

Land O'Lakes Bioanalytical Conference

Posters 101-111 presented in 2006

Posters 112-126 presented in 2007

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The Impact of Analytical Column Particle Size on a Turbulent Flow Chromatography Method for Cortisone and Cortisol.

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Objective: Recent advances in silica particle technology have permitted the development of stable particles of less than 2 μ in diameter. As the particle diameter decreases the active surface per gram of packing material increases, resulting in increased separation power. Recent advances in both pump and column technology have expanded pressure range of pumps to 600 bar. We compare a direct injection method for the analysis of cortisol and cortisone in raw urine. **Methods:** The system is a Cohesive Technologies TLX1 in focus mode, utilizing a Turboflow column followed by an analytical column. For the comparison the original analytical column, a Supelco Sil 4x50mm, 3 μ , was replaced by a new Agilent HR XDB C18 3x100mm, 1.8 μ . **Results:** The original method, while providing a substantial amount of separation, cannot fully resolve all the interferences in the MS transition monitored for cortisol. The test column ran under the same chromatographic conditions, with the exception of a higher flow rate and operational pressure. It successfully resolved the interferences and the compounds of interest in the same time span. Further development to optimize the method has produced a very efficient and robust method. Within the context of an in-line system, upgrading the analytical column to a high resolution 1.8 μ column was very easy, requiring only minor method optimization. **Implications:** With no changes to the turbulent flow aspects of the method, the method gained significantly in robustness. The TurboFlow chromatography brings real samples and sample sizes to Ultra chromatography columns.

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Quantitative Determination of Imatinib and N-Desmethyl Imatinib in Human Plasma: an Application of High-Performance Liquid Chromatography / Electrospray Ionization (ESI) Tandem Mass Spectrometry.

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Objective: The objective was to develop an LC/MS/MS method with ESI interface for the determination of Imatinib and its metabolite, N-Desmethyl Imatinib, in human plasma. Imatinib is a signal transduction inhibitor for the treatment of chronic myeloid leukemia. **Methods:** A solid phase extraction (SPE) method using a Waters MCX plate was developed utilizing 0.05 mL of plasma and an internal standard working solution

containing Imatinib-d₈ and N-Desmethyl Imatinib-d₈ (90 ng/mL). A solution containing 5% Ammonium Hydroxide in Methanol was used as the eluent. HPLC separation was carried out on a Waters Exterra RP18 4.6 x 50 mm, 3.5 µm LC-MS column at a flow rate of 0.5 mL/min of 90:10 Methanol:10mM Ammonium Acetate in H₂O. Tandem mass spectrometric detection was performed using a Micromass Quattro LC. **Results:** A between batch test was performed with nine levels of calibration standards (50, 100, 250, 500, 750, 1000, 1500, 2300, 2500 ng/mL) and four levels of quality control samples (150, 1250, 2000, 600 ng/mL). The between batch precision and accuracy of the standards were 1.68-5.47% and 96.6-110.5% respectively for Imatinib and 2.82-6.25% and 95.0-112.7% respectively for N-Desmethyl Imatinib. The precision and accuracy of the quality controls were 2.81-5.29% and 99.5-103.0% respectively for Imatinib and 3.53-4.66% and 100.3-103.4% respectively for N-Desmethyl Imatinib. In addition, a matrix blank test was performed with six lots of plasma to demonstrate selectivity and a blank matrix. **Implications:** A sensitive and accurate method was developed for the simultaneous determination of Imatinib and N-Desmethyl Imatinib in human plasma.

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Evaluation of Waters® Acquity UPLC™ for Use in Metabolite Profiling using Radiochemical Detection

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Objectives: Metabolite profiling involves complex chromatographic separations of radioactive components in biological matrices. Acceptable separations may require long run times on a conventional HPLC, and generate large quantities of radioactive mixed waste. A Waters® Acquity UPLC™ was evaluated for use in metabolite profiling in an attempt to reduce analytical run times, cost of analysis, and solvent waste. **Methods:** A conventional HPLC method for radiochemical metabolite profiling was scaled down for UPLC™. Urine and bile samples were analyzed by UPLC™ with on- or off-line radiochemical detection. The resulting radiochromatograms were compared to profiles generated by conventional HPLC with on-line radiochemical detection. Waste generation and the use of consumables were tracked. **Results:** A complex 130 minute HPLC method was reduced to 35 minutes on the UPLC™. UPLC™ radiochromatograms compared favourably with those generated by HPLC when adequate radioactivity was profiled. On-line radiochemical detection using UPLC™ was less sensitive than conventional HPLC. Off-line profiling was sensitive, but required the use of expensive solid scintillation plates and extra analysis time for scintillation counting. Waste generation was reduced 9-fold (on-line) and limited to solid scintillation plates and injector rinse (off-line). **Implications:** UPLC™ significantly reduced run time, solvent use and waste compared to HPLC. A loss of sensitivity occurs with on-line radiochemical detection compared to conventional HPLC. Off-line detection is sensitive but requires longer analysis times and is more costly. Radiochemical metabolite profiling using the UPLC™ is currently best suited for high activity samples.

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Development of LC-APPI-MS/MS for Clinical Analysis of Steroids

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Introduction: As part of the ongoing development of MS based tools for clinical analysis, we have evaluated and utilized the latest APPI interfaces for the AB Sciex 4000 and 5000 MS systems, together with the Cohesive ARIA TX4 platform. **Methods:** Optimization of APPI sensitivity and comparison to APCI was performed using API4000 and API5000 systems. Multiple 96-well mobile phase module plates were analyzed in FIA mode to establish propensity of well content for ionization efficiency. Both pseudomolecular protonated ion and radical cation formation were evaluated with RP and NP HPLC solvents. A total of 24 HPLC columns were screened using an automated column selector indexed to the ARIA TX4-API4000 system. Selection was based upon k' and peak asymmetry. The final APPI methodology for estrogens was validated and compared against a number of established in-house LC-MS/MS assays, together with electron capture APCI. **Abstract:** A multi-parametric approach was employed to empirically probe potential solvent and dopant conditions to enhance positive ion formation using APPI. This paper describes comparison of APCI and APPI interfaces for steroid structures relating to 4 major classes of functionality and structural similarity. Bioanalytical details regarding assay linearity, interface ruggedness, reproducibility and matrix effects will be further discussed for the estrogen APPI methodologies. Correlation to RIA results and benefits of LC-APPI-MS/MS techniques will be described.

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Quantitative Determination of Letrozole in Human Plasma: an Application of High-Performance Liquid Chromatography / Atmospheric Pressure Photoionization (APPI) Tandem Mass Spectrometric Analyses.

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Objectives: To develop an LC/MS/MS method with APPI interface for the determination of Letrozole, a non-steroidal aromatase inhibitor, in human plasma. **Methods:** Solid phase extraction (SPE) was applied to 0.1 mL of plasma sample and internal standard Letrozole- d_4 using acetonitrile as eluent. HPLC separation was carried out on a Zorbax SB-CN, LC-MS column, (2.1 x 50mm, 5 micron) at a flow rate of 0.2 mL/min with 2.5-minute gradient starting at acetonitrile/water 40:60(v/v). Mass spectrometry detection was carried out with a Sciex API 3000 LC-MS-MS system equipped with an APPI interface. Toluene was introduced into the ionization chamber with a syringe pump at 20 μ L/min. Mass spectra were acquired in positive ion mode with selective reaction monitoring. **Results:** Preliminary detection of Letrozole in human plasma after SPE with electrospray showed low signal response. Switching to the APPI interface where toluene ionizes under irradiation of UV and further assists ionization of analyte either by charge and/or proton transfer, the signal response increased by ≥ 10 fold. Eight levels of

calibration standards (0.5-50ng/mL) and four levels of quality control samples (1.2, 15, 25, 40 ng/mL) were prepared. Two doublet standard curves, each with six replicates of the four quality control levels, were run for the method development. The between-batch precision and accuracy of the standards and quality controls were 0.95-6.89% and 97.8-101.6%, and 2.10-4.43% and 94.5-100.6%, respectively. **Implications:** The LC/MS/MS equipped with APPI provides an enhanced signal response for the analysis of Letrozole at low concentrations in human plasma compared with electrospray.

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Matrix Removal and Matrix Effect Elimination by HTLC/MS/MS.

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Objective: Phospholipids present in biological samples, are a component of matrix interference in LC MS/MS analysis. These interferences affect analyte ionization resulting in loss of reproducibility and precision during quantitative analysis. Turbulent Flow Chromatography (TFC) can be utilized to minimize phospholipids in a similar manner. Herein we investigate the extent of matrix removal by TFC and discuss conditions for minimizing phospholipids. **Methods:** Phospholipid MRM transitions were monitored with an API4000 (Sciex) and a TLX-2 (Cohesive Technologies). Samples of whole and acetonitrile precipitated rat plasma and acetonitrile blank were analyzed and compared. Elution and peak intensity changes were monitored. **Results:** TFC columns eliminated the vast majority of phospholipids in samples. A small amount is retained on the column and transferred to the analytical column. Higher loading pH appears to further reduce the retained phospholipid fraction. The TFC column, washed with a mixture of isopropanol, acetonitrile, and acetone between samples, shows complete removal of phospholipids prior to the next sample. Loading velocities (2, 4 and 6 ml/minute) appear to have no effect on phospholipid abundance, but do favor the elimination of higher molecular weight compounds. Injections of acetonitrile precipitated serum show significant baseline suppression when bypassing the TFC column while similar injections of acetonitrile blank show no suppression. Conversely, injections of raw serum and acetonitrile blanks show no suppression when injected through a TFC column. **Implications:** The TFC column effectively removes the vast majority of compounds responsible for matrix effect in serum samples.

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Evaluation of the Waters Acquity UPLC System for the Analysis of 1-OH Midazolam in *In Vitro* Inhibition Studies

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Objectives: To evaluate the Waters Acquity UPLC system for the evaluation of 1-hydroxymidazolam from *in vitro* inhibition incubation samples. A current validated

method allows chromatographic resolution of the isobaric 1-hydroxy, and 4-hydroxy metabolites of midazolam, but has a cycle time of approximately 6.5 minutes. The robustness and reproducibility of the Acquity system was evaluated to determine time and cost savings that could be made by this technology. **Methods:** The existing methodology was modified by 'direct transfer' onto the Acquity system using a BEH C18 (50 x 2 mm; 1.7 μ m) column. The method was then optimised to minimise retention times but maintain resolution. The reproducibility and robustness of the method was then investigated by the analysis of 300 samples. **Results:** 300 samples were analysed in less than 12 hours compared to nearly 36 hours by the existing method. Chromatographic retention time and peak shape were maintained throughout the batch with resolution of the hydroxy metabolites preserved. The precision of peak parameters from replicate injections throughout the analytical batch were found to be less than 9.00 % at all concentrations. **Implications:** The use of the Acquity system could significantly increase sample throughput and reduce costs for future analytical work.

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Evaluation of a Multiplexed Enhanced Pressure Pumping System for Clinical Diagnostics

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Introduction: This paper describes the successful implementation of steroid hormone assays utilizing ARIA-TranscendTM multiplexed LC systems capable of staggered parallel LC and 2D LC operation, to enable high throughput (>1000 samples per day per MS/MS system) quantitative hormone analysis. **Methods:** An on-line methodology incorporating 2D LC-LC-MS/MS was used to improve assay performance. Final optimization was performed using enhanced pressure compatible pumps in a 2D LC-LC-MS/MS mode for separation of isobaric interferences and optimal assay cycle time of 3 minutes per sample (>1800 samples/day/MS/MS system). **Preliminary results:** A rational development program utilizing 1D LC, 2D LC and 2D LC with enhanced pressure enabled improvement of bioanalytical assay performance in resolution. In neat solutions of comparative concentrations, the 1D LC assay provided excellent separation (selectivity's >1.2 between isobars), however, when analyzing extracted patient samples, loss of chromatographic fidelity required further optimization. Optimization methodology overcame some of the resolution constraints observed in 1D mode, however, did not facilitate a final run-time of 5 minutes per channel (1000 samples per day). Thus, a rational LC-LC method was developed and validated. Further optimization of key parameters such as chromato-focussing, columns selectivity (i.e. affecting analytical selectivity using multiple analytical columns in series for separation) and gradient time enabled an assay that fitted our multiplexing criteria.

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A Sensitive LC-MS/MS Method for the Quantitation of 4-Aminobiphenyl Hemoglobin Adduct in Human Erythrocytes.

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Objectives: The objective of this project was to develop and validate a bioanalytical method for the quantitation of 4-aminobiphenyl hemoglobin adducts in human erythrocytes. The validation data should demonstrate the assay is selective, precise, accurate, rugged and reproducible. **Methods:** Erythrocytes were collected and washed from whole blood samples. After overnight hydrolysis with sodium hydroxide, samples were extracted by a liquid-liquid procedure. The extracts were evaporated to dryness and reconstituted with an acidic polar organic solvent mixture. The chromatographic conditions utilized a Hypersil BDS C18 precolumn and a Varian Pursuit Diphenyl analytical column. A linear gradient mobile phase delivery system was used. The AB/MDS Sciex API 5000, using an ESI interface, was employed. Positive ions were monitored in the multiple-reaction monitoring mode. **Results:** The method was linear (weighted $1/x^2$) over a concentration range of 1.00 to 100 pg/mL. Interday precision (C.V.%) and accuracy (R.E.%) of middle and high levels of quality controls were less than or equal to 6.0% and 7.0%, respectively. Interday precision (C.V.%) of the incurred quality controls was less than or equal to 8.3%. Accurate recovery from 9 out of 10 human erythrocyte lots spiked at the concentration of the LLOQ and 10 out of 10 spiked at the concentration of the high QC demonstrated sensitivity and lack of significant matrix effect. Stabilities established were short-term stability, freeze-thaw stability, processed sample integrity, and post-preparative stability. **Implications:** The assay for the analysis of 4-aminobiphenyl hemoglobin adducts in human erythrocytes has met the criteria established for a validated bioanalytical method.

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Evaluation of Non-Specific Binding in Several Different Matrices: A Case Study.

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Objectives: Non-Specific Binding (NSB) is an issue that is most often seen in association with bioassays, but can be just as problematic in small molecule quantitation. NSB can cause nonlinear standard regressions, inability to achieve a low limit of quantitation, poor accuracy and precision of quantitation, as well as many other problems. We present a case study in which NSB of a proprietary compound was evaluated by LC/MS/MS following extraction from 3 different matrices where we have encountered this problem in the past. Assessment of any sample effects was necessary to ensure that the correct sample pretreatment was formulated ahead of collection during an upcoming clinical study. **Methods:** NSB was measured by comparing a fresh analytical solution to one that had undergone several transfer steps in polypropylene tubes. Analyte concentrations were calculated after extraction and LC/MS/MS analysis using validated bioanalytical methods. **Results:** Although we anticipated NSB to polypropylene from human cerebrospinal fluid no such problems were seen. Compound A showed marked NSB to polypropylene from urine, but this was prevented with the addition of 0.2, 2.0% Formic acid or 15 mM CHAPS. A similar degree of NSB to polypropylene was observed

from Dulbecco's PBS (plasma dialysate surrogate). Pretreatment with 15 and 30 mM CHAPS equally prevented the NSB. Furthermore, addition of CHAPS (15 mM final concentration) was sufficient to recover most of the lost material when NSB had been observed in samples. **Implications:** This preventative step allowed the sponsor to collect study samples with appropriate modifiers present to preserve the ability to accurately quantitate analyte concentration.

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Strategies for Overcoming Dosing Vehicle Effects Observed During LC/MS/MS Bioanalysis of Incurred Study Samples.

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Objectives: Study the effect of dosing vehicle observed during sample analysis via LC/MS/MS for a Pfizer preclinical toxicokinetic (TK) study and discuss the impact on calculated TK parameters. **Methods:** An LC/MS/MS assay was developed in monkey serum for the purpose of measuring Pfizer drug (P) concentrations in a preclinical study. Serum samples were extracted via protein precipitation with acetonitrile containing internal standard (IS, a structural analog of compound P). Erratic response of the IS signal was observed only in the incurred study samples, suggesting a dosing vehicle effect. This prompted modification of the analytical assay to eliminate the effect. Strategies to correct the analytical method include alternative chromatographic programs, alternative extraction schemes and LC/MS/MS parameter optimization. Additionally, vehicle-containing matrix, spiked as a QC sample, was utilized to assess accuracy and to mimic the incurred study samples. **Results:** In the case study presented, elimination of the vehicle effect was achieved via analytical method modifications. TK parameters calculated pre- and post-assay modifications show an approximately 2-fold increase in the C_{max} and AUC. **Implications:** Bioanalytical methods applied to study samples should always be monitored for dosing vehicle effect. If present and uncorrected for, the effect can introduce significant error into the calculated TK parameters, which in-turn could lead to erroneous conclusions about the TK data.

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Accurate Mass Measurements of Fragment Ions on a Triple Quadrupole in Selected Reaction Monitoring Mode

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Objectives: Accurate mass measurements are used to determine the elemental composition and formulae of molecules to confirm their identity or to assist in their characterization. In particular, exact mass measurement of fragment ions are commonly a useful tool for fragmentation pathway elucidation. The herein work reports an original

method for accurate mass measurements of fragment ions by means of triple quadrupole mass spectrometer. **Method:** Accurate mass determinations were carried out on an enhanced mass-resolution mass spectrometer. The accurate mass measurements were carried out on a chromatographic time scale by injecting 5 μL of the parent compound working solution on a Waters C₁₈ Sunfire (20 x 2.1 mm, 5 μm) column. Exact mass determinations were carried out by electrospray ionization (ESI) in selected reaction monitoring. The mass spectrometer was operating in enhanced mass-resolution mode (peak width of 0.1 Th FWHM). **Results:** Accurate mass determinations in ESI-MS/MS provided information on urapidil fragment ions formed through collisionally-induced dissociation allowing for the determination of the most probable fragment ion structures. The differences between the accurate mass measurements and exact masses calculated for the proposed fragment ions were ranging from -1.24 and 1.05 ppm. **Implications:** The ability of the enhanced mass-resolution triple quadrupole mass spectrometer to provide accurate mass measurements of fragment ions, to a level able to assist in structural elucidation, will make this technique very useful in areas such as metabolite identification.

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Ultra Low Level Endogenous Peptide Quantitation using Two-Dimensional Reverse-Phase / Normal-Phase LC-LC-MS/MS

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Introduction: An ultra-sensitive assay was developed for the endogenous decapeptide, Gonadotropin Releasing Hormone (GnRH). Circulating levels of GnRH are typically too low to measure accurately via radioimmunoassay and quantitation of peptides via traditional LC-MS/MS can be challenging. The high polarity of the GnRH molecule makes plasma extraction difficult, necessitating the use of non-conventional HPLC. Plasma extraction with organic solvents results in poor recovery. SPE results in low yield and exacerbates chromatography issues. Utilizing buffered solvents with reverse-phase chromatography enhanced selectivity by eliminating the presence of matrix interferences. Normal-phase (non-traditional) chromatography in the second-dimension led to phenomenal sensitivity gains and even greater selectivity. **Methods:** GnRH was measured by mass spectrometric detection after protein precipitation and 2-D multiple-phase liquid chromatography. A Cohesive-Thermo Electron ARIA TX-4 HPLC System was used for the two-dimensional liquid chromatography. Analyte detection was performed on an AB/Sciex API5000 triple-quadrupole mass spectrometer, operating in positive ion electrospray (ESI) mode. **Preliminary results:** The quantitative method for the determination of GnRH in human plasma using reverse-phase / non-traditional normal-phase 2D-LC-MS/MS detection was validated in compliance with CLIA and FDA guidelines. Experimental method development and validation data will be shown. Cross-validation of samples analyzed by radioimmunoassay (GnRH RIA) against the 2D-LC-MS/MS method resulted in a mean bias of 1.08%. A scatter plot of the cross-validation data gave a slope of 0.9736 and a correlation coefficient of 0.9829.

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Simultaneous Determination of Scopolamine and Dextroamphetamine in Human Plasma by UPLC/MS/MS

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Objectives: Scopolamine and dextroamphetamine are often co-administered for the therapeutic management of motion sickness in space flight. We sought to develop and validate an UPLC/MS/MS method to simultaneously determine scopolamine and dextroamphetamine in human plasma for assessment of their bioavailability. **Methods:** A Waters ACQUITY ultra-performance liquid chromatography (UPLC) system and Quattro MicroTM API mass spectrometer were used. The UPLC method involved eluting the sample on a 1.7- μ m 2.1 \times 50 mm Waters C18 column using acetonitrile:methanol:10 mM ammonium acetate 625:375:150, v/v/v, at a flow rate of 0.2 ml min⁻¹ as the mobile phase. Positive ions were monitored in the MRM mode for the determination of scopolamine m/z = 304.2 \rightarrow 138.1, dextroamphetamine m/z = 136.2 \rightarrow 91.0 and internal standard hyoscyamine m/z = 290.2 \rightarrow 124.1. **Results:** The method had a linearity range of 20–2000 pg ml⁻¹ for scopolamine and 100–10000 pg ml⁻¹ for dextroamphetamine, and correlation coefficients for the standard curves of > 0.99. The intra- and inter-day precision, as percent relative standard deviation (RSD), was < 8.5 %. **Implications:** This novel UPLC/MS/MS method is suitable for the simultaneous determination of scopolamine and dextroamphetamine in human plasma. The assay is sensitive, efficient, and reproducible.

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Challenges of Modifying an Existing Quantitative Assay: Adding a New Metabolite

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A method for a 5-hydroxytryptamine 1B receptor subtype selective antagonist being developed for treatment of patients with Major Depressive Disorder (MDD) was developed and validated. Subsequently, a method for its 2-OH metabolite was required. A stable label internal standard for metabolite was not available at the time of method development. An LC/MS/MS method was developed to combine both compounds into a single assay. During development of this assay, numerous challenges occurred, including impurity of metabolite reference material, matrix effects, sensitivity, non-specific binding and carryover. Due to the interdependency of the challenges faced, correcting one could not be done without consideration of the others. Development methods varied in order to overcome the observed challenges.

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Investigation of Conditions Necessary to Stabilize a Polypeptide Therapeutic in Plasma to Support Early Drug Development and Analysis by LC-MS/MS

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Objective: Stabilize the protein therapeutic MJK-007, which degrades as much as 90% in untreated plasma by proteolytic enzymes, in rat, dog, pig, rabbit and human plasma to ensure accurate analysis of samples, stored frozen for at least one month. **Method:** Blank plasma from the 5 species were treated with each of the following stabilizing reagents: 1% protease inhibitor cocktail, 1% protease inhibitor solution in 0.5 % trichloroacetic acid (TCA), 20 mM diisopropyl fluorophosphate (DFP) and 20 mM DFP in 0.5% TCA. Quality Control samples were prepared at 30 ng/ml and 375 ng/ml of MJK-007 in each of the 20 species-treatment combinations (n= 40 stability indicating QC samples). The QC sample were frozen at -70°C until analysis at a 1-week, 2-week and 4-week intervals. MJK-007 and internal standard were extracted from a 50 µL plasma sample by solid phase extraction (SPE) and analyzed using LC-MS/MS. **Results:** The overall accuracy % ± CV% of QC samples by treatment over the 4-weeks (n=120) was 113 ± 13.4 for 1% protease inhibitor cocktail, 114 ±12.5 for 1% protease inhibitor cocktail + 0.5% TCA, 106± 9.4 for 20 mM DFP and 110 ± 10.6 for 20 mM DFP + 0.5% TCA. **Implications:** Based on overall accuracy and precision of the QC samples tested, the 20 mM DFP was the optimal treatment to stabilized MJK-007 for at least 1-month storage at -70°C in all of the species evaluated. The 20mM DFP treatment has been used to support early drug development studies performed at Covance.

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Quantitative Analysis of Haloperidol in Biological Samples Using Chip-based NanoMate with Mass Spectrometer

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Objectives: To develop a fast, sensitive and automated chip-based NanoMate bioanalytical method for the determination of haloperidol in dog plasma. **Methods:** This method utilized a chip-based technology, Advion Biosystem's Triversa NanoMate to quantitatively analyze haloperidol in biological matrix. Twenty microliters of dog plasma fortified with haloperidol were extracted with two automated SPE methods. Strata-X polymeric SPE plate and OMIX SPE tips for Tomtec were evaluated for through-put and extraction efficiency. Sample analysis was performed on an Applied Biosystems API 4000 triple quadrupole mass spectrometer operating in the positive ESI mode. The Strata-X automated SPE method and the OMIX sample aliquoting procedure were processed utilizing a Tecan Genesis Freedom 200 liquid handling system. The OMIX SPE sample extraction procedure was performed using a Tomtec Quadra 3NS. A stable labeled isotope internal standard was used for the quantitation. **Results:** For both extraction procedures, the results showed that the calibration range was linear from 1 – 2000 ng/mL. A 3-curve intra-assay experiment was used to evaluate the method

precision and accuracy. The results showed that the precision and accuracy met Good Laboratory Practice (GLP) criteria. The method was specific at the 1.00 ng/mL LLQ. There was no carry-over or contamination observed. **Implication:** An automated and sensitive chip-based method was developed to analyze haloperidol in biological matrix. Compared with the traditional bioanalytical methodology based on liquid chromatography, this chip-based method demonstrated significantly reduced carry-over, decreased sample consumption, increased calibration curve range and decreased run times.

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HPLC-LTQ-Orbitrap MS Applied to the Quantitation of Small Molecules in Biological Samples

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Objectives: The purpose of this study was to evaluate the utility of high resolution accurate mass MS for the quantification of small molecules in biological samples. Selective extraction of full scan accurate mass data using very small mass ranges (e.g. 5-10 mDa), based on the exact masses of the protonated analytes, offers an alternative approach to SRM quantification. The analytical performance on an LTQ-Orbitrap was compared with triple quadrupole SRM quantitation in the analysis of small molecules for pharmacokinetic profiling. **Methods:** Two compounds A & B, which differ in molecular weight by 20mDa, were dosed to rats subcutaneously. The plasma samples were processed by simple protein precipitation with acetonitrile. LC/MS analysis was performed on a Thermo U-HPLC coupled to LTQ-Orbitrap. The same samples were also analyzed with a Sciex API-4000 MS using the SRM approach. **Results:** Similar precision and accuracy was observed at each QC concentration for both compounds when the analysis was performed either by the LTQ-Orbitrap or a Sciex API-4000. 90% of unknown concentrations reported from LTQ-Orbitrap analysis differed less than 15% from those reported by API4000. The other 10% agreed within $\pm 22\%$. **Conclusion:** In conclusion, there was no need to optimize SRM parameters when using a high resolution accurate mass instrument for quantitative work. Useful information on metabolites and endogenous components are generated simultaneously. The full scan MS/MS capability of the instrument could also be investigated in the future to address more complicated quantitative applications.

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An Automated OMIX Tips Extraction for the Analysis of Compound A in Dog Serum Supporting GLP Toxicokinetic Studies.

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Objectives: Develop a fully automated GLP compliant extraction method using OMIX tips and the Tomtec Quadra 96. **Methods:** Dog serum (25 uL) was extracted using a

Tomtec Quadra 96 fitted with MP1 OMIX tips. The OMIX tips were conditioned with methanol and water, respectively followed by multiple sample loading cycles. The OMIX tips were then washed twice, followed by two basic elutions of acetonitrile:ammonium hydroxide (95:5). The extract was then evaporated under N₂ at 40°C and reconstituted in 0.1 mL of acetonitrile:water (1:1 by volume). Samples were injected (10uL) into an API4000 tandem mass spectrometer for detection in MRM mode. An isocratic HPLC separation (1 mL/min) was carried out on a ThermoElectron Hypersil GOLD PFP, (50 x 2.1 mm ID) using water and acetonitrile acidified with 0.1% acetic acid containing 5 mM ammonium acetate. The run time was 2.5 minutes per sample. The analyte was quantified by measuring peak area ratios. **Results:** The inter-day precision (%CV) and accuracy (%Bias) of the validated MP1 OMIX tips extraction were 2.9 to 9.1 and -2.9 to -1.8, respectively. To date, this walk-away extraction method has been used to analyze samples from two GLP toxicokinetic. **Implications:** The overall efficiency of the extraction is maximized by significantly reducing the manual labor aspects (vacuum box, changing solvents, etc.) of routine sample analysis. Further efficiency gains can be achieved by incorporating robotic liquid handling systems for aliquoting and sample manipulation.

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Study of the Impact of Drug-to-Drug Interaction on Quantitative Analysis of Combination Drugs in Human Plasma Using LC-MS/MS

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Objectives: There is a trend in the pharmaceutical industry for companies to market combination drugs. The purpose of the work presented here was to study a drug-to-drug interaction, and develop a strategy to overcome any impact on the quantitation of these combination drugs in human plasma. **Methods:** Drugs A and B in human plasma were determined by Methods A and B, respectively. QC samples (Test) (containing both drugs A and B) were extracted and detected using a Sciex API 3000. The concentrations of drugs A and B were compared with the respective QC samples (Control) (containing only drug A or B). **Results:** Concentrations of drug A in Test samples were found to be about 50% lower than those in the respective Control samples. As drug B was found to elute within the retention time window of drug A and its ISTD, the composition of mobile phase was modified to resolve drugs A and B. Under these conditions, the results showed that the plasma concentrations of drug A in the presence of drug B were comparable to those with drug A alone. Therefore, the impact of drug B on drug A was eliminated by resolving these analytes chromatographically. The impact from the metabolites of drugs A and B was also investigated and successfully resolved. **Implications:** For the combination drugs, it is necessary to assess the impact of one drug on the other if these drugs co-elute chromatographically. Hence the determination of these drugs in clinical samples is not compromised.

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An Approach for Absolute Quantification of Therapeutic Proteins in Plasma Using 2D-SPE coupled with LC-MS/MS.

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Objectives: In this presentation, an LC-MS/MS method for quantifying plasma levels of recombinant human growth hormone (rhGH) and a therapeutic monoclonal antibody is discussed. **Methods:** Plasma samples were spiked with bovine fetuin as a protein internal standard, then digested with trypsin. The digests were cleaned and concentrated using 2-dimensional (cation exchange and reverse phase) solid phase extraction. The samples were then separated using a 5-minute gradient on a reverse phase column, and analyzed by LC-MS/MS. **Results:** The results from the LC-MS/MS analysis showed that sensitivity was improved about tenfold with the use of 2D-SPE compared with 1D-SPE. The introduction of a protein as an internal standard improved the accuracy of the sample analysis. Using the procedure described above, concentrations of 0.5 µg/mL of rhGH in plasma were detected on LC-MS/MS with S/N > 3. The application of this LC-MS/MS approach to quantifying a therapeutic monoclonal antibody will be discussed and presented. As part of the evaluation, the method will be applied to an animal study and the results compared with those obtained using ELISA. **Implications:** This simple approach allows quick method development, and has shown good sensitivity and accuracy. The use of a protein internal standard allows the monitoring of the entire process, including the digestion step. It is applicable to early development projects where labeled internal standards are not available. It can provide an effective method for evaluation of candidate drug proteins without the time consuming process of developing an ELISA assays for multiple compounds.

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Trace Quantity Determination of an EP4 Receptor Agonist Prodrug and Active Metabolite in Human Plasma by HPLC-MS/MS.

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Objectives: Develop and validate a highly sensitive HPLC-MS/MS method to support low clinical ocular doses of an ester prodrug of the potent, selective EP4 agonist (acid metabolite) developed for lowering of intraocular pressure associated with glaucoma. **Methods:** Plasma samples were treated with dichlorvos and phosphoric acid to inhibit esterase activity, preventing in vitro conversion of prodrug to the acid metabolite. The calibration range for both compounds was 20-200 pg/mL. The 20 pg/mL lower limit of quantitation and the analogue internal standard employed for the metabolite method required highly selective, sensitive assays. Sample preparation techniques for the ester and metabolite utilized semi-automated liquid-liquid extraction of 250-µL of pH-adjusted plasma. Reverse phase chromatography was employed for the prodrug and HILIC

chromatography for the metabolite. An organic flush step after the elution of the ester prodrug provided adequate selectivity from endogenous late-eluting peaks. Tandem mass spectrometry with electrospray ionization using multiple reaction monitoring was utilized for detection of both compounds. The ester prodrug and stable-labeled internal standard ions were detected in positive ionization mode while the metabolite and analogue internal standard ions were detected in negative mode. **Results:** Recovery was approximately 100% for both compounds. In both methods precision (CV%) was less than 6.6% and accuracy was within 4.5% of nominal in five different lots of plasma. **Implications:** Methods to quantitate the ester prodrug and acid metabolite were validated and applied to the analysis of human plasma samples. Additionally, no significant conversion of the prodrug to the acid metabolite occurred during analysis.

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An Automated Method for the Identification of Non-Specific Binding in Protein-Free Matrices

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Objective: Non-specific binding (NSB) can have a major impact on the accuracy of quantitation during analysis of protein-free clinical samples. This presentation demonstrates an automated approach for the identification and measurement of NSB. It includes an assessment of user selected modifiers in controlling NSB. **Method:** A known concentration of analyte and one of a series of modifiers are added to the matrix and mixed thoroughly. The automated system performs multiple transfers of the samples across a series of tubes to maximize the potential losses during sample processing. Internal standard is then added to all samples and extraction of all samples, including NSB controls, is done using the same automated workstation. Samples are analyzed using a validated plasma LC/MS/MS assay. Area ratio data allow the comparison of the degree of NSB and to monitor the effects of a range of modifier concentrations. **Results:** Urine samples were spiked with the test compound at the LLOQ level and then with an aliquot of a reagent to prevent adsorption to the sample containers. For compound A, addition of 15 mM CHAPS surfactant was sufficient to prevent adsorption to large sample containers. This was used as the positive control during assessment of the ability of other urine treatments to effectively reduce NSB. In this instance, urine treated with water showed 90% losses compared to 15 mM CHAPS and concentrations of CHAPS >5 mM showed that NSB was under control. **Implications:** This automated approach provides a rapid assessment of NSB giving more robust methods for accurate quantitation of clinical samples.

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Case Studies in Chromatography with LC/MS/MS Detection

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Objectives: Historically, reversed phase techniques based on pH dependent hydrophobic interactions were utilized for chromatographic bioanalysis. The advent of routine LC/MS/MS bioanalysis promised unprecedented selectivity and sensitivity compared to conventional HPLC. In the haste to take advantage of this selectivity, methods were developed without regard to chromatographic retention. Alternatively, methods were transferred without regard to the efficiency gains that a high level of selectivity could provide. Balancing efficiency and effectiveness through application of appropriate chromatography to LC/MS/MS bioanalysis will be presented using real-life examples. **Methods:** LC-MS/MS bioanalytical methods received from a variety of sources, representing overly complex or simplistic chromatographic approaches, and their redeveloped counterparts will be compared. **Results:** An overly complex gradient method was converted to a simpler isocratic system, improving sample throughput by 340%. A method employing a linear gradient with an extended runtime was replaced by an efficient isocratic system with significant retention ($k' = 7.7$) improving throughput by 560%. An isocratic method ($k' = 2.2$) with a “busy” baseline was modified to increase retention ($k' = 4.5$) and slightly decrease runtime with superior baseline characteristics. A method with little chromatographic retention and problematic downfield matrix suppression was redeveloped to provide adequate retention while eliminating downfield suppression. **Implications:** LC/MS/MS is the powerful tool of choice to increase efficiency in bioanalysis, but intelligent choices in chromatographic development are required to produce a reliable method. Matrix suppression, secondary retention, and efficiency are critical factors to consider when developing a chromatographic system to couple with MS detection.

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The Use of ‘Semi’ UPLC-MS/MS Technology in Bioanalytical Applications.

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Objectives: HPLC-MS/MS is the primary method of separating and detecting analytes in biological samples. The requirement for lower detection limits, increased throughput and resolution have resulted in new tools to enable bioanalysts to achieve these goals. Our objective was to achieve these goals without the need for wholesale changes in equipment. **Methods:** The advent of Ultra Performance Liquid Chromatography (UPLC) has been a valuable addition to industry. UPLC enables faster separations with greater resolution; making it ideal for complex mixtures. UPLC is not without drawbacks, however; it requires relatively long 1.7 μ m particle columns with pumps and injection valves capable of withstanding extremely high pressures and flow rates. We have found it possible to incorporate most benefits of UPLC without the need for new equipment. This ‘semi’ UPLC approach uses similar sub-2 μ m particles but utilizing 2 and 3 cm column lengths that typically keep the back pressure at less than 4000psi at flow rates optimal for MS/MS, thus allowing short run times with k' values >5 . **Results:** We have been able to achieve UPLC results on standard HPLC equipment. Some of our recent applications will be discussed, including the use of dual injection semi-UPLC. **Implications:** This technology has enabled us to drastically reduce our analysis times and

lower the limits of quantification compared to more conventional methods of chromatography, whilst improving capacity factors (k') and reducing cycle times. Combined with dual injection systems, throughput has been further increased.

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“On Column” Derivatization for the Quantitation of Ibandronate and Other Bisphosphonates by LC/MS/MS in Human Serum

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Objectives: Measurement of bisphosphonates in human matrices at clinically relevant concentrations has presented a challenge to bioanalytical scientists. The method presented herein for measurement of bisphosphonates utilizes a solid-phase extraction with a novel on-column derivatization, developed previously at MDSPS Lincoln to measure alendronate and risedronate (Zhu L *et al*, RCMS, 2006). This approach was used for analysis of ibandronate and other bisphosphonates at clinically relevant (low pg/mL) concentrations. **Methods:** Human serum containing the analyte of interest was extracted using an anion-exchange solid phase extraction procedure. The analytes were derivatized with diazomethane directly on the SPE sorbent prior to elution with methanol or acetonitrile. Extracts were analyzed via LC-MS/MS (AB/MDS Sciex API 4000). The validated range was 0.200 – 100 ng/mL. **Results:** For ibandronate analysis in serum, the inter-batch precision (CV) and accuracy (% Bias) for quality control (QC) samples were from 3.3 to 6.1% and from -0.7 to +5.7, respectively. Method robustness was demonstrated through the use of multiple extraction scientists, platforms, matrix lots, and columns. The method maintained ample signal to noise (>30:1) while employing just 0.100 mL of serum. The results indicate that this approach may be applied to the quantitative analysis of other bisphosphonates. **Implications:** This novel method demonstrated the versatility of “on-column” diazomethane derivatization for the quantitative analysis of ibandronate and other bisphosphonates in human serum by LC-MS/MS, allowing for measurement of clinically relevant concentrations with a relatively small volume of sample.

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Study of Dried Blood Spots Technique for Determination of Dextromethorphan and its Metabolite Dextrorphan in Human Whole Blood by LC-MS/MS

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Dried blood spots (DBS) technology has been evaluated in an assay for the quantitation of dextromethorphan (DEX) and its metabolite, dextrorphan (DXO), in human whole blood by high performance liquid chromatography with tandem mass spectrometry method (LC-MS/MS). Both parent drug and metabolite were spiked in the blood matrix and subsequently allowed to dry onto a specimen collection card. The dried blood spots were punched out by a manual punch and then extracted into methyl *tert*-butyl ether (MTBE). The organic supernatant was transferred and

evaporated; the residue was reconstituted in 20% acetonitrile. The recovery of DEX and DXO was 62% and 56%, respectively. The assay was linear over the concentration range of 0.2-200 ng/mL for both analytes. Several factors that potentially affect DBS assay quantitation had been investigated, such as punch size, DBS sample punch-out location, and blood sample volume pipetted on the specimen collection cards. Our study has found out that punch size does not affect assay quantitation accuracy. Indeed, larger punch size increases the sensitivity due to the larger sampled blood spots. Sampling from different location on the specimen collection cards shows no significant variation for both drugs. Our study also shows that different blood sample volume pipetted onto the collection cards causes some variation for both drugs, which indicates a more accurate blood sample volume is necessary for assay quantitation. To achieve the similar LLOQ as plasma assay, several blood spots at the same concentration level have been stacked together and extracted. The advantage and limitation using DBS technology for bioanalytical assays will be further discussed in the presentation.

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Gaining Metabolite Information From First-In-Human Plasma And Urine Analysis

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Objectives: First in Human (FIH) studies are designed primarily to investigate the safety and toleration of a drug in humans and secondly to investigate its pharmacokinetics. Generally plasma and urine sample analysis from FIH studies provides only quantitative concentration data on parent and sometimes known metabolites. Obtaining *in vivo* human metabolite data as early as feasible in the drug development process has become particularly important in light of the FDA MIST guidelines. **Methods:** Modern mass spectrometers have the capability to provide much more information, such as scouting for potential unknown metabolites (on the basis of preclinical *in vitro* data). **Results:** We have several examples of proactively examining preclinical metabolism data and subsequently obtained valuable human metabolism data long before the definitive human dose balance studies are performed. **Implications:** This data has helped gain valuable information which would otherwise have been missed. Whilst this information can be useful, care must be taken in using such information.

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Investigation of Impact of Ionization Polarity to Regression Model of Calibration Curve in a LC-MS/MS Method

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Objectives: Establishing appropriate regression models for calibration standards is one important aspect in bioanalytical method development/validation. Usually, a structure analogue compound is used as IS when stable-labeled compound is unavailable. This poster reports the impact of ionization polarity to the regression model in a gemcitabine assay using floxuridine as IS. **Methods:** Both compounds, eluted with capacity factors of 2.4 from a reverse phase column using ammonium acetate in water and methanol as mobile phase solvents, were monitored in MRM transition in both positive and negative ESI modes at the optimized mass spectrometer parameters. **Results:** The curve ranges of gemcitabine are 0.0100-5.00 ng/mL and 1.00-500ng/mL in positive and negative mode, respectively, where compound signals are comparable within linear response range of the instrument. In positive mode, calibration standards are well characterized in a linear regression model (weighted 1/x) in the 500 fold dynamic range, while in negative mode, a quadratic regression model (weighted 1/x) is required. The same regression

models are applied without adding IS in the solution. Using 10mM ammonium acetate in water, the linear regression model can characterize a calibration curve with 100 fold dynamic range (1.00-100ng/mL) in negative mode. Although the gemcitabine sensitivity decreases when ammonium acetate concentration reduced to 2mM, the dynamic range with linear regression model extends to 200 fold (1.00-200ng/mL) and the calibration curve becomes less quadratic in the 500 fold dynamic range. The observation indicates that ammonium acetate could assist ionization in negative mode. It may also compete with gemcitabine during ionization.

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Validation of a Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of Desmopressin in Human Plasma

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Objectives: To develop and validate an LC-MS/MS method for the quantitative determination of desmopressin in human plasma. Desmopressin is a synthetic analogue of the neurohypophysal peptide hormone vasopressin used in the treatment of diabetes insipidus and nocturnal enuresis. **Method:** Desmopressin and d_8 -Desmopressin (internal standard) were extracted from 1 mL human plasma using SPE followed by evaporation and reconstitution. The analyte was chromatographically separated on a Zorbax XDB-C₈ (4.6 x 50 mm) column using gradient elution with a mobile phase composed of 0.1mM ammonium formate/formic acid (0.05%) and methanol/formic acid (0.005%), at a flowrate of 0.6 mL/min for a total runtime of 6.5 minutes. LC-MS/MS was performed using electrospray ionization (ESI) in the positive mode. Detection and quantitation were carried out by multiple reaction monitoring (MRM) scan at 535.3 to 328.3 (Desmopressin) and 539.3 to 328.2 (d_8 -Desmopressin). **Results:** The method was validated over the range of 4 to 50 pg/mL. Inter-batch accuracy (%RE) and precision (%CV) for standards and quality control samples ranged from -8.7 to 3.6 and 2.6 to 13.3 respectively. The mean (n=5) correlation coefficient was 0.9978 ± 0.0015 . Negligible matrix effects were observed (<30%) despite the large aliquot volume. Mean assay recovery was at $74.4 \pm 6.8\%$ (n = 15) for the analyte and $73.3 \pm 4.7\%$ (n=10) for the IS. Desmopressin stability in solution and in matrix was established. **Implications:** A sensitive, accurate and reliable method to quantitate desmopressin in human plasma was developed, validated and successfully applied for the quantitation of desmopressin in clinical samples.

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An LC-MS/MS Method for the Quantitation of Diazepam and N-Desmethyldiazepam in Human Plasma (EDTA)

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Objective: The objective of this project was to develop a high-throughput, automated bioanalytical method for the quantitation of diazepam and N-desmethyldiazepam in human plasma (EDTA) requiring minimal sample volume. **Methods:** 100 μ L plasma samples were extracted in a 96-well plate by a liquid-liquid procedure using a Zymark/Caliper Sciclone automated sample handling system. The extracts were evaporated to dryness and reconstituted with acetonitrile. A SCX analytical column was utilized along with a mobile phase consisting of acetonitrile and ammonium formate buffer to achieve separation. An AB | MDS Sciex API 4000, using and ESI interface, detected positive ions in the multiple reaction monitoring mode.

Results: The method was linear (weighted $1/X^2$) over a concentration range of 2.00 to 500 ng/mL. Inter-batch precision (C.V.%) of diazepam and N-desmethyldiazepam quality control samples was less than or equal to 6.5% and 7.4%, respectively. Inter-batch accuracy (R.E.%) of diazepam and N-desmethyldiazepam quality control samples was less than or equal to 7.3% and 7.0%, respectively. Assay selectivity was demonstrated by accurate quantitation of standard spikes at the LLOQ and high quality control level into ten separate lots of blank human plasma (EDTA). Adequate short-term and freeze-thaw stabilities were established. **Implications:** The assay for the analysis of diazepam and N-desmethyldiazepam in human plasma (EDTA) met the criteria established for a validated bioanalytical method and required only a 100 μ L of plasma sample. The low sample volume allowed for the extraction to be automated which greatly increased projected production throughput.

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Bioanalytical LC/MS/MS Assays for the Quantitative Analysis of Amikacin, Neomycin and Gentamicin in Plasma and Tissue

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Jenny McKinnell, Bob Cass and Dane Karr, Achaogen, Inc. South San Francisco, CA

Objective: The objective was to develop LC/MS/MS bioanalytical methods for the analysis of aminoglycosides from animal plasma and tissue. These compounds are very hydrophilic due to the large number of amino and hydroxyl groups in their structures. This presents a challenge when developing an HPLC/MS/MS method because the use of traditional reversed-phase HPLC will not retain these compounds on the column. Here we report on an accurate, precise and specific HPLC/MS/MS assay for the determination of amikacin, neomycin and gentamicin from rat, dog and mouse plasma as well as various tissues. **Method:** Bioanalytical methods were evaluated using HPLC/MS/MS on a Sciex API3000 or API4000. Several HILIC methods and reversed-phase ion pairing methods were investigated. In addition, various organic precipitation methods were investigated to determine which method provided the most efficient extraction from plasma and tissue. **Results:** Bioanalytical methods were developed for the LC/MS/MS bioanalysis of amikacin, neomycin and gentamicin in rat, dog and mouse plasma as well as various tissues. The best combination of retention and peak shape for these compounds was found using mobile phase containing the ion pairing reagent HFBA and a C18 reversed-phase column. Organic precipitation using methanol containing 1% formic acid provided the best extraction from plasma. The methods provided accurate and precise data in less than 4 minutes per injection with LLOQs at 5 ng/mL. **Implications:** This work demonstrates that LC/MS/MS can be used for the development of bioanalytical methods for the analysis of aminoglycosides from biological matrices.

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A Strategy to Develop Sensitive and Rugged 96-Well Formatted Bioanalytical Assays Rapidly

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Pharmaceutical companies are under a lot of political and economic pressure to develop more innovative treatments rapidly with less resource. This trend impacted all phases of the drug discovery and drug development including bioanalysis. In the past three years, we increased the number of drug candidates in development several folds with minimal change in resources. It was achieved through improved personnel training; process improvement, communication, better

LC-MS equipment, automation, and increasing the use of bioanalytical CROs for later phased studies. The increasing number of drug candidates also makes the assay development and validation a greater portion of the total workload. How to streamline the development and also improve the quality of the validated methods for repeatability of incurred sample results becomes a challenge that many of us are pursuing. The following are our strategies. This poster describes the usage of physical and chemical properties of the drug candidates as a guide to narrow the field of search for each segments of bioanalytical method development. In combination with high-throughput screening based on 96-well plates and on-line mobile phase preparation, this approach will identify the outline of the bioanalytical method in a short time. To help us focus, we surveyed the assays developed between 2004 and 2007 in our department to verify the relationship between the structures and the final assays. The results indicated that HPLC column and mobile phase are correlated with the physical property. However, the correlation between sample preparation method and physical property is poor. On the other hand, the sample preparation method seems to have a relationship with the on-column amount required for the sensitivity. We would present our progress in the poster to initiate discussions with our colleagues.

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Chromatographic improvements and overcoming matrix effects and carryover for a previously validated nine analyte LC/MS/MS assay using UPLC.

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Objectives: In order to improve sample throughput, we explored the possibility applying UPLC technology to a previously validated LC/MS/MS assay in which one parent drug and eight metabolites were quantified simultaneously using a single analogue internal standard and a cycle time of 12 minutes. **Methods:** Extracts were prepared by protein precipitation (PPE). The LC/MS/MS system was a Sciex API5000 under ESI (+) mode using either a Waters Alliance 2690 HPLC or Acquity UPLC system. The same basic pH mobile phases were used in both methods using different gradient conditions. A Waters Xterra C18 2x100mm column was used in the HPLC method and a Waters BEH 50x2 mm column was used for UPLC method. **Results:** The precipitation extraction method was maintained because of stability issues. The cycle time was reduced from 12 minutes to 4.5 minutes. However, matrix effects emerged using the UPLC method because of difficulty in using column switching. The original method used column switching to back-flush phospholipids from the HPLC column following each injection. The UPLC method used a forward flush program to achieve the same result. Initially, carryover was more severe with the UPLC method using the manufacturer recommended "Partial loop" mode. It was reduced sufficiently by using "Partial loop needle overfill" mode. **Implications:** An investigation into converting an existing HPLC/MS/MS assay to a UPLC method to quantify nine compounds using a single analogue internal standard was performed.

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The Development of a Method for the Determination of Niacin in Dog Plasma using LC/MS/MS

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Objective: To develop a reliable method for the determination of niacin in heparinized dog plasma using LC/MS/MS. Niacin, vitamin B3, is commonly used to lower cholesterol levels; this dog method is being developed to support toxicokinetic studies comparing niacin and a potential cholesterol lowering drug in development stages. **Methods:** The extraction techniques explored

were protein precipitation using acetonitrile, liquid-liquid extraction using MTBE and solid phase extraction with the Oasis MCX plate. Also, a comparison between two HPLC columns (Thermo Silica and SIELC Primesep B2) in isocratic and gradient mode was made. **Results:** Due to the low transitions being monitored (m/z 124 to m/z 80), high baseline and endogenous interferences presented a problem. Using the SPE method with the Primesep B2 column in gradient mode, we were able to achieve a good signal to noise ratio at the LLQ, good separation between niacin and metabolites (including nicotinuric acid, nicotinamide, 1-methyl-nicotinamide and nicotinamide-N-oxide) and maintain a consistent baseline allowing us to develop a method that was linear from 50 ng/mL to 25000 ng/mL. **Implications:** A reliable and reproducible method for the analysis of toxicokinetic samples to determine niacin concentrations in heparinized dog plasma was developed. Comparing our method with other published dog plasma methods, we achieved a shorter run time using a smaller i.d. column while still maintaining good chromatography and separation of metabolites.

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Simplified Protein and Lipid Depletion in Bioanalysis

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Objective: Here, we present a simplified approach to sample preparation through the use of a lipid depleting protein precipitation plate. This approach removes ion-suppressing phosphatidylcholines and other matrix interferences from plasma samples and is easily implemented. **Methods:** Simply, plasma samples spiked with a range of analytes were precipitated in well and filtered through a prototype lipid depleting filter. Samples were analyzed by LC/MS for both phosphatidylcholine removal and analyte recovery. Post column infusion was used to quantify improvements in the ion-suppression compared to protein precipitation alone. **Results:** Protein precipitation conditions were identified where both Lysophosphatidylcholines and phosphatidylcholines are >90% removed. At the same time, relative responses of the analytes tested were improved over protein precipitation alone. **Implications:** This novel approach to sample preparation takes protein precipitation a step up as far as cleanliness is concerned, while maintaining its ease of use. Also, this approach is useful in reducing matrix carryover in the high throughput environment, and can facilitate the use of newer high speed sub 2 micron columns.