

Abstract 137

Clinical Sample Treatment, Absolute Matrix Effects and Chromatography: A Look at the Impact on Bioanalytical Results

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Objectives: Compound **I**, investigated for treatment of Type 2 Diabetes, required sample treatment with phosphoric acid to stabilize a potential glucuronide metabolite observed in animal species. During sample analysis for the first clinical trials, the sample treatment procedure and analytical protocol needed to be modified to minimize variable response due to sample pH and absolute matrix effects. **Methods:** Analyte and stable labeled internal standard were isolated from acidified human plasma via liquid-liquid extraction. Samples were quantified by HPLC-MS/MS using electrospray ionization under negative polarity. Initial chromatographic conditions utilized a C18 column with a polar embedded phase. Clinical sample analysis results using this method revealed a highly variable internal standard response. **Results:** Clinical protocol procedure was modified to ensure consistent treatment of plasma samples, as recovery was highly dependent on sample pH. The analytical protocol was modified to minimize the absolute matrix effect by utilizing the atmospheric pressure chemical ionization source and by changing the chromatographic conditions such that absolute matrix effect was minimized. **Implications:** Potential issues with validated bioanalytical methods often do not take form until the first in man and subsequent clinical studies. It is important to consider all aspects of bioanalysis, including sample treatment at the clinic, in order to isolate and solve these issues. Collection procedure, ionization source, and chromatography were all necessary points of investigation in order to minimize variable response.

Abstract 138

Internal Standard Response Variation during Incurred Sample Analysis by LC-MS/MS: How Much Is Too Much?

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Objectives: Internal standard (IS) responses can directly impact the accuracy of reported concentrations in bioanalysis as most methods are based on analyte/IS response ratios for quantitation. Due to the complexity of incurred sample matrices, variable IS response is quite common upon applying a validated method to the analysis of incurred samples. But how much variation in IS response is acceptable? **Methods:** Firstly, different real cases of IS response variations observed during the analysis of incurred samples were investigated for root-causes. Then, the impact of these variations on the accuracy of reported concentrations was evaluated. **Results:** Twelve cases were investigated, such as error in addition of IS, random and sharp drop in IS response, gradual decrease/increase of IS response, high/low IS response for incurred samples only, less IS response variation observed but accuracy was affected, randomly scattered low IS responses for incurred samples only and not repeated during re-analysis, deuterated IS not following the analyte and reinjection results not matching those of 1st injection. **Implications:** IS response variation could be caused by many different factors and therefore, each case should be dealt individually. Neither no criterion at all nor a set cut-off criterion is appropriate. Ideally, the difference in IS response between an incurred sample and calibration standards and quality controls should be evaluated first to determine whether it was a random occurrence or there were patterns/trend. Accordingly, the identified samples could be reanalyzed or the results could be accepted with an investigation to demonstrate that the accuracy was not affected.

Abstract 139

Identifying And Overcoming Bioanalytical Challenges Associated With Chlorine-Containing Dehydrogenation Metabolites.

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Objectives: Assess strategies to minimize quantitative interference of co-eluting dehydrogenation metabolites during the bioanalysis of chlorinated small molecules. **Methods:** LC-MS/MS experiments were conducted using Shimadzu pumps and a CTC Analytics HTS PAL autosampler, coupled to an Applied Biosystems API 4000 tandem quadrupole mass spectrometer equipped with a TurboIonspray™ source. Reverse phase chromatography was performed on an ACE 3 C18 column. Plasma samples were extracted by acetonitrile precipitation. **Results:** During LC-ESI-MS/MS analysis of plasma samples from a rat exploratory toxicology study two unexpected interfering peaks were observed to nearly co-elute with the chlorine-containing analyte. Q1 precursor ion scans of study samples indicated that these peaks contained putative [M-2] (loss of 2 Dalton) metabolites. Strong apparent isotopic contributions at [M+2] were noted in both cases, suggesting that the putative metabolites retained the chlorine atom of the parent compound. The potential isotope-related impact of these metabolites on quantification of the parent compound was assessed. Several alternate precursor ion and product ion combinations were evaluated and shown to minimize the quantitative impact of the interfering metabolites without having to rely on their chromatographic resolution from the parent compound. **Implications:** These results indicate that when quantifying chlorine- or bromine-containing small molecules from *in vivo* samples or *in vitro* metabolic incubations, 1) efforts to detect potential dehydrogenation metabolites should be undertaken, and 2) if such metabolites are detected, the judicious choice of alternate multiple-reaction monitoring (MRM) transitions can limit their impact on quantification of the parent molecule without the need for robust chromatographic resolution.

Abstract 140

Quantitative Interference by Cysteine and N-Acetylcysteine Conjugates During the LC-MS/MS Bioanalysis of an Electrophilic Small Molecule

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Objectives: Identify interfering metabolites discovered during bioanalysis of a small molecule in human urine samples, assess their quantitative impact, and develop strategies to minimize the interference. **Methods:** Quantitative LC-MS/MS experiments were conducted using Shimadzu pumps and a CTC Analytics autosampler, coupled to an Applied Biosystems API4000 triple quadrupole mass spectrometer equipped with a heated nebulizer probe or a TurboIonspray™ probe. Metabolite identification experiments were carried out on both Orbitrap and TSQ systems (Thermo). Reverse phase chromatography was performed on Phenomenex Onyx C18 columns. Urine samples were extracted by acetonitrile precipitation. **Results:** During LC-APCI-MS/MS analysis of a small molecule in urine samples from a human pharmacokinetic study, an unexpected interfering peak was observed to nearly co-elute with the analyte. Chromatographic separation revealed the presence of at least 3 metabolites, which were identified as two diastereomeric cysteine conjugates of the analyte, as well as their N-acetylated (mercapturic acid) derivatives. Quantitative comparison using the original chromatographic method and the improved separation method indicated that the original method substantially over-estimated the analyte concentration in many study samples. The quantitative over-estimation in the original method was shown to be due to in source fragmentation of the metabolites to the analyte. **Implications:** These results 1) demonstrate the potential for compromised quantification of electrophilic small molecules in biological matrices due to co-eluting thiol metabolites, 2) underscore the need to carefully evaluate LC-MS/MS methods for electrophilic small molecules to ensure that they are not susceptible to such interferences during quantification of *in vivo* samples.

Abstract 141

Development and Validation of a LC-MS/MS Method for the Determination of Salmeterol in Human Plasma

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Objective: Salmeterol is a long-acting β_2 -adrenergic receptor agonist drug that is currently prescribed for the treatment of asthma and chronic obstructive pulmonary disease. Published methods for the determination of salmeterol are not sufficiently sensitive to measure levels typically present at the later phases of elimination. A more sensitive method (lower limit of quantitation 1 pg/mL) was therefore developed. **Methods:** A solid phase extraction (SPE) method was developed for the extraction of salmeterol from plasma samples (250 μ L). The method employed a polymeric mixed-mode cation exchange medium. Salmeterol- d_3 was used as internal standard. HPLC separation was achieved on an ODS silica column using a mobile phase gradient comprising a formic acid - methanol mixture, delivered at a flow rate of 400 μ L/minute. Tandem mass spectrometric detection was performed using an MDS Sciex API 5000 instrument using a Turbo IonSpray interface (positive ion mode). **Results:** The method was validated over a range of 1 to 200 pg/mL. Inter-run precision was \leq 9.5% while bias ranged from -2.5% to 8.0%. The method was determined to be specific for the determination of salmeterol and free from matrix effects investigated in six different individual plasma samples and in lipaemic and haemolysed plasma. The method does not suffer from interference from co-administered fluticasone propionate. **Implications:** A sensitive, accurate and robust method for the determination of salmeterol in human plasma was developed, validated and successfully applied for the quantitation of salmeterol in clinical samples.

Abstract 142

Process Optimization of Bioanalytical Support for Merck Development Compounds; Leveraging One Analytical Assay to Generate Data for Multiple Analytes and Multiple Matrices.

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Objective: A semi-automated liquid-liquid extraction method was developed and validated to quantitate a Merck compound (Compound A) in human plasma to support pharmacokinetic studies. This assay was then applied to determine levels of drug in human urine and levels of metabolite in human plasma with minimal additional development or validation effort. **Method:** The analytical method was automated to extract 300 μ L of plasma samples with 0.8 mL ethyl acetate in a 96-well plate. Ethyl acetate was evaporated and samples were reconstituted with 0.150 mL of 45:55 methanol:water. Compound A and its hydroxylated metabolite were separated by reversed phase HPLC isocratically with a mobile phase of 55:45 methanol:0.1% formic acid. Tandem mass spectrometry in Multiple Reaction Monitoring mode with a turbo ionspray interface in positive ion mode was used for detection. Compound A had a dynamic range of 0.2-200 nM. Metabolite ranged from 1-1000 nM. Control urine and urine samples were diluted 100 fold with control plasma and analyzed using the validated plasma assay. **Results:** For the metabolite, three standard curves were extracted and analyzed. Intraday precision varied from 3.23%-7.00% and accuracy ranged from 94.64%-107.14%. Recovery of metabolite in plasma was 94.15%. For the urine assay, replicate QC's (n=5) were analyzed. Precision was 1.54%, accuracy ranged from 96.98%-100.68%, and recovery was 98%. **Implication:** By leveraging one analytical assay across various matrices and analytes, method development time and resources were saved. The data generated was then used to determine the necessity of further developing metabolite and urine assays.

Abstract 143

A Sensitive LC-MS/MS Method for the Quantitation of Fluticasone Propionate in Human Plasma

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Objective: Fluticasone propionate (FP) is a potent, synthetic corticosteroid used to treat asthma and allergic rhinitis. Literature methods for FP in plasma have lower limits of quantitation which are often too high (20 - 50 pg/mL) to allow quantitation of FP in plasma more than a few hours after administration. The objective of this work was therefore to develop and validate a sensitive LC-MS/MS method for the determination of FP in human plasma, with a lower limit of quantitation of 3 pg/mL. **Methods:** A solid phase extraction (SPE) method utilising a C₁₈-modified silica sorbent was developed (sample size 500 µL). FP-d₃ was used as an internal standard. HPLC: ODS silica column; mobile phase gradient comprising ammonium formate (25 mM, pH 5) - methanol, 300 µL/minute. Tandem mass spectrometry: MDS Sciex API 5000 instrument, TurboIonSpray (positive ion mode). **Results:** The method was validated over a range of 3 to 200 pg/mL. Inter-run precision was ≤10.0% while bias ranged between -1.0% to 15.0%. The method was demonstrated to be specific for the determination of FP, free from matrix-related modification of ionisation effects investigated in six different individual plasma samples and to be unaffected by lipaemia and haemolysis. **Implications:** A sensitive, accurate and robust method for the determination of FP in human plasma over the range 3 - 200 pg/mL was developed, validated and successfully applied to the quantitation of FP in clinical samples. The method is suitable for the detection of FP in samples at least 24 hours after an inhaled dose of 100 µg.

Abstract 144

Development and Validation of an LC-MS-MS Method for the Quantitation of Anastrozole in Human Plasma (K₂EDTA)

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Objective: The objective for this project was to develop and validate a method for quantitation of anastrozole in human plasma. **Method:** Anastrozole and an internal standard (letrozole) were extracted from 100 µL of human plasma using 1.0 ml of methyl tert-butyl ether. Following a flash freeze in a dry ice-alcohol slurry, the organic layer was poured into a second tube and evaporated. Samples were reconstituted in 1.0 mL of water:acetonitrile, 70:30, v:v and 10 µL of each sample was injected onto an LC-MS system. The analytes were eluted from a Waters Symmetry C18 column (3.5 µm, 2.1 x 50 mm) with a mobile phase of 0.1 % glacial acetic acid in water:acetonitrile, 68:32, v:v, followed by a column wash of 0.1 % glacial acetic acid in water:acetonitrile, 20:80, v:v between each injection. Detection was performed using an Applied Biosystems API 4000 using electrospray ionization in the positive ion mode. **Results:** The method was validated over the range of 2.00 to 500 ng/mL. Inter-batch accuracy (%RE) for quality control samples of anastrozole ranged from 0 to 4%, while inter-batch precision (%RSD) ranged from 6 to 8%. Acceptable accuracy and precision was shown at the LLOQ level. Specificity was shown in six lots of plasma by infusion experiments and ion suppression experiments. Short-term stability in plasma at ambient conditions and following three freeze-thaw cycles was also shown. **Conclusion:** A sensitive, accurate, and reproducible method was developed and validated for the analysis of anastrozole in human plasma and was applied to clinical sample analysis.

Abstract 145

Analysis of Drug Metabolites in Biological Fluids using Mixed-Mode Solid Phase Extraction and UltraPerformance Liquid Chromatography-Tandem Mass Spectrometry

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Objective: The development of a bioanalytical method for the determination of morphine and compounds. **Methods:** A method using mixed-mode solid phase extraction and Ultra Performance liquid chromatography-tandem mass spectrometry was developed for the analysis of morphine, morphine-3 β -glucuronide, morphine-6 β -glucuronide, 6-acetylmorphine, morphine N-oxide, and 10-hydroxymorphine in porcine plasma. All six compounds, along with four deuterated internal standards, were simultaneously extracted using mixed-mode strong cation exchange SPE in a 96-well μ Elution plate format. Due to analyte instability, a neutralizing solvent was used during the elution step to minimize degradation of 6-acetylmorphine. Separation was performed on a 2.1 \times 100 mm, 1.8 μ m C₁₈ column designed for retention of extremely polar compounds using a formic acid and methanol gradient. Analytes were detected by positive electrospray ionization in multiple reaction monitoring mode. **Results:** Recovery was 77-120 % depending on the analyte, and inter-day variability was less than 6 %. Linearity was determined in porcine plasma by spiking the analytes prior to SPE. Correlation coefficients were \geq 0.998, and % deviation from the actual concentrations was less than 15 %. The lower limit of quantitation for all compounds was between 0.1 and 0.25 ng/mL. **Implications:** A method was developed to simultaneously extract and preserve analyte structural integrity during sample preparation and subsequent quantification of parent drug and associated polar compounds.

Abstract 146

Dextromethorphan Metabolism by Human and Rat Liver Microsomes by UPLC QTOF Accurate Mass MS/MS with Isotopically-Labeled Parent and Metabolite Standards.

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Objectives: To investigate the *in vitro* metabolism of dextromethorphan by human and rat liver microsomes by UPLC QTOF MS/MS analysis with and without stable-isotope labels for comprehensive metabolite structural elucidation. **Methods:** Dextromethorphan and major metabolites, native and deuterated, were incubated with UDPGA- and NADPH-fortified liver microsomes from human and rat. Samples were analyzed by UPLC and tandem accurate mass spectrometry with electrospray ionization. Metabolite profiling and characterization for all incubated compounds were performed. **Results:** In addition to the expected major metabolites, several unexpected hydroxylation metabolites of dextromethorphan were detected. Comprehensive fragmentation pathways for dextromethorphan and metabolites were determined from accurate mass data acquired for the native and stable isotope-labeled compounds. Comparison of fragmentation data acquired for the native and labeled compounds facilitated enhanced structural elucidation of the metabolites. **Implications:** Dextromethorphan is of interest as both a widely available over-the-counter drug and as an FDA-approved *in vivo* and *in vitro* probe substrate for the polymorphically-expressed cytochrome P450 enzyme CYP2D6. Profiling of its major metabolites has been previously published, but metabolite structural elucidation has been limited by unusual MS fragmentation behavior. Incubation of a mixture of stable isotope-labeled and unlabeled drug for metabolite profiling has been widely reported for various test compounds, but separate incubation of native and labeled drug and metabolite standards is not currently used to improve metabolite structural elucidation. This study illustrates the value of such an approach.

Abstract 147

High Throughput Screening GSH Adducts Using Hybrid Linear Ion Trap Systems Coupling with Fast chromatography at Clinically Relevant Dose Concentration

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Objective: The detection of reactive metabolites such as GSH adducts has become a common practice in early drug toxicity screening. In order to detect all potential GSH adducts, two or more injections were often used. Higher substrate concentrations (10-50 μM) which may be higher than clinical relevant concentration and complex sample preparation were often needed in order to achieve the sensitivity desired. This paper investigates combining LC/MS/MS scan modes on a hybrid linear ion trap for high throughput (HT) screening of GSH adducts at clinically relevant concentrations in a single injection.

Methods: UPLC-like conditions with an acquisition time of 5 to 10 minutes. Two MS information dependent acquisition (IDA) methods were explored. The first combines neutral loss (+129, +307) and precursor ion (-272) scan modes for survey scans. The second method uses the characteristic fragmentation pattern of GSH to build predictive MRM (pMRM) transitions. Both methods make use of fast polarity switching between scan modes. **Results:** Trazadone was used as a model compound to investigate GSH formation at 2 and 20 μM in human liver microsomes. The two MS approaches were able to detect GSH conjugates at trace levels not easily observed using standard LCMS workflows. The pMRM approach proved to be more sensitive, but relies on the ability to predict the biotransformation.

Implications: A sensitive HT method for complete GSH detection and structural confirmation at clinically relevant concentrations ensure better *in vivo* prediction.

Abstract 148

Development and Validation of a Simple, Cost Effective LC-MS/MS Method for the Quantification of Mevalonic Acid (a Biomarker for Cholesterol Synthesis) in Human Urine.

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Objective: To develop a simple, cost effective LC-MS/MS method for the quantification of Mevalonic acid (MVA) in human urine. **Methods:** A liquid/liquid extraction technique was developed using tert-butyl methyl ether (TBME). The calibration curve was prepared in water due to endogenous MVA present in urine. The linear range was 10.0 – 2500 ng/mL. Mevalonolactone-1,2-¹³C₂ was used for the internal standard. Mevalonic acid lactone was spiked into human urine at three concentrations for quality control samples. Samples were acidified to convert any MVA present to lactone form, and allowed to sit for one hour at room temperature. TBME was added, the samples were vortexed and centrifuged. The organic layer was collected and dried under nitrogen then reconstituted using a basic solution to convert the lactone into the acid form for analysis. Separation was achieved on a Varian Monochrome C18 column (100 x 4.6, 5 μm) at a 0.700 mL/min flow using a gradient. Analysis was done on an AB Sciex 4000 mass spectrometer using TurboIon spray. Detection was by negative ion mode with multiple reaction monitoring. **Results:** The method was linear 1/x with correlation coefficients >0.9900. Intra-run mean accuracies ranged from 93–107% over three separate days, with inter-run accuracies from 95–104%. Precision ranged from 1.0–3.1 %CV. **Implications:** MVA is an important biomarker of cholesterol and bile acid synthesis. This validated method is simple, cost effective, robust and repeatable, thus making it an attractive method for evaluating changes of the rates of cholesterol synthesis post cholesterol lowering treatments.

Abstract 149

Strategies to Increase the Throughput of Drug Quantitation in Tissues for Discovery Support

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Objectives: The goal of this work was to explore streamlined approaches to 1) homogenize several rodent tissue types such as lung, skin, trachea, bronchi, liver, kidney, brain, harderian glands and 2) evaluate the success rate of a plasma standard curve as surrogate matrix for quantitating tissue drug levels. **Methods:** Several commercial tissue homogenization devices were evaluated for their ease of use, speed and ability to effectively homogenize tissues. Device types included bead beating, polytron and ultrasonic systems. Tissue homogenates from liver, kidney and lung were analyzed using standards prepared in a tissue matrix as well as standards prepared with a plasma matrix supplemented with controls spiked into pooled tissue extracts. UHPLC-MS/MS was used for all quantitation. **Results:** The Fastprep™ tissue homogenization system showed superior efficiency at homogenizing the most difficult tissues such as skin, trachea and bronchi. The Genogrinder™ in a 96well format showed the highest throughput and best possibility for automation. 88% of the compounds tested could be quantitated with a plasma standard curve with less than 30% deviation from the tissue curve results. The use of controls spiked in pooled tissue extracts did not provide additional benefit. **Implications:** Increased throughput and versatility is achieved by using the Fastprep™ and Genogrinder™ tissue homogenization systems. A reduction in use of laboratory and animal resources was demonstrated by using a plasma calibration curve as a surrogate for tissues.

Abstract 150

Quantitation of Abbott-Compound In Biological Matrices By MALDI/MS/MS As Compared To LC/MS/MS.

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Objectives: A MALDI-MS/MS method has been developed for the quantitation of Abbott-Compound, a small drug molecule. An investigation was conducted to evaluate MALDI FlashQuant as compared to a validated LC/MS method for the analysis of biological samples. **Methods:** Abbott-Compound and its (d-IS) in rat and human plasma were extracted using 96-well LLE with 1/1 ethyl acetate/hexane. For MALDI, the dried extracts were dissolved into 50 µL α -cyano-hydroxycinnamic acid matrix, (6 mg/mL in 60% acetonitrile/40% water/0.1% TFA). 1µL sample was deposited in triplicate onto MALDI 384-plate, analyzed at 1.5 mm/sec. Optimized MRM method with +/ions was used for both techniques. The rat (AP) and human (HP) plasma STDs ranged from 0.025 to 2.03 µg/mL and 0.116 to 25.8 ng/mL respectively. Matrix Effect (MEQCs) (21 ng/mL) in HP were assessed in six lots. **Results:** For AP, mean bias: LLOQ 2.8% with CV 11.1%, QCs between -0.4% and 6.4% and CV \leq 11.0%. 80% of analyzed rat study samples by MALDI matched the values by LC/MS with difference of +/- 20% and CV \leq 16.8%. For HP, mean bias: LLOQ -10.4% and 17.7% CV, QCs between 68.2% and 1.73% with CV \leq 36.9%. MEQCs spots, mean % bias between -1.16% and 1.14% of theory with CVs \leq 3.4%. The MALDI run time was 22 minutes for 288 spots, and LC/MS was ~ six hours for 98 samples. **Implications:** MALDI was 20X faster than LC/MS acquisition time and assay reproducibility, with acceptable accuracy and precision, was in the range of 21 to 2000 ng/mL.

Abstract 151

Validation of a Chiral LC-MS/MS Assay for R- and S-Warfarin in Human Plasma to Support Drug-Drug Interaction Studies

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Objective: Warfarin is used extensively as an oral anticoagulant for the prevention and treatment of thromboembolic disease. Results from a study published in 2006 reveal that 14.7% of patients who participated in the study experienced a hemorrhage event within one week after beginning warfarin therapy. It has been well documented that these adverse events typically are the result of a pharmacokinetic interaction between a concomitant drug and warfarin involving inhibition of cytochrome P450 2C9. The FDA requires that new potential drugs must be evaluated for potential drug metabolism and interactions with warfarin. A bioanalytical method was needed to separate concomitant drugs from warfarin to minimize them as potential interferences in the detection of warfarin extracted from plasma. **Method:** The chiral LC-MS/MS assay was validated using a Chirobiotic V™ analytical column. Warfarin was extracted from plasma using a MCX 96-well plate to trap basic amine drugs on the extraction bed while eluting warfarin from the neutral phase. **Results:** A full validation of the chiral LC-MS/MS assay for R- and S-warfarin in the concentration range of 5 to 2500 ng/mL was carried out successfully. **Implications:** Having a bioanalytical method which separates basic drugs from warfarin during the plasma sample extraction reduces the risk of these drugs as being potential interferences. In turn, this greatly increases the ruggedness of the method.

Abstract 152

Validation of a Chiral LC-MS/MS Assay for R(+)- and S(-)-Bupivacaine in Human Plasma to Support Bioequivalency Studies.

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Objective: The first objective was to validate a robust and rapid chiral bioanalytical assay for clinical bioequivalency studies of bupivacaine using low sample volumes. The second objective was to probe the mechanisms of chromatographic chiral recognition. **Method:** The chiral bioanalytical assay was validated using supported liquid extraction and HPLC/MS/MS. The Chirobiotic-V™ column was selected for method development studies due to its broad chiral recognition mechanisms. **Results:** A full validation of the chiral bioanalytical method for the analysis of bupivacaine enantiomers within 14 min in the range of 1-1000 ng/mL using a sample volume of 50 µL human plasma was completed successfully. The affects of flow rate, organic strength, buffer strength, and pH on chiral recognition were evaluated for optimization of enantiomeric resolution. Baseline resolution of 3.1 was achieved. The affect of the alkyl chain length of the analyte was also evaluated using the series N-(2,6-dimethylphenyl)-2-piperidinecarboxamide mepivacaine, ropivacaine, and bupivacaine. **Implications:** Bupivacaine is used extensively in the clinic as a local anesthetic, nerve blocker, and for epidural and intrathecal anesthesia. The cardiotoxic effects of local anesthetics are well documented and it was demonstrated that bupivacaine's toxicity is due to the R(+) enantiomer. Future clinical bioequivalency studies will require robust chiral assays such as that developed and validated by MPI research. The low volume and fast run time of this chiral assay coupled with a thorough understanding of the mechanisms of chiral recognition yield a method ideal for such studies.

Abstract 153

Identification of the *in vitro* Glucuronidation and Sulfation Pathways of ARQ 501 (β -lapachone) Using Human UDP-Glucuronyltransferases and Sulfotransferases.

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Objective: ARQ 501 (β -lapachone) is an investigational compound for the treatment of advanced solid tumors. The *in vivo* metabolism of ARQ 501 observed in humans, and also in human hepatocytes, is primarily conjugation followed by biliary excretion. The identification of the specific UDP-glucuronyltransferase (UGT) and sulfotransferase (SULT) forms responsible for conjugative biotransformation of ARQ 501 is currently being explored. **Methods:** The *in vitro* assays are conducted using a variety of commercially available enzymes. The assays are also conducted in the presence and absence of potential activating compounds and enzymes to determine what role, if any, they have in the metabolism of ARQ 501. **Results:** Our goal is to identify the specific isoforms of UGT's and SULT's responsible for biotransformation of ARQ 501. Studies are currently ongoing, but preliminary data suggests that one or more co-factors not normally required to initiate glucuronidation *in vitro* may be necessary in the case of ARQ 501. **Implications:** Identifying the specific enzymes which metabolize ARQ 501, and potentially any additional co-factors or components required, will allow an understanding of the pathways involved in its metabolism and elimination. Additionally, it may shed light on the metabolism of other compounds that share the same structural motif.

Abstract 154

Utilizing Fast LC-MS/MS for High-Throughput Bioanalytical Analysis In GLP Environment

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Objectives: Fast LC has recently gained a lot of popularity by reducing the LC/MS/MS cycle time during bioanalysis. One risk associated with fast LC is the loss of resolving power under high flow rate. Two columns designed to achieve fast separation without compromising resolution were used for 4 development compounds in 7 validations. The success and challenges of using both the superficially porous column (also called as fused core silica column) and the small particle column (2.5 μ m) in GLP environment will be presented. **Methods:** All the validations were performed on an API 4000 mass spectrometer. A Shimadzu SIL HT autosampler was used with a Shimadzu 10ADvp HPLC. The run time was less than a minute with a total LC cycle time of less than ~1.5 minutes for all methods. All validated methods were used to support GLP tox studies or clinical studies. ISR (Incurred Sample Reproducibility) was performed for all the studies analyzed with fast LC-MS/MS methods. **Results:** Both the superficially porous and the small particle column provided satisfactory validation results and acceptable ISR results. However, the superficially porous column (Halo C18) is more robust compared to the small particle column (Phenomenex Max-RP). An interfering conjugated metabolite peak was observed during GLP tox sample analysis. A modification of mobile phase had to be made to resolve the two peaks for accurate integration. Therefore, risks associated with running fast LC should be taken into account when evaluating methods to be used for regulated studies.

Abstract 155

A uHPLC-MS/MS Method for the Quantitation of a Drug Candidate in Rat Plasma with Three Drug-related Compounds Present

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Introduction: Ultra High Performance Liquid Chromatography (uHPLC) using sub-2 μm columns has become a popular technique for bioanalytical assays using LC-MS/MS. One reason is that it can greatly increase sample throughput but still maintain the required separation for a given analyte. Here we report a fast uHPLC-MS/MS bioanalytical method for the quantitation of a drug candidate in rat plasma. Based on the discovery data, three drug-related compounds may be present in incurred samples. Therefore, a good separation of the analyte from these interference compounds is critical for the success of this method.

Methods: This method utilizes a Leap 4x Ultra (Flux) pump to deliver the mobile phases.

Chromatographic separation was achieved with gradient elution on a Waters Acquity UPLC BEH C8, 1.7 μm , 2.1 \times 50 mm column. The mobile phase contained water, acetonitrile, ammonium bicarbonate and ammonium hydroxide. Detection was accomplished using a Sciex API 4000 tandem mass spectrometer in positive ion electrospray SRM mode. **Results:** In this method, four compounds were well separated with a run time of 1.5 minutes. This assay showed excellent accuracy, precision and reproducibility. The intra-assay and inter-assay precision was within 4.4% CV and 4.8% CV respectively. The assay accuracy was within 3.3% of the nominal concentration values. In the incurred sample reanalysis test, the reanalysis results were within 10% of the mean of the repeat and the initial values for all 20 samples tested. This assay has been successfully applied to multiple GLP toxicology studies.

Abstract 156

Validation of a Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of Latanoprost Acid in Plasma From Pediatric Subjects

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Objective: To develop and validate a sensitive LC-MS/MS method (10 pg/mL) to determine latanoprost acid (LA) concentrations in plasma from pediatric subjects. Latanoprost is the ester prodrug of latanoprost acid used to treat ocular hypertension. **Methods:** Liquid-liquid extraction was applied to 0.5 mL of plasma fortified with latanoprost acid-d4 as internal standard (IS). The organic layer was applied to a SPE column and LA was eluted with a solution of formic acid, hexane, and isopropanol 2:40:60 (v/v/v). Using column switching, HPLC was performed with an Aquasil 2.1 mm x 50 mm, 3 μm loading column and a Restek 2.1 mm x 50 mm biphenyl, 5 μm elution column at a flow rate of 250 μl per minute with a 12 minute gradient starting at 50:50 acetic acid (0.005%) / acetic acid (0005%) in methanol (v/v). Upon elution of LA from the HPLC, detection and quantification was achieved with a Sciex API 5000 mass spectrometer using electro-spray ionization (ESI). Negative ions were monitored in MRM mode with m/z transitions for LA at 389.3 to 146.9 and IS at 393.3 to 147.1. **Results:** The method was validated over a range of 10 to 600 pg/mL with correlation coefficients of >0.99. Inter-batch accuracy (%RE) and precision (%CV) for quality control samples ranged from -7.16% to -0.392% and \leq 10.1% for accuracy and precision, respectively. **Implications:** A sensitive and accurate method to determine latanoprost acid in human plasma was developed, validated and successfully applied to the quantification of LA in clinical samples from pediatric subjects following ocular administration of latanoprost.

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A Novel Technique for Monitoring the Matrix and Metabolites while Simultaneously Detecting Ibuprofen by MRM

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Objective: The detection of ibuprofen while simultaneously monitoring the matrix for potential metabolites or interferences. **Methods:** A bioanalytical assay was used for the analysis of ibuprofen in urine. Urine was taken from 2 subjects 8 hours after dosing with 400mg of ibuprofen. Simple sample preparation by centrifugation and dilution was employed prior to injecting the samples on to an LC/MS/MS system. Separation was performed on a 2.1 x 50mm, 1.7 μ m C column. The analytes were detected by negative electrospray ionization in multiple reaction monitoring mode for the selective detection of ibuprofen. The MS conditions were also set to allow the simultaneous acquisition of full ms scan data. **Results:** The full scans acquired for each volunteer demonstrated the variability of matrix between the two subject's urine. Furthermore, the full scan was mined for parents of likely metabolites. Peaks with m/z values which corresponded to common metabolites were identified. The ibuprofen was detected using the transition 205->161. **Implications:** Use of novel technology to simultaneously scan the matrix of a bioanalytical sample whilst detecting, an analyte of interest will allow the scientist to be aware of any changes in matrix which might affect the ruggedness or accuracy of a bioanalytical assay and also to monitor for any unexpected metabolites of the analyte or of over the counter medications.

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Evaluation of 96-well Phospholipid Depletion Plates for LC-MS/MS Bioanalysis.

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Objectives: Determine the effectiveness of phospholipid depletion (PLD) plate technology and assess their utility for routine use in regulated bioanalytical assays. **Methods:** Human plasma was precipitated on a standard 96-well Strata Impact protein precipitation (PPT) plate, a 96-well Varian ND^{lipids} plate or a Supelco Hybrid SPE plate. In each plate, 100 μ L of human plasma was added to 300 μ L precipitation solvent (acetonitrile). The sample eluent was collected, evaporated to dryness under N₂ at 40°C and reconstituted in 0.2 mL of appropriate reconstitution solvent depending on chromatography conditions. Samples were injected into an API4000 tandem mass spectrometer using positive electrospray ionization in MRM mode. Hydrophilic interaction chromatography (HILIC) was used to determine the percent depletion of the most prevalent phospholipids found in protein precipitation extracts. Reversed-phase chromatography was used to assess the impact PLD plates have on ionization and matrix effect experiments routinely conducted during assay validations. Data was quantified by measuring peak area ratios. **Results:** Nearly 100% of endogenous phospholipids can be removed from protein precipitation extracts. Pfizer test compounds demonstrated that ionization and matrix effect data could change depending upon the use phospholipid depletion technology. Data indicate PLD plates significantly improve matrix effect and ionization effect data when using an analog IS for drug quantitation. **Implications:** Currently available phospholipid depletion plates do offer a practical solution for removing phospholipids from precipitated human plasma samples. Using depletion plates to assay samples for routine regulated bioanalytical assays can lead to improved ionization and matrix effect data.

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Method Development Strategies For Ultra High-Throughput LC-MS/MS Analysis of Small Polar Molecules Utilizing HILIC Mechanisms

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Introduction: HILIC has proved to be a robust and selective tool (from isobars and phospholipids) for analysis of small polar molecules, enabling ultra-high-throughput-LC-MS/MS methodologies (>2000 samples/instrument/day). This paper describes strategies wherein functional groups, sorbent-bed selection, and non-intuitive mobile phase chemistries can be utilized to provide selective, suppression-free mass spectrometric detection of small polar analytes. **Methods:** Nicotine (NN), Succinic Acid (SA), and Orotic Acid (OA), (all <200 amu) containing carboxylic acids and/or amines, were spiked into DI water and human plasma at 500 ng/mL. 300 μ L sample was precipitated with 1200 μ L acetonitrile; samples were vortexed, centrifuged, and 10 μ L supernatant was injected. 52 mobile phase modifications on 8 HILIC-style columns were screened; comparison of retention times for analytes and phospholipids were used to elucidate developmental workflows for the selective shifting of phospholipids and isobaric interferences. **Results:** Chromatographic selectivity based on modifications in acid, buffer, and protic solvent concentrations was evaluated by monitoring the retention times of analytes, and phospholipid classes (phosphatidyl/lysophosphatidyl cholines). Protic organic solvent elution on silica and HILIC sorbent beds contributes to the early elution of phospholipids and analytes lacking amines (SA), whereas the retention of aminated (NN/OA) molecules is relatively unchanged with protic solvents in the presence of buffering materials. Selectively shifting isobaric interferences and phospholipids from carboxylic acids, regardless of amine content, is achieved by utilizing ion exchange mechanisms derived from volatile buffers, charge state modifications of phospholipids in solution and aqueous content in gradient chromatography. Stepwise developmental workflows will be shown; enabling these mechanisms to be elucidated.

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The Gyrolab: A New Frontier in Immunoassay?

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Objectives: The use of enzyme-linked immunosorbent assay (ELISA) has been the gold standard for the quantitation of biomolecules from complex media. In general an ELISA is carried out over a two day period of time where multiple reagents are incubated for varying lengths of time in a 96-well plate. ELISA methods are normally very selective and sensitive however the range is often limited to less than two orders of magnitude making multiple dilutions mandatory for the analysis of toxicokinetic studies where plasma concentrations can exceed 1 mg/mL. **Methods:** The Gyrolab is a compact disc based system that performs all steps of an immunoassay for 112 samples in 50 minutes with detection by laser induced fluorescence and allows ranges on the order of four orders of magnitude. **Results:** To test the utility of the system, we converted an in-house ELISA method for the quantitation of a humanized monoclonal antibody (range 100 to 2500 ng/mL) to the Gyrolab platform. The Gyrolab allowed a range of 10 to 10,000 ng/mL in rat plasma (with a maximum %CV of 14.1% and a maximum Bias of -11%) and 250 to 100,000 ng/mL in human plasma (with a maximum %CV of 8.3 and a maximum Bias of 13.6%). **Implications:** The Gyrolab is an easy to use fully automated way to run immunoassays. It allows the user to speed up method development time, increase sample throughput and decrease the amount of time spent diluting samples.