput in Drug Discovery Applications**

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In analyzing lead molecules by LC/MS, the large numbers of samples precludes optimization of methodology for each compound. Since most molecules will never make it through the process, the goal is to get meaningful results on the first injection. This work describes an ion source capable of generating ions by both electrospray and atmospheric pressure chemical ionization simultaneously over a broad HPLC flow range. By combining this source with polarity switching modes, there is a high probability of getting ions indicative of molecular weight in one injection. Using a range of pharmaceutical products, the source was evaluated for use in compound confirmation, sample purification and unknown identification by accurate mass measurement using a time-of-flight mass spectrometer. Since both ionization processes are occurring simultaneously, it was evaluated for high throughput applications where short run times and narrow LC peaks are produced. Since the operating range of the source was up to 2 mL/min, it was combined with short small particle size columns that are operated in this flow range. Overall system performance was tested for the ability to get the maximum information in the minimum time.

**WP-13: A Novel High-Content Screening Technology for the Characterization of Disease Models, Drug and Side Effects: Case Study of Characterization of the Metabolic Syndrome**

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Metabolomics, i.e. the systematic detection and quantification of marker metabolites in a given compartment, cell, tissue or body fluid, is increasingly recognized as the most relevant approach to depicting the functional end-points of physiological or pathophysiological processes. Yet, a reliable and standardized technology platform rendering metabolomics applicable to routine diagnostics or quality-controlled pharmaceutical R & D at various stages of preclinical and clinical development has not been in place so far. Based on a long-standing experience in mass spectrometry-based neonatal screening for inborn disorders of metabolism and on a strong scientific background in analytical chemistry, clinical pharmacology and laboratory medicine, BIOCRATES life sciences has established an integrated metabolomics platform comprising proprietary automated sample preparation

procedures, high-resolution quantitative mass spectrometry and innovative bioinformatics tools for process control, data management, analysis and biochemical interpretation. For the present study focussing on the Metabolic Syndrome, hundreds of metabolites have been quantified via internal standards from different compartments. The results of the subsequent multivariate statistical analysis were linked to our proprietary pathway information system to elucidate the biochemical implications. According to this approach candidates for novel biomarkers for early diagnosis and detailed sub-typing of complex diseases were identified, thus demonstrating the huge potential of comprehensive quantitative metabolomics.

**WP-14: Managing ADME/Tox Laboratory Workflow with Thermo's LeadStream Orchestrator Software**

Rob Dunn-Dufault, Brian Daniels, Blair Leduc, Andreas Stelzer, Joe Senteno, HansJoerg Haas  
Thermo Electron Corporation, Burlington, ON, Canada

Thermo Electron has introduced a suite of products for ADME/Tox preclinical profiling laboratories, whose operations are managed and coordinated by Orchestrator; a new enterprise lab automation workflow software. Orchestrator makes use of contemporary Services Oriented Architecture, and provides the flexibility of creating complex lab solutions through the coordination of specialized automated sub-systems; Leadstream Reformatter(TM), LeadStream WorkCell(TM), and LeadStream LC/MS(TM). Within the Orchestrator Services Oriented Architecture, commercial off-the-shelf business software components are leveraged for the first time in an ADME/Tox screening environment. An integration engine, collaboration software, and information access software provide a stable environment for managing ADME/Tox activities. Through modern software standards, Orchestrator makes available the ability to interconnect with other systems, as well as the data and access security, needed in high-productivity drug discovery. In this poster we will describe the operation of a highly integrated ADME/Tox laboratory managed with LeadStream Orchestrator and will describe the key benefits of automated process and information management.

**WP-15: Evaluation of LeadStream's High Capacity Performance Characteristics in Multiple ADME/Tox Assays**

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A suite of high throughput assays have been implemented on the LeadStream ADME/Tox Solution, and have been shown to produce equivalent results to semi-automated methods that screen for drug-drug interactions, metabolic stability and artificial membrane permeability. In order to assess LeadStream's performance characteristics, the system was challenged with hundreds of compounds, multiple times, including replicates and standards. Compounds were selected to span a large diversity of

chemical structures, with purity above 90%, but with no advance knowledge of how they would behave in each of the assays. In parallel, the same compounds were analyzed with equivalent semi-automated methods. This poster reports performance characteristics of LeadStream, including throughput, turn-around time, as well as measures of technical robustness, accuracy, precision and ease-of-use.

**WP-16: Serotonin Measurement in Platelets – A Report on Analytical Methodology and Influence of PK/PD in Study Design**

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An analytical method utilizing LC-MS/MS has been developed for the measurement of serotonin/5HT in platelets. The method consists of a simple protein precipitation sample extraction procedure and a reverse phase gradient chromatography with mass spectrometric detection. A stable labeled internal standard is used to compensate for ionization suppression/matrix effect. The method has a dynamic range of 5-5000 ng/mL and acceptable accuracy and precision as reflected by the relative error and relative standard deviation  $\leq \pm 15\%$  respectively. This method is more selective and sensitive when compared to the currently reported methods that use fluorescence or electrochemical detection. Serotonin levels have been measured in rat and dog platelet rich plasma and in human blood using this analytical method. Previous studies have shown that peripheral measurements of serotonin reuptake inhibition to be indicative of central serotonin reuptake inhibition<sup>1</sup>. Studies using fluoxetine, a known selective serotonin reuptake inhibitor, in rats show serotonin modulation in platelets is dose dependent and frequency dependent. Use of PK/PD modeling suggests that the exposure above the EC<sub>50</sub> of the drug is essential to measure modulation in serotonin reuptake. Experimental data also suggest that this should be considered in conjunction with the rate of platelet turnover in vivo to obtain meaningful data. The impact of analytical technology in combination with PK/PD will be presented in this poster with emphasis on how modeling can aid study design and minimize/ guide experimental work.

Narayan M et al. (1998) Serotonin transporter-blocking properties of nefazodone assessed by measurement of platelet serotonin. *J Clin Psychopharmacol.* 18(1):67-71.

**WP-17: Global Metabolite Profiling of NSAID Effects and Novel Metabolite Discovery**

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Recent technological advancements of MS instruments, such as that used in this study (Applied Biosystems/MDS

Sciex QSTAR XL system) enable the acquisition of high resolution and high mass accuracy MS data suitable for novel metabolite discovery. By perturbing cell systems with drugs or environmental disturbances, opportunity for such novel metabolite discovery is presented; in the current study, we applied four NSAID drugs to HT29 human colon cancer cell lines, and then analyzed global metabolome changes in the search for novel metabolites. The measurement of global changes represents the opportunity to quantify the phenotypic consequences of perturbations, and metabolites found to correlate with phenotypic changes can be utilized as biomarkers for applications including disease diagnosis, treatment efficacy tracking, and drug dose finding. The combination of the Applied Biosystems QSTAR system and Phenomenome Profiler data analysis software, allowing the identification of significant metabolite biomarkers of phenotypic events is discussed.

**WP-18: Drug Metabolite Discovery in Human Colon Cancer Cell Lines**

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Human cancer cell lines are indispensable tools for many aspects of basic cancer research ranging from purely investigational studies to drug efficacy and toxicity studies. Traditionally, drug metabolite discovery work is done using GC/MS and LC/MS instrumentation. Currently available instrument-based software is limited in its utility for discovering and tracking drug metabolites across a biological sample set, especially across a range of chromatographic retention times; Phenomenome Profiler™ software has been developed to systematically address this challenge. We have undertaken to understand the effects of low and high doses of four different commercially available NSAID drugs in the HT29 human colon cancer cell line, both from the perspective of endogenous metabolism effects, and simultaneously measuring drug metabolites. Data has been generated using an Applied Biosystems/MDS Sciex 4000 QTRAP system. The simple to use software tools combined with the ability to read data from any mass spectrometry platform should appeal to a large audience base, encompassing researchers in the fields of metabolomics as well as proteomics.

**WP-19: Off-line Combination of the Multi-dimensional HPLC and the MALDI-TOF-MS for Proteomic Analysis Using a High-Throughput Spotting System**

Steve Wishnies Shimadzu Biotech, USA

MALDI MS is a powerful tool in imaging molecules from relatively small populations of cells in specific regions of heterogeneous tissue architectures. To generate MALDI imaging matrix solution is deposited onto tissue sections in discrete digital micro-droplets using a piezoelectric delivery device (using a Chemical Printer) and each droplet is analyzed. The spectra from

specific tissue map locations are then compiled into image analysis software which can then be linked to database tools to compare between sample types and identify the statistical significance between tissue types and further probe for biomarkers of disease. In this pilot study, human oral tumor samples were used as model system to develop methodology for MALDI tissue imaging using a chemical printer to print matrix solutions at specific cell locations. Conventional light based microscopy was also employed to check printing.