

Abstract 145

Species Relative Plasma Protein Binding.

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Objectives: The measurement of the free drug concentration in plasma protein binding assays can be very difficult when dealing with highly bound compounds (greater than 98%). However, knowledge of the relative concentrations of the free fraction amongst species is often critical in the drug development process to assure that *in vivo* dosing produces drug levels that are efficacious, but also within toxicity limits of the species to be studied. There is an inherent error in the determination of the free fraction in each individual species due to analytical challenges in the measurement of a low concentration, and this error can multiply when comparing the free fraction of multiple species. **Methods:** This poster will discuss the successes and challenges we have encountered while implementing a species competitive assay within our laboratory, applying the assay to a known highly bound reference compound, as well as to an internal compound. Also discussed will be the re-design of parts of the assay to make it practical, as well as the tailoring of commercially available 96-well dialysis plates for this experiment. Depending upon the experiment, either scintillation counting (for radio-labeled compounds) or LC/MS/MS was used to quantitate the amount of drug present in each matrix. **Results:** In summary, we will report the successful development of a species relative protein binding assay that can be used to compare the relative binding in plasma for up to four species, for even highly protein bound compounds. **Implications:** Relative binding among species can be determined in one single experiment.

Abstract 146

Using Design of Experiments as a Tool in Bioanalytical Method Development: Case Studies

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Objectives: Bioanalytical laboratories employ a variety of assay methods and technologies as tools to support biotherapeutic drug development. During assay development, improved assay robustness and quality with efficient timelines can be achieved by using Design of Experiments (DoE). DoE is a statistical approach to experimentation for determining cause/effect relationships between input factors and output responses. DoE identifies and optimizes significant factors affecting bioanalytical methods, and is a troubleshooting tool when problems arise. **Methods:** DoE was used in developing different bioanalytical assay types; a cell-based reporter gene assay, a quantitative ELISA, an anti-drug antibody bead-based assay, and a flow cytometric assay. **Results:** In the different bioanalytical assays discussed, DoE was useful in identifying critical factors in the methods and uncovering interactions between factors. In the reporter gene assay, utilizing DoE maximized drug response and minimized intra-assay variability. In other assay formats, DoE resulted in reduction of significant serum interference, rapid optimization of bead/reagent concentrations and the development of a successful flow cytometric assay. **Implications:** The results show that assay factors in a range of bioanalytical methods were quickly identified as critical to assay performance and thus incorporated in the assay development. Some factor interactions were identified that would have been missed if one-factor-at-a-time experimentation had been used. The use of DoE software programs was important to ensure the statistical significance of the effects on assay responses and appropriate data modeling.

Abstract 147

Comparison of *In Vitro* Methods for Assessing CYP 3A4 Inhibition

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Objective: Cytochrome P450 based inhibition can affect bioavailability as well as clearance and can be an underlying cause of adverse drug-drug interactions. More rapid and sensitive methods for detecting CYP inhibition are continually being sought. **Methods:** Three *in vitro* methods for assessing CYP 3A4 inhibition were investigated using Abbott compounds and commercially available controls. A fluorescent method using 7-benzyloxyquinoline (BZQ), a novel bioluminescent method using luciferin-IPA, and the FDA recommended mass spectrometry based method using midazolam were examined. **Results:** All assays were reproducible, amenable to high throughput and correlative to the midazolam method but the bioluminescent assay had advantages over the others. The use of a bioluminescent substrate negates the problem of fluorescent interference of test compound which was a limitation of the BZQ assay. Additionally, the ease of analysis was far superior compared to the use of mass spectrometry in the midazolam assay which increased analysis time and sample preparation. Furthermore, the use of a Schedule IV controlled substance complicates the procedure. **Conclusion:** The luciferin-IPA bioluminescent assay offers a rapid, specific and reproducible method of screening for CYP 3A4 inhibitors and correlates well with the FDA recommended midazolam assay, making it an excellent ADME screening tool.

Abstract 148

Changes in Bile Salts Affect Metabolism and Elimination of Xenochemicals in Rats

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Objectives: Standard practices for bile duct cannulation (BDC) studies include either replacement or non-replacement of bile-salts. Non-replacement is a simpler model; no infusion pumps or additional second cannula surgery. We compared these BDC methods for effects on metabolism and drug elimination in bile. **Methods:** Test compound 1 (which migrated with bile-salts and was extensively metabolized) and Diclofenac were compared under both methods. Bile was analyzed by LC-MS and/or flow scintillation radioactivity detection. Rat physiology was assessed by visual, clinical blood chemistry, and RNA microarray analysis of the liver. **Results:** Without bile-salt replacement, peak separation, metabolite ion intensity, and quality (MS/MS) of ESI mass spectra were improved. However, changes in metabolite elimination rate and metabolite profile were observed. With bile salt replacement, test compound 1 had predominant metabolites: O-glucuronide (M3) and the sulfate mono-oxide (M6). Without replacement, M3 was greatly reduced and a carboxylic acid metabolite (M1) became one of the most abundant metabolites. Phase II metabolism was impaired and drug elimination was slowed in animals not receiving replacement bile salts. Without bile salt replacement, rats dosed with Diclofenac produced novel metabolites and exhibited decreased drug elimination. **Implications:** Bile salt replacement is needed to avoid changes in metabolism and increased drug exposure. The presence of new metabolites and altered metabolism indicates that changes in bile-salt homeostasis can have acute effects on liver function. Diclofenac's acute liver toxicity may result from formation of new reactive metabolites caused by changes in liver bile salt homeostasis.

Abstract 149

Comparison of RapidFire® Ultra High Throughput MS with Conventional LCMS/MS for Cytochrome P450 Inhibition Testing

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Objective: Assessment of CYP inhibition has moved into earlier phases of drug discovery/development. Coupled with recent guidance from FDA recommending testing with at least six P450 isoforms, demand for higher throughput solutions has increased. The objective of this study was to apply RapidFire mass spectrometry technology (BioTrove, Woburn MA) to *in vitro* CYP inhibition testing and compare results to conventional LC/MS/MS. **Methods:** Seven-point IC₅₀ values for a range of inhibitors were determined in individual incubations in human liver microsomes using the FDA recommended drug probe substrates with validated assay methods [Perloff ES et al (2009) *Xenobiotica* 39:99-112]: CYP1A2/ phenacetin (tacrine for RapidFire); CYP2B6/ bupropion; CYP2C8/ amodiaquine; CYP2C9/ diclofenac; CYP2C19/ S-mephenytoin; CYP2D6/ dextromethorphan; CYP3A4/ testosterone, midazolam. Samples were split and analyzed either by RapidFire technology or conventional LC/MS/MS methods. Stable-labeled isotope internal standards were used for all probe substrate metabolites except for 1-hydroxytacrine. **Results:** IC₅₀ values obtained using RapidFire analyses (~ 7 sec cycle times) were consistent with the data obtained using conventional LC/MS/MS methods. Greater than 90% of corresponding IC₅₀ values were within 2.0-fold of each other. **Implications:** The increased analysis speed represents a >20-fold improvement in cycle time, thereby permitting rapid data delivery to project teams and clients at lower cost. Importantly, the ultra-rapid analysis allows acquisition of more data points per unit time. Thus, RapidFire analysis provides the option of conducting robust, multipoint drug-development style assays in the drug discovery stage.

Abstract 150

Assessment of P-gp, BCRP and MRP Activity in Caco-2 and MDR1-LLC-PK1 Cell Monolayers

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Objectives: Drug intestinal permeability and transporter interactions are often characterized using *in vitro* models Caco-2 and LLC-PK1 transfected with MDR1 (MDR1-LLC-PK1). Prominent efflux transporters expressed in Caco-2 cells are P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) whereas MDR1-LLC-PK1 cells predominantly express human P-gp. **Methods:** In this study, the activity of transporters was characterized in Caco-2 and MDR1-LLC-PK1 cells by measuring bidirectional permeability and efflux ratios of radiolabeled probe substrates in the presence or absence of efflux transporter inhibitors. Substrates were digoxin for P-gp, estrone-3-sulfate (E3S) for BCRP and leukotriene C4 (LTC4) for MRP. **Results:** In Caco-2 cells, digoxin efflux ratio was 15, which was significantly attenuated upon addition of P-gp inhibitors ketoconazole, elacridar, quinidine and verapamil. Similarly, E3S efflux ratio of 19 decreased considerably upon addition of BCRP inhibitors novobiocin and fumitremorgin C (FTC). An efflux ratio of 1.1 was observed for LTC4, suggesting negligible MRP activity present. Similarly, in MDR1-LLC-PK1 cells, digoxin efflux ratio was 14 and attenuated by P-gp inhibitors. Qualitative and quantitative P-gp inhibition was similar across the two cell lines. In MDR1-LLC-PK1, efflux ratios of 1.2 and 1.0, were found for E3S and LTC4, respectively. Control LLC-PK1 cells, showed negligible transporter mediated efflux in all 3 transporters examined. **Implications:** Caco-2 cells showed strong P-gp and BCRP mediated efflux activity, whereas MDR1-LLC-PK1 cells exhibited strong P-gp, but negligible BCRP activity. Neither cell line exhibited MRP mediated efflux. Because of relatively high BCRP activity in Caco-2 cells, these data suggest caution when interpreting Caco-2 data as a sole means for characterizing substrates or inhibitors P-gp.

Abstract 151

Elucidate CYP3A4-Catalyzed Hydrocarbon Hydroxylation and N-dealkylation Mechanisms using Docking and Quantum Chemical Calculations

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Objectives: Molecular docking and quantum chemical calculation methods were applied in combination with *in vitro* and *in vivo* metabolite identification to study CYP3A4-catalyzed biotransformation of several Pfizer drugs, including dirlotapide, a selective microsomal triglyceride transfer protein inhibitor, torcetrapib, a cholesteryl ester transfer protein inhibitor, and CP-533,536, a selective prostaglandin E2 agonist. In particular, the mechanisms of regioselectivity for hydrocarbon hydroxylation and N-dealkylation were investigated. **Methods:** The geometries of molecule structures were optimized before docking performed within the three-dimensional structures of CYP3A4. The searching was based on a genetic algorithm and scoring functions combined the calculation of van der Waals, hydrogen bonding and electrostatic interactions between substrates and CYP3A4. For quantum chemical calculations, the energy difference initiated from hydrogen atom abstraction to form radicals was calculated by density functional theory. **Results:** Although hydrocarbon hydroxylation has a relative higher oxidation potential than N-dealkylation of the studied compounds, the substrate accessibility to heme iron of CYP3A4 plays a more significant role in determining the reaction outcome. For example, the hydroxylation of the *tert*-butyl moiety of CP-533536 and the ethyl group of torcetrapib were found to be the major metabolic pathways instead of the N-dealkylated products. **Implications:** While CYP3A4 has a relatively large active site cavity when compared to other P450 cytochromes, the interactions between the substrate and protein structure within its active site cavity and the chemical reactivity of potential oxidation sites on the molecule must be considered together when predicting sites of metabolism and the consequent considerations for drug design.

Abstract 152

Single Incubation Approach for the Determination of Michaelis-Menten Constants for CYP-Mediated Biotransformations

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Objective: the determination of enzyme kinetic parameters, K_m , V_{max} , and CL (V_{max}/K_m), for CYP-catalyzed reactions is an important aspect in drug discovery and development. In discovery, these *in vitro* data can be used in compound design to optimize human pharmacokinetic (PK) behavior of new chemical entities. In early stages of development, these data can be helpful in predicting non-linear PK due to saturation of a drug metabolizing enzyme, or modeling a potential drug-drug interaction. **Methods:** a single incubation automated approach for determining the Michaelis-Menten kinetic constants for a single recombinant CYP and one substrate, using metabolite(s) monitoring (method 1) or substrate depletion (method 2). In this experiment, initial enzyme concentration and incubation time linearity are also determined from the same incubation. In the case of metabolite(s) monitoring, synthetic standards are required to quantify formation of product(s). During the discovery and early development stages these standards or the information on the metabolites formed are usually not available. Therefore, multiple substrate depletion curves can be utilized to derive the K_m and V_{max} using a modified Michaelis-Menten equation. **Results:** this approach was validated using midazolam hydroxylation, a known CYP3A4/5 substrate using methods 1 and 2. An unknown compound (ABT-xyz) was also tested using the same methods. The K_m value for midazolam from methods 1&2 were in agreement with the literature values. The Michaelis constants for ABT-xyz from both methods were consistent. **Implications:** this single incubation method is a practical approach for discovery and early development to obtain enzyme kinetics parameters for lead compounds.

Abstract 153

Comparative Analysis of CYP450 Activity and mRNA Expression in a Hyper-Induced Liver Environment

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Objectives: Standard cytochrome P450 enzyme induction analysis examines the induction of an individual P450 enzyme, CYP1A2, CYP2B6, or CYP3A4. If a patient is taking multiple drugs, the possibility exists that multiple P450 enzymes are induced simultaneously resulting in a hyper-induced liver environment. The current study examines the feasibility of utilizing a cocktail method containing known positive control inducers of CYP1A2, CYP2B6, and CYP3A4 in fresh primary human hepatocyte cultures, thereby producing a hyper-induced liver. **Methods:** Primary human hepatocytes were cultured in the presence of vehicle (0.1% DMSO), omeprazole (OMP; 50 μ M), 3-methylcholanthrene (3-MC; 2 μ M), Phenobarbital (PB; 1000 μ M), rifampicin (RIF; 10 μ M), Cocktail 1 (OMP, PB, RIF), or Cocktail 2 (3-MC, PB, RIF) for 48 and 72 hours prior to mRNA and enzyme activity analysis, respectively. Total RNA was isolated for each induced sample. qRT-PCR was utilized to quantify CYP1A2, CYP2B6, and CYP3A4 mRNA content. Individual P450 enzyme substrates for CYP1A2 (100 μ M phenacetin), CYP2B6 (500 μ M bupropion), and CYP3A4 (200 μ M testosterone) were incubated with hepatocyte monolayers (*in situ*) to determine cytochrome P450 enzyme activities. Incubation samples were extracted and analyzed using LC-MS/MS. **Results:** Preliminary results indicate that hepatocytes remain viable in the presence of the positive control inducer cocktail. Further analysis will involve comparison of the data from the hyper liver induction analysis with that derived from the traditional singly induced P450 analysis (mRNA and P450 enzymatic activities). **Implications:** Hyper liver induction could provide a method for examining potential drug-drug interactions of new chemical entities.

Abstract 154

Metabolite Profiling of 14 C-labeled Drugs in Different Matrices using UPLC and Quantitation with Accelerator Mass Spectrometry (AMS)

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Objective: The use of AMS to identify and quantify carbon-14 labeled compounds in the fg/mL range for both parent and metabolites relies on chromatography that is rugged across both matrices and species. We summarize here our experience in comparing UPLC analysis of metabolites across various matrices and species. **Methods:** We have reviewed the extraction and chromatography performance of several profiling methods involving human, rat and dog plasma, urine and fecal matrices. We have also evaluated the effectiveness of several extraction techniques in terms of radiocarbon recovery and UPLC chromatography in terms of temporal resolution, separation of metabolites found and percent recovery throughout the process. **Results:** We have found that UPLC-AMS is applicable to both matrices, and that agreement and reproducibility between matrices for the same analyte is excellent, using UPLC-AMS. Urine can be prepared by filtration, but plasma - particularly at the later time points - can require exhaustive extraction techniques. **Implications:** We find that UPLC-AMS is rugged, reproducible and reliably enables comparison of metabolite profiles across matrices and species. The temporal resolution from UPLC is excellent and provides a reliable platform for AMS quantitation of complex metabolism.

Abstract 155

Enantio-selective and Species-Dependent Carbonyl Reductase Metabolism of LX6171 in Preclinical Models

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Introduction: An internally-developed small molecule drug candidate, LX6171, for cognitive disorders has demonstrated improved learning and memory in healthy and aged mice. It has also exhibited an acceptable profile in toxicology and safety studies and has completed a Phase II clinical trial. In preclinical studies, LX6171 is well behaved pharmacokinetically, with the in vivo metabolism being reflected by in vitro studies. The predominant in vitro and in vivo metabolic biotransformation involves the keto-reduction of the parent compound to a chiral metabolite "M1". **Objectives:** Studies were conducted to determine: a) what enzyme was involved in the metabolism to "M1", b) whether there was species-dependent metabolism to "M1", and c) whether there was enantio-selective metabolism to "M1", both in vitro and in vivo and in multiple species. **Methods:** LX6171 was incubated with mouse, rat, dog, monkey, and human liver microsomes and S9. To specifically investigate the keto-reduction, incubations in the presence and absence of inhibitors of carbonyl reductase were performed. Plasma samples from companion pharmacokinetic studies in the same species were also analyzed. An LC/MS method employing a chiral stationary phase was used for identification and semi-quantitation of each "M1" enantiomer. **Results:** Our results demonstrate that a) the keto-reduction to "M1" is mediated by a cytosolic carbonyl reductase, b) there is species-dependent metabolism to "M1" in vitro, and c) there was a consistent correlation of this enantioselective metabolism between the in vivo and in vitro data in all of the preclinical species studied.

Abstract 156

Luciferin Isopropyl Acetal: A New, Highly Selective and Sensitive Bioluminogenic CYP3A4 Substrate for Induction and Inhibition Assays

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Abstract: CYP3A4 induction and inhibition by drugs and other xenobiotics is a significant cause of adverse drug-drug interactions. To predict the potential for these outcomes early in drug discovery, compounds are tested for their capacity to induce or inhibit the conversion of a probe substrate by CYP3A4 to a specific product. We have synthesized a new, highly selective and sensitive CYP3A4 probe substrate, luciferin isopropyl acetal (luciferin-IPA), for use in rapid bioluminescent cell-based and cell-free CYP3A4 assays. With a panel of 21 recombinant human CYP enzymes luciferin-IPA only showed activity with CYP3A enzymes. Selectivity for CYP3A4 was 14 and 137 fold over CYP3A5 and CYP3A7, respectively. CYP3A4 selectivity was confirmed in a rapid, 96-well, intact hepatocyte assay where activity was increased by known CYP3A4 inducers and inhibited by CYP3A4 selective inhibitors. The luciferin-IPA/CYP3A4 hepatocyte assay was easily configured in a multiplex application with a cell viability assay. Cell free luciferin-IPA enzyme assays were sensitive to a wide range of known CYP3A4 inhibitors. The reaction was competitively inhibited by the CYP3A4 probe substrates midazolam, testosterone and nifedipine, suggesting it will detect inhibitors also detected by each of these commonly used probe assays. While luciferin-IPA/CYP3A4 induction and inhibition data was virtually identical to data from conventional CYP3A4 testosterone 6-beta hydroxylation assays, the luminescent assays were simpler, more sensitive and substantially quicker.