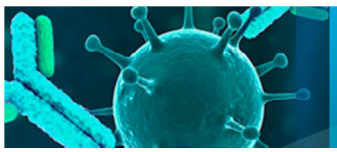

Comprehensive Metabolite Identification–A One-Stop Solution

Analysis of HepatoPac® co-cultured hepatocytes using SWATH™ Acquisition on a TripleTOF® 6600 System delivers a one-stop solution for qual/quant across multiple species in a single experiment

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Key challenges of metabolite identification in drug development

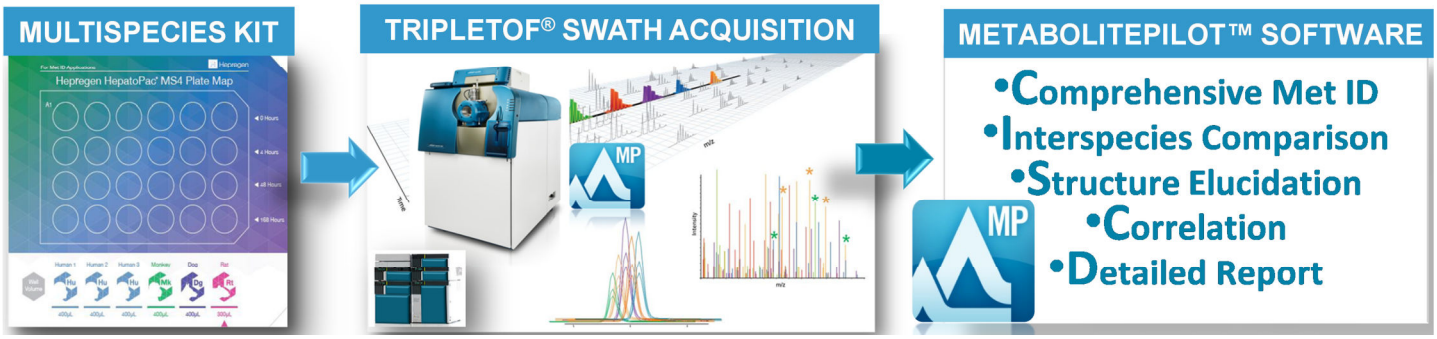
- **Incomplete metabolite profiles** – Variable *in vitro* enzyme expression (Cytochrome P450 and Phase II enzymes, drug transporters) and limited culture lifetimes constrain the production of all clinically relevant metabolites
- **Limited applicability of *in vitro* multi-species models** – Comparing metabolite profiles across different animal species is challenging, making it difficult to extrapolate relevant data from preclinical toxicology studies to humans.
- **Inefficient sample acquisition –** Gaps in metabolite information require sample re-analysis and repeated injections, decreasing productivity and impeding definitive structural assignments.
- **Too many met ID platform options –** Selecting the best, most comprehensive drug metabolism model for each new drug candidate can be overwhelming and time-consuming.

Key benefits of comprehensive metabolite ID

- **Full metabolite coverage for all species** – Hepregen's micro-patterned HepatoPac co-culture is a long-lived bioengineered liver model that delivers complete multi-species profiles, including all phase I, phase II and secondary metabolites formed during preclinical animal studies (human, monkey, dog, and rat).
- **Assured relevance of metabolite profiles –** The improved longevity of hepatic co-cultures (relative to primary hepatocyte cultures, microsomes, and S9 fractions) supports long-term drug incubations for more extensive investigations of pharmacokinetics, superior identification of slow-forming toxins, and increased applicability for toxicology modeling.
- **Simplified discovery of unknown metabolites –** Use of a generic, data-independent acquisition method (SWATH™ Acquisition) identifies both predicted, *in vivo* relevant and low-level metabolites, reducing surprises in phase I trials.
- **Consolidated and efficient metabolite ID strategy** – Concurrent bioanalysis of multiple species in a single-plate format reduces laboratory overhead costs and improves throughput. Quantitative identification of all metabolites—both known and unknown—ensures confident metabolite profiling.

Key features of comprehensive met ID

- **Extended hepatocyte longevity** – The hepatic phenotype is stabilized by the unique architecture of co-cultured cells, improving viability from a few days to up to four weeks.
- **Streamlined, industry-standard platform –** Micro-patterned hepatocyte co-cultures are plated in a single 24-well format, a design suitable for the simultaneous analysis of multiple species and high-throughput screening.
- **Optimized hepatocyte performance –** Metabolic activity is cultivated by enhanced cell-cell, cell-matrix and soluble-factor interactions, yielding the most accurate metabolic phenotypes.
- **Comprehensive, inter-species metabolite correlation –** MetabolitePilot™ Software SWATH algorithm deconvolutes complex MS/MS data for both qualitative and quantitative analysis with built-in structural elucidation and cross species comparison



A comprehensive solution for complete, across-species metabolite identification: The Multi-Species HepatoPac® Kit, SWATH™ Acquisition on a TripleTOF® 6600 System, and data analysis with MetabolitePilot™ Software.

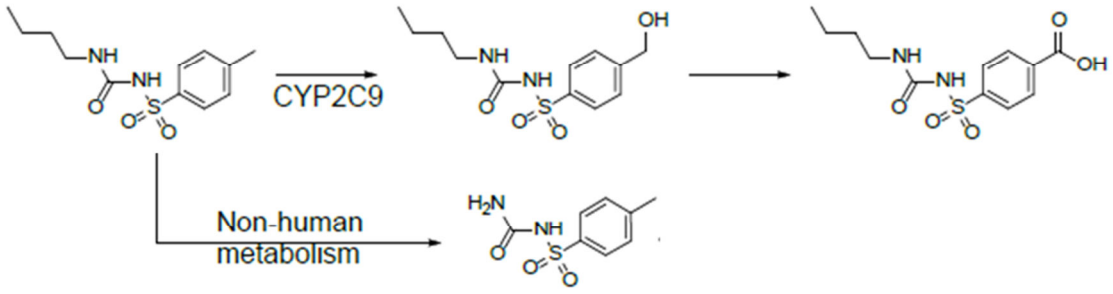


Figure 1. Tolbutamide metabolism. Human and non-human metabolic pathways for tolbutamide are shown (figure excerpted from Gee *et al*, 1984 and Thomas and Ikeda, 1966).

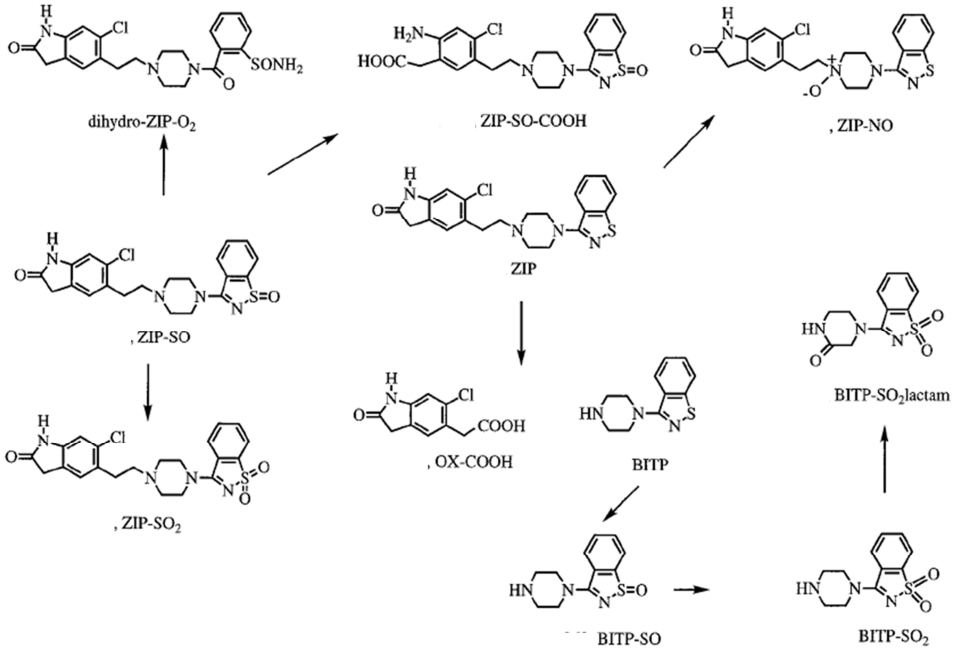


Figure 2. Ziprasidone metabolism. The pathways for the formation of the major metabolites of ziprasidone are shown (figure excerpted from Prakash, *et al*., 1997).

SUMMARY

The determination of metabolic stability and the accurate prediction of major human metabolites are critical for the optimization of drug candidates. Traditional models for evaluating drug metabolism such as sub-cellular fractions and suspended hepatocytes are short-lived and have a limited applicability for monitoring multi-generation metabolites or drug clearance kinetics, particularly for slowly metabolized compounds. Furthermore, traditional LC/MS instrumentation approaches lack the sensitivity to measure metabolites within the therapeutic dosing range, often resulting in the need to generate metabolite profiling and drug clearance data from separate samples. In this study, we simultaneously identified and quantified metabolites, as well as evaluated drug clearance kinetics from the same sample using 1 and 10 µM concentrations. Multi-generational drug metabolites obtained from a multi-species hepatocyte co-culture model (HepatoPac) were analyzed on a high-resolution TripleTOF® 6600 instrument with data-independent SWATH acquisition for simultaneous metabolite ID and relative quant across multiple time points

INTRODUCTION

One of the primary objectives of pre-clinical safety and toxicity studies is to identify human metabolites that differ from those in animal models. The FDA Guidance for Industry (MIST, FDA 2008) recommends that comparisons of *in vitro* inter-species metabolites occur early in the drug development process to enable more accurate predictions of a drug candidate's safety performance during clinical trials. Understanding the major pathways of a drug's metabolism makes it easier to evaluate parameters surrounding drug clearance, drug-drug interactions, and possible inter-patient differences in drug response. This

generates a clearer picture of a drug's stability and toxicity profiles and reduces the possibility of discovering unforeseen, major metabolites in a clinical setting, a situation that could potentially jeopardize a drug's continued development.

Preclinical studies are largely conducted in animal models, and the applicability of toxicity data derived from non-human studies to humans depends largely on whether humans are exposed to parallel chemical entities. An *in vitro* approach that can reliably predict *in vivo* human metabolite profiles is highly desirable due to the expense and logistical challenges of animal studies; however, many current *in vitro* animal model systems cannot accurately replicate human metabolic activity. Additionally, those *in vitro* systems (*e.g.,* isolated primary hepatocytes, microsomes, S9 fractions) that express analogous drug metabolism enzymes or require supplementation with cofactors to simulate metabolic activity are unsuitable for long-term drug studies due to rapid decline in liver-specific functions within a few hours or days.

The Multi-Species HepatoPac® Kit is a novel tissue-engineered hepatic co-culture model that retains phenotypic stability over several weeks, enabling long-term biotransformation studies and comprehensive, multi-generational metabolite production. A head-to-head study of 27 diverse compounds with known *in vivo* human metabolite profiles revealed that HepatoPac outperforms microsomes, S9 fractions, and primary human hepatocyte suspension cultures.[1] These HepatoPac metabolic identification studies found up to 75-80% of the *in vivo* excretory and circulating drug metabolites, while also identifying 41% more metabolites and 76% more secondary metabolites during long-term incubations.[1] Providing extensive coverage of interspecies metabolites in a stable cell-culture environment addresses a major drawback of *in vitro* metabolite studies and offers a viable solution for reducing the costs and timeframes surrounding early-stage, inter-species metabolic profile comparisons.

By coupling high-resolution, sensitive bioanalysis with inter-species drug incubations in a long-term co-culture system, we developed a strategy for simultaneously quantifying and identifying human metabolites from drugs with highly varied structures and biotransformation pathways (tolbutamide[2-3], ziprasidone[4], and linezolid[5], **Figures 1, 2, and 3**, respectively) in a relevant pharmacokinetic timeframe. To evaluate known and unknown metabolites, a comprehensive, data-independent acquisition method, MS/MS^{ALL} with SWATH™ Acquisition, was used to collect high-resolution MS and MS/MS data on a high-speed TripleTOF 6600 LC-MS/MS System, providing the detailed information needed for the accurate identification of inter-species drug derivatives.[6] Additionally, the longevity of the co-cultured hepatocytes permitted extended studies of

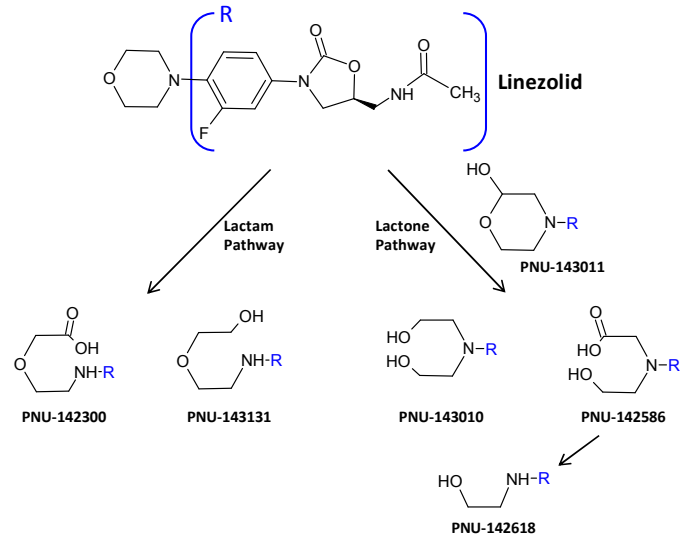


Figure 3. Linezolid metabolism. The biotransformation of linezolid occurs via 1) the lactam pathway or 2) the lactone pathway (figure excerpted from Slatter, *et al*., 2001).

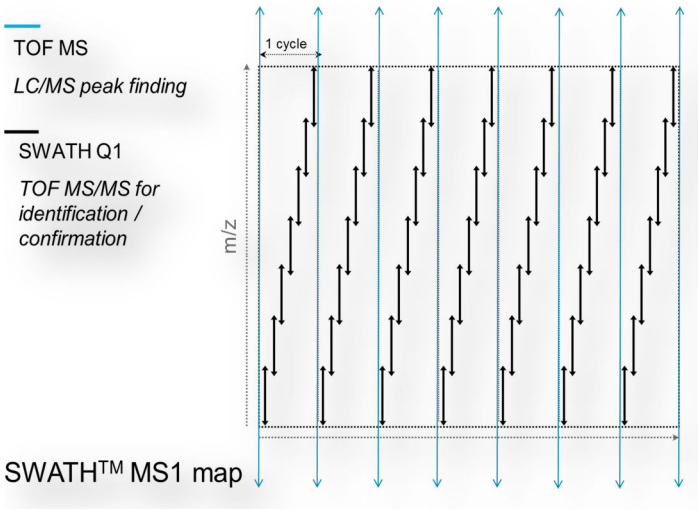


Figure 4. SWATH™ Acquisition strategy. During each TOF MS cycle, wide isolation windows that stepped across the mass range in 25 Da *swaths* are used to collect high-resolution, composite MS/MS spectra.

biotransformation pathways for each drug, yielding in-depth kinetic assessment of inter-species drug turnover.

MATERIALS AND METHODS

Experimental conditions and sample preparation

Tolbutamide, ziprasidone, and linezolid were purchased commercially, and 24-well multi-species hepatocyte co-culture plates were from Hepregen Corporation. Varying drug concentrations (1 µM and 10 µM) were incubated for 0, 4, 48, and 168 hr (7 days) in human hepatocyte co-culture. To end

each reaction, cells were treated with ice-cold acetonitrile (400 µL) and lysed prior to collection of the total cell and media fraction and transferred to a collection reservoir (with scraping/disruption of the cell samples). Samples treated with 1 µM drug, were injected (10 µL) directly into the LC/MS for metabolite analysis.

LC/MS conditions

LC/MS analysis was performed on SCIEX TripleTOF® 6600 LC-MS/MS System and Exion LC system fitted with an IonDrive™ Turbo V Source. Below is a summary of LC/MS conditions:

LC System:	Exion System
Column:	Phenomenex Kinetex C18 (50 × 2.1 mm), 2.6 µM
Column Temp.:	40 °C
Injection:	10 µL
Flow Rate:	600 µL/min
Mobile Phase:	A) Water, 0.1% formic acid
	B) Acetonitrile, 0.1% formic acid

Gradient:

Time/min	*A%*	*B%*
0	95	5
1.0	95	5
3.0	5	95
4.0	5	95
4.1	95	5
5.0	95	5

Mass Spectrometry

System:	TripleTOF® 6600 LC-MS/MS System
Interface:	IonDrive™ Turbo V Source used in high-mass, positive-ion mode
Ion Source Gas 1 (GS1):	40
Ion Source Gas 2 (GS2):	70
Curtain Gas (CUR):	20
Temperature (TEM):	500 °C
IonSpray Voltage:	5500
CAD:	High
Scan Type:	SWATH, 25 Da windows, 100-800 Da

Data processing

MetabolitePilot™ 2.0 Alpha Software was used to process high-resolution TOF-MS survey scans and data obtained using MS/MS^{ALL} with SWATH™ Acquisition (**Figure 4**). Post-acquisition fragment ion XICs collected used this strategy to quantify and confirm the presence of all chemical entities in the sample, preserving the sample in a digital record that can be evaluated retrospectively as needed. Several algorithms such as principal component variable grouping (PCVG), multiple mass defect filtering (MMDF), isotope pattern filtering (IPF), common product ion and neutral loss and generic background subtraction were used to identify and confirm both predicted and unpredicted metabolites using sample control comparison logic. Structure elucidation was performed using a built-in semi-automated interpretation module for both MS/MS fragmentation interpretation and soft-spot identification.

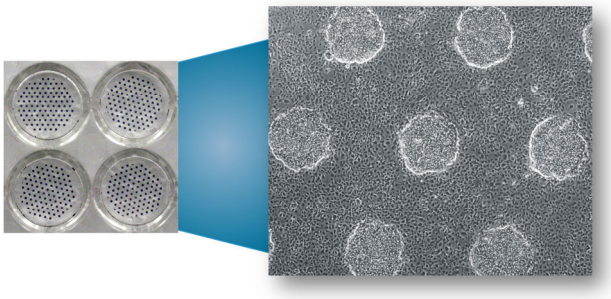


Figure 5. Micro-patterned, multi-species hepatocyte co-culture plates for long-term ADME/toxicology studies. Hepatocytes from multiple animal species (human, monkey, dog and rat) were co-cultured on a standard 24-well tissue culture plate in the Multi-Species HepatoPac® Kit (Hepregen Corporation). Cells are applied using a micro-patterning technique so that multi-species metabolite profiles can be captured in a single experiment under optimized cell-cell, cell-matrix, and soluble factor interactions.

Results and Discussion

Comparative metabolite studies with tissue-engineered, multi-species, hepatocyte co-culture plates

The major objective of *in vitro* metabolite comparison studies is to identify trends in metabolite formation across animal species, which requires comprehensive detection of the drug and its derivatives throughout all stages of drug development. Access to accurate multi-species drug profiles allows for easier elucidation of unique or disproportionately formed metabolites for a particular animal model. Traditional hepatocyte cell-culture conditions are unsuitable for long-term drug metabolism studies, and the challenging logistics of animal studies (*e.g.*, prolonged regulatory approval, limited lab space, escalating costs) can hinder the pace of development.

To address these challenges, a novel cell-culture model, the Multi-Species HepatoPac® Kit, was used to study the biotransformation of drugs of multiple animal species on a single, 24-well tissue culture plate (**Figure 5**). In this format, micro-patterned primary hepatocyte cells from three distinct human donors, as well as cells from monkey, dog, and rat donors were co-cultured with non-parenchymal cells, enhancing the longevity of *in vitro* models to support the study of both slowly and rapidly metabolized drugs. The longevity of the model is attributed, in part, to the use of controlled extracellular matrix cues that allow for precise mimicry of cellular ratios and cell-type specific interactions. These tissue-engineered co-cultures secrete albumin, synthesize urea, and metabolize compounds using active phase I and II drug metabolizing enzymes.[7]

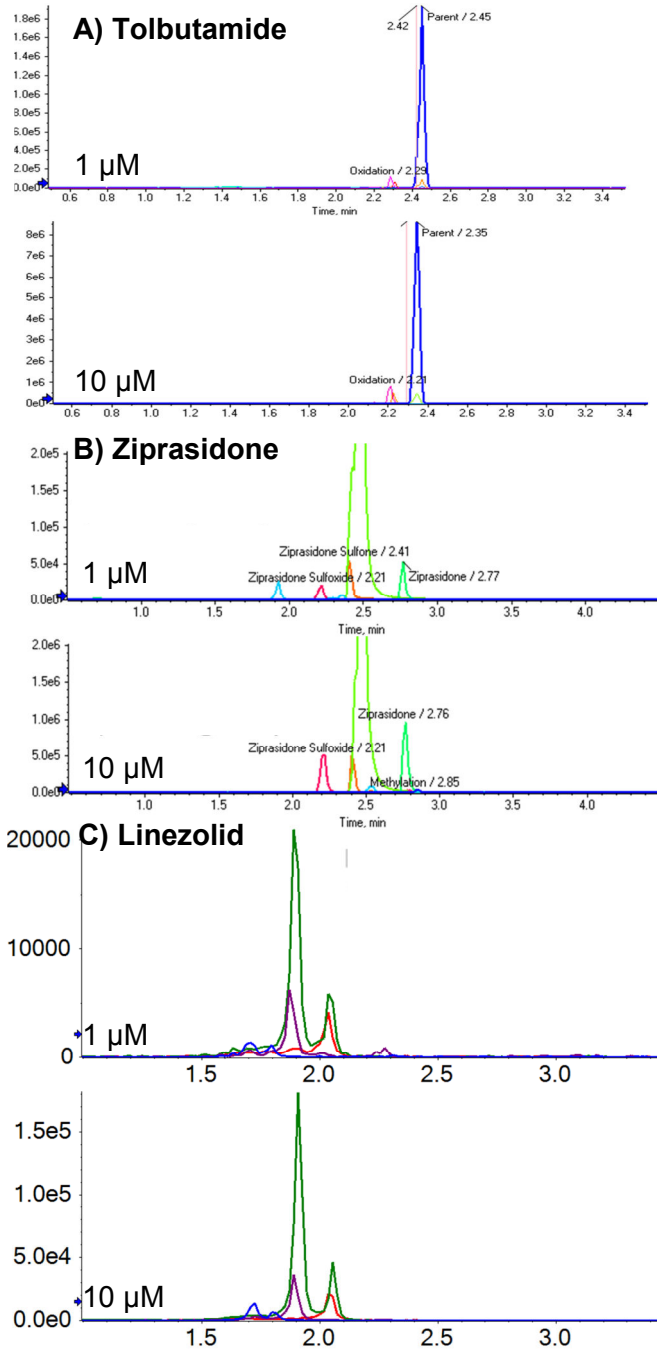


Figure 6. Comparisons of human metabolic profiles (pretreated with 1 µM vs. 10 µM drug). Multi-Species HepatoPac® hepatocyte co-cultures were treated with various drugs: (A) tolbutamide, (B) ziprasidone, and (C) linezolid at either 1 µM (*top panels*) or 10 µM (bottom *panels*). Metabolite profiles for human cells treated for 48 hr are compared across concentrations for each drug to evaluate instrument sensitivity and the concentration-dependence of metabolite generation.

In this study, we evaluated the capacity of the Multi-Species HepatoPacKit to generate distinct arrays of metabolites for human and pre-clinical species. Compounds with diverse chemical structures and biotransformation pathways that generate metabolites through a variety of species-specific reactions (tolbutamide[2-3] ziprasidone[4], and linezolid[5], **Figures 1, 2, and 3**, respectively) were incubated separately with co-cultured cells for a week-long period. Compound concentrations of 10 µM (an industry standard concentration traditionally used in metabolism ID studies) and 1 µM (a more physiologically relevant concentration) were used for the simultaneous generation of multiple inter-species metabolic profiles

At the completion of compound incubations, metabolites were detected and quantified using high-resolution, data-independent survey scans that provided comprehensive metabolite identification and structure elucidation with high sensitivity. These studies confirm the utility of MS/MS^{ALL} with SWATH acquisition for assessing metabolite profiles obtained from a co-culture hepatocyte model, generating data that 1) identify all possible phase I, phase II metabolites, and 2) quantify the kinetics of metabolite formation for the correlation of clearance rates of parent compound with the corresponding appearance of its derivatives.

Comprehensive metabolite identification using MetabolitePilot™ Software

The concentration-dependence of metabolite formation was assessed by comparing profiles obtained from incubations of co-cultured hepatocytes with two levels of parent compound. Additionally, these dual-concentration profile comparisons were used to assess instrument sensitivity and ensure that even low-level metabolites obtained from lower, more physiologically relevant doses, can be detected. Evaluation of human metabolic profiles for the drugs at 1 µM and 10 µM incubations reveals consistent metabolite coverage for each dose, with XICs for each concentration displaying the same metabolite profiles for each drug (**Figure 6**). Furthermore, these profiles demonstrate that that there is sufficient sensitivity to evaluate low-level metabolites formed during physiologically relevant dosing regimens.

To highlight which metabolites are conserved or unique across animal species, the human metabolite profiles were further compared to profiles generated from monkey, dog and rat studies. Inter-species metabolites and the pathways responsible for their formation are captured in **Figure 7, Table 1** and **Figure 8** for each drug incubated in the hepatocyte co-culture model. The results demonstrate a range in outcomes, with some drugs retaining metabolites across species, and some drugs displaying increased profile variability between animal species. Overall, the high-resolution LC-MS/MS system used in these studies was highly robust, demonstrating a capacity for detecting a wide-diversity of drug metabolites in various animal models.

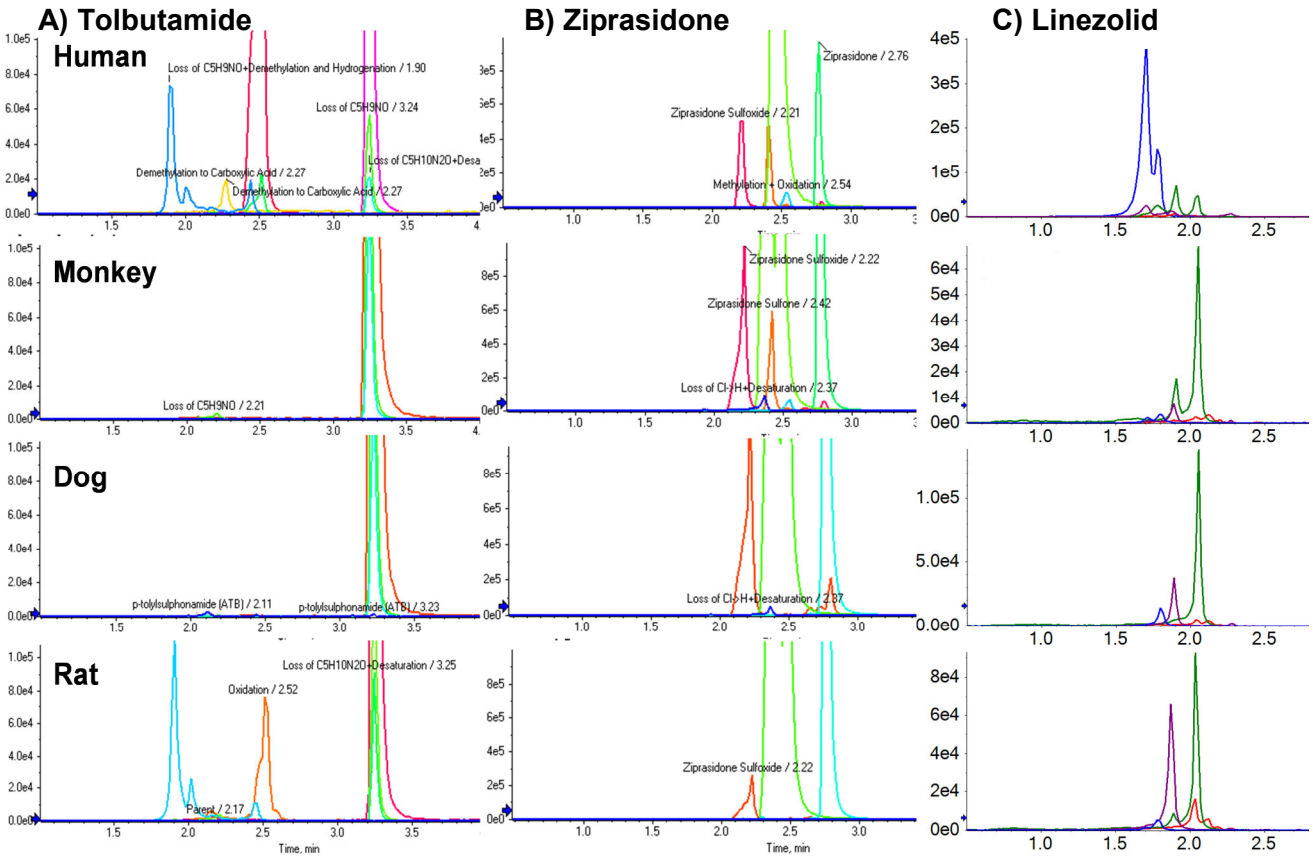


Figure 7. Multi-species metabolic profiles. These chromatograms provide an overview of the metabolic profiles for A) Tobutamide, B) Ziprasidone, and C) Linezolid after 7-day HepatoPac® incubations in human (*top panels*), monkey (*second-from-top panels*), dog (*second-from-bottom panels*), and rat (*bottom panels*) hepatocytes.

Table 1. Major metabolites formed after 7 days during multi-species drug metabolism studies on HepatoPac® co-culture plates

Compound	*In vivo* human metabolites	Main human enzymes involved	Metabolites detected in Multispecies HepatoPac			
			Human	Monkey	Rat	Dog
Tolbutamide	*Hydroxy-tolbutamide*	CYP2C9	*Yes*	*Yes*	*Yes*	*Yes*
	Carboxy-tolbutamide		*Yes*	*ND*	*ND*	*ND*
	Amide-hydrolysis product	*Non-human pathway*	*ND*	*Yes*	*Yes*	*Yes*
Ziprasidone	*Ziprasidone-sulfoxide*	*CYP3A4, A0*	*Yes*	*Yes*	*Yes*	*Yes*
	Ziprasidone-sulfone		*Yes*	*Yes*	*Yes*	*Yes*
	S-methyl-dihydro-ziprasidone		*Yes*	*Yes*	*Yes*	*Yes*
Linezolid	*PNU-142586*	*Lactone*	*Yes*	*ND*	*ND*	*ND*
	PNU-143010		*Yes*	*Yes*	*Yes*	*Yes*
	PNU-142618		*ND*	*ND*	*Yes*	*ND*
	PNU-142300	*Lactam*	*Yes*	*ND*	*ND*	*ND*
	PNU-143131		*Yes*	*Yes*	*Yes*	*Yes*

*Data for **Table 1** was extracted from **Figure 7** (multi-species metabolic profiles) and summarized above.*

Tolbutamide

Multi-Species HepatoPac co-cultures incubated with 10 µM Tolbutamide for 7 days revealed species-dependent metabolic profiles (**Figures 7A and 8A**). Hydroxy-tolbutamide (**Figure 7A**, *red,* 2.51 min) and carboxy-tolbutamide (**Figure 7A**, *yellow*, 2.27 min) were representative metabolites of tolbutamide after 168 hr incubation in human hepatocyte co-culture, as expected.[2] The amide hydrolysis product (**Figure 7A**) of tolbutamide was generated in rat, monkey, and dog hepatocytes, and not in human hepatocytes, as expected since this metabolite forms via non-human pathways.[3]

Ziprasidone

In contrast, long-term incubations with ziprasidone revealed a consistent array of metabolites formed across species (**Figure 7B and 8B**). Three major human circulating and excreta metabolites, ziprasidone sulfoxide (**Figure 7B**, *fuchsia (human and monkey), orange (dog and rat)*, 2.22 min), ziprasidone sulfone (**Figure 7B**, *orange*, 2.42 min), and *S*-methyl ziprasidone (**Figure 7B**, *lime*, 2.48 min) were generated in human, monkey, dog co-cultures.[4]

Linezolid

Cross-species metabolites that formed post-incubation with linezolid were similar for monkey, dog, and rat co-cultures with the drug derivative, PNU-143131 (**Figure 7C,** *forest green*, 2.05 min and **Figure 8C**, *orange bar*), consistently represented for each species. In contrast, human metabolic linezolid incubations contained the co-eluting linezolid derivatives, PNU-142586 and 142300 (**Figure 7C**, *blue, 1.72 min*), the major human metabolites in urine and feces.[5] Rat showed the strongest presence of the PNU-142586 breakdown product, PNU-142618 (**Figure 7C**, *violet, 1.89 min*. and **Figure 8C**, *violet bar).*

To evaluate and interpret the considerable number of high-resolution MS and MS/MS data files generated during SWATH acquisition of complex biological extracts, high-level data filtering, and deconvolution are needed to extract relevant information on the parent compound and its metabolites. To accomplish this, MetabolitePilot™ Software provides an intuitive user interface in two user workspaces. One workspace correlates metabolites across multiple samples, overlaying chromatograms and permitting the review of MS and MS/MS data in the same screen (**Figure 10A**). The other user workspace interprets and correlates high-resolution structural data for each metabolite, comparing experimental MS/MS data to theoretical data for verification of structural assignments (**Figure 10B**). These tools provide an intuitive software-user

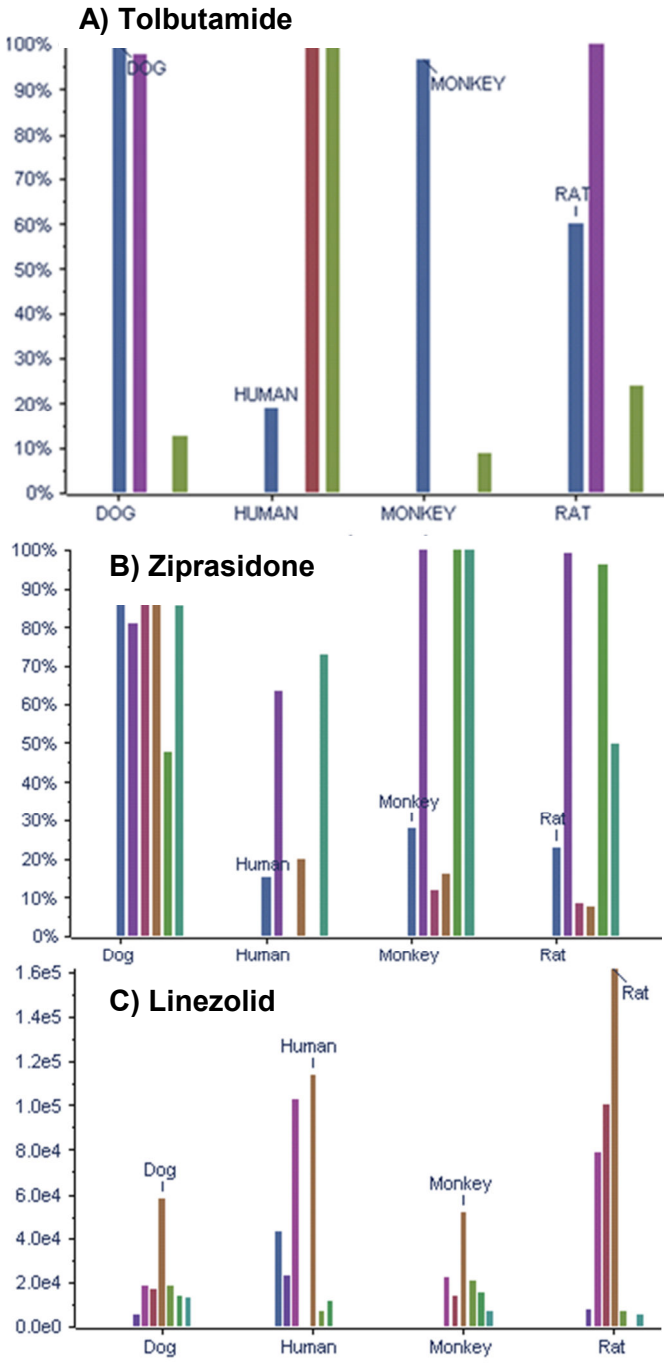


Figure 8. Selected metabolites for various animal species. Metabolites for three drugs, A) tolbutamide, B) ziprasidone, and c) linezolid, were quantified after a 7-day incubation in co-cultured hepatocyte cells from different animal species. Bar graph shows a selection of metabolites that were generated from each different drug incubation, but not the full drug metabolite profile for each species. (See **Appendix A** for identification of drug metabolites.)

interface that not only consolidates data compilation, but also eases the transition between data sets for different metabolites.

Correlating time-dependent metabolite identification and drug clearance in MetabolitePilot™ Software

The determination of metabolic stability and the accurate prediction of major human metabolites is critical for the optimization of drug candidate selection. Comprehensive metabolite identification can be accomplished using SWATH Acquisition on the TripleTOF® 6600, and the data can be mined to identify relevant *in vivo* human metabolites across multiple species, as well as correlate the appearance of metabolites with the clearance of parent compound over time.

These examples highlight the capacity of ultra-sensitive detection achieved with TripleTOF® SWATH acquisition and the longevity of the Multi-Species HepatoPac co-cultures to evaluate the kinetics of low and intermediate turnover compounds along with the generation of human relevant metabolites using physiologically relevant dosing concentrations. Cross-correlation data linking the appearance of metabolites to the disappearance of parent compound is shown below (**Figure 9**) for tolbutamide, ziprasidone, and linezolid over time in human hepatocytes.

Tolbutamide

The relative levels of metabolites obtained from tolbutamide incubations with multi-species HepatoPac co-cultures can be obtained by plotting the % peak area for each detected metabolite per time point in culture. As the parent compound (*blue*) disappeared over time in human cell co-culture (decreasing from 87% at 48 hr to 50% by 168 hr), relevant *in vivo* metabolites appeared concurrently in cell extracts (**Figure 9A**). Oxidation (*fuchsia*), demethylation (*Kelly green*), and other drug derivatives are all shown increasing from 10-30% at 48 hr to 100% at 168 hr, indicating that tolbutamide metabolite levels are following a typical kinetic trajectory that provides relevant pharmacokinetic information for the drug development process.

Ziprasidone

Parent compound levels (*violet*) diminished over time for ziprasidone, but compared to tolbutamide, this drug was metabolized more rapidly in human co-cultures (**Figure 9B**), decreasing from 80% at 4 hr to <10% after 48 hr. After 48 hr, ziprasidone (*violet*) was nearly cleared from the hepatocytes, and its major (phase I) metabolite, *S*-methyl-dihydro ziprasidone (*blue*) was fully formed (**Figure 9B**).

Linezolid

The metabolism of linezolid's phase I and phase II metabolites is more complex, with parent compound derivatization occurring

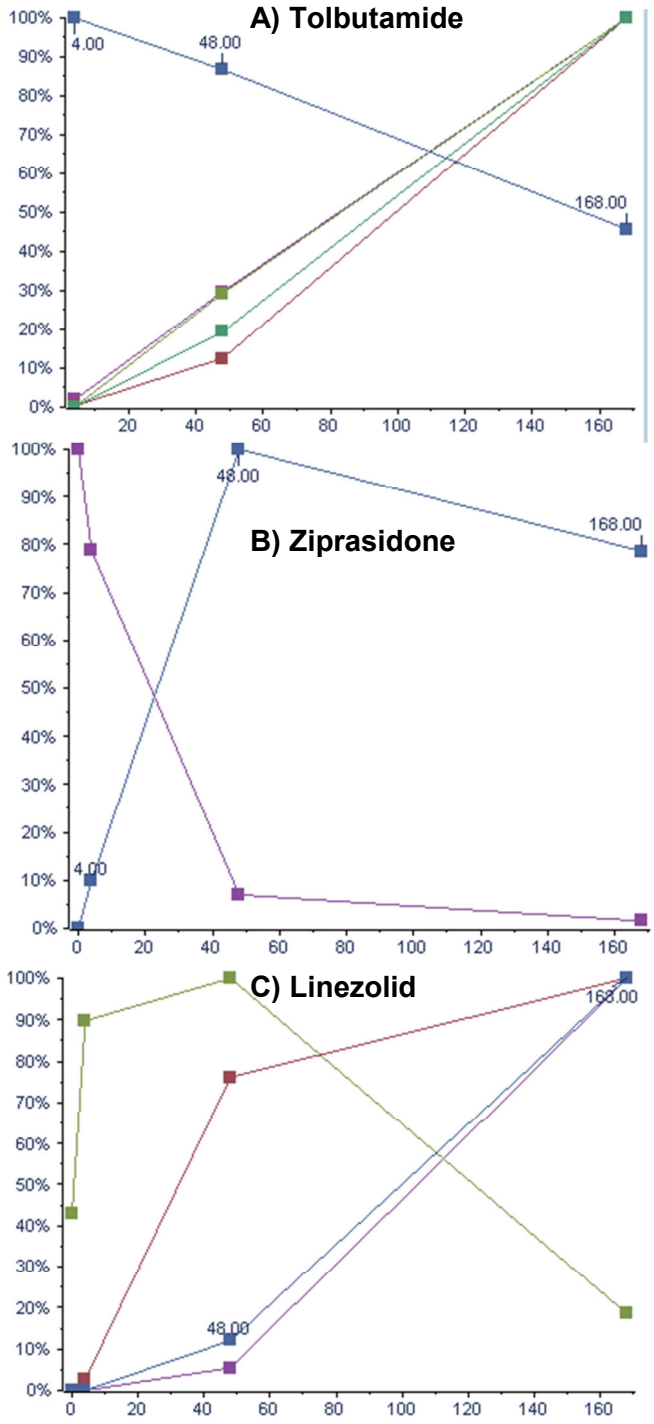
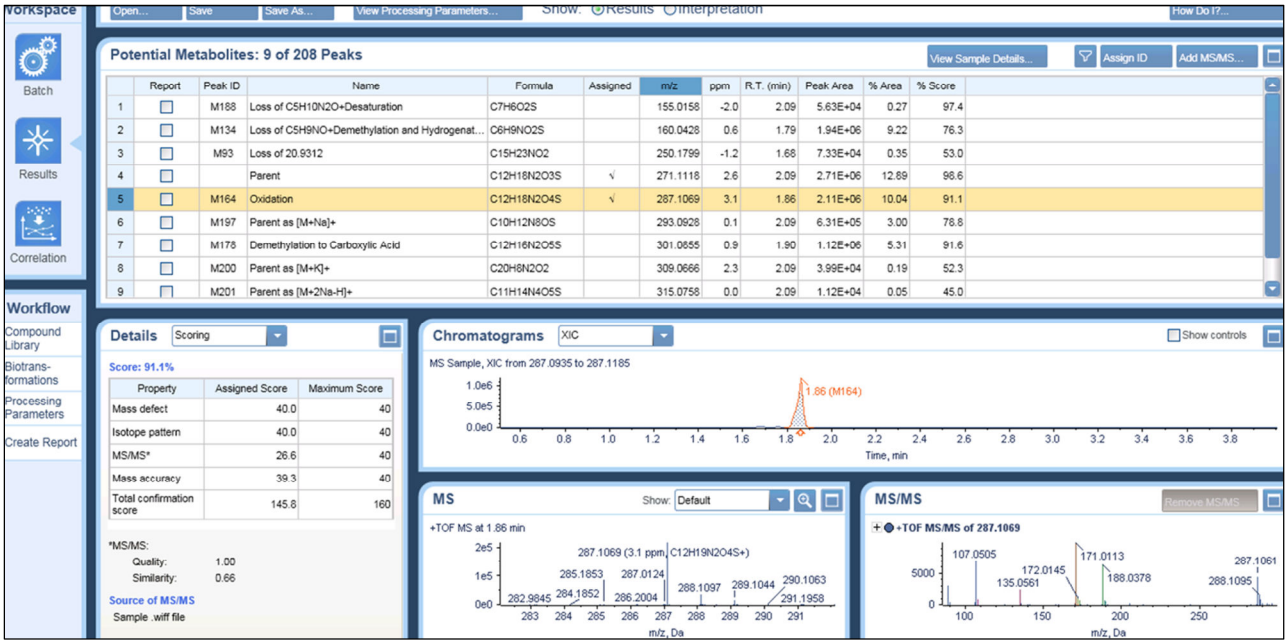


Figure 9. Pharmacokinetics of various drugs in multi-species hepatocyte co-cultures. Various drugs, A) tolbutamide (1 µM), B) ziprasidone (1 µM) and C) linezolid (1 µM), were incubated with hepatocyte co-cultures (Multi-Species HepatoPac®) and evaluated for human metabolite across multiple time points: 0, 4, 48 and 168 hr. (See **Appendix B** for identification of drug metabolites.)

through several biotransformation pathways (lactone and lactam, **Figure 3**).[5] The primary metabolite, PNU-142586 (*violet*), is formed via the lactone pathway through the decomposition of a

A) Results module



B) Structural interpretation module

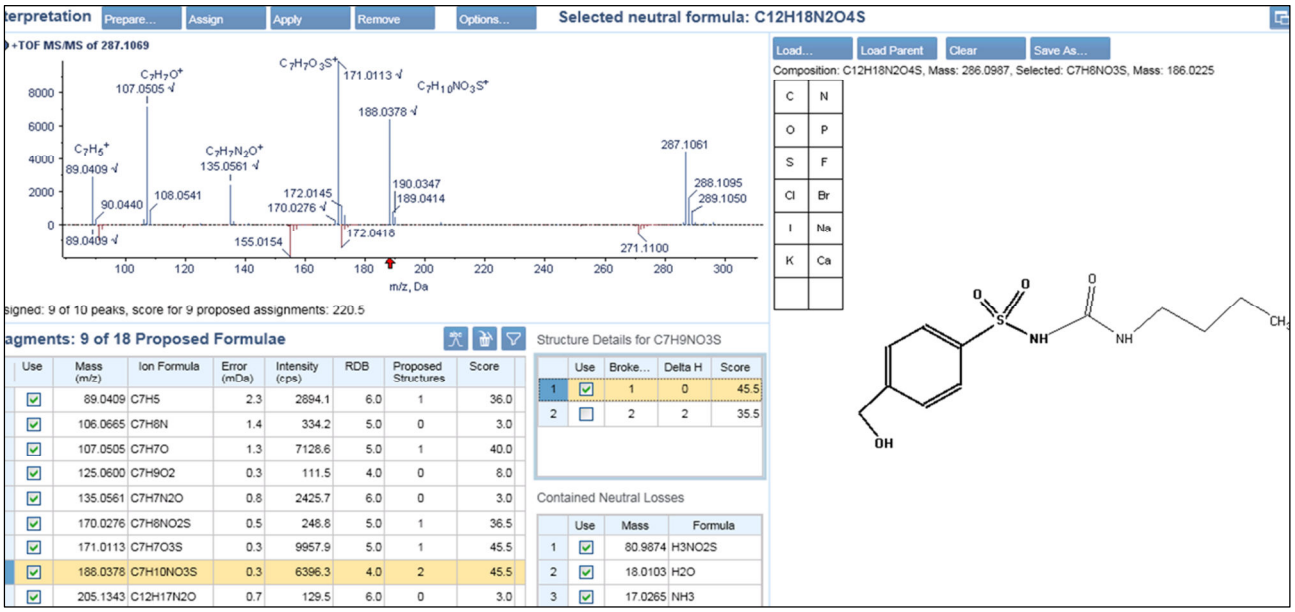


Figure 10. MetabolitePilot™ Software user interface. A) MetabolitePilot™ 2.0 Alpha Software was used to evaluate the high-resolution TOF-MS survey scan and MS/MS data obtained using SWATH™ Acquisition. Several algorithms such as principal component variable grouping (PCVG), multiple mass defect filtering (MMDF), isotope pattern filtering (IPF), common product ion and neutral loss and generic background subtraction were used to identify and confirm both predicted and unpredicted metabolites using sample control comparison logic. B) Structure elucidation was performed using a built-in, semi-automated interpretation module for both MS/MS fragmentation interpretation and soft spot identification.

precursor metabolite, resulting in less rapid formation of PNU-142586 (and its downstream metabolite PNU-142618 (*blue*) in the early stages of the experiment (10% and 30%, respectively, at 48 hr) that are fully formed by 168 hr. Another intermediate, PNU-143131 (*fuchsia*), is dependent on the enzymatic decomposition of linezolid through the lactam pathway. The levels of this intermediate rise in the early stages of the time course (100% at 48 hr), but then decrease (50% at 168 hr) as PNU-143131 is broken down to another, more stable, metabolite.

Conclusions

The studies herein demonstrate how drug clearance and structural elucidation can be investigated in a single injection

using a novel multi-species, long-term tissue-engineered hepatic co-culture model and a data-independent acquisition strategy.

- The Multi-Species HepatoPac® Kit provides a long-term, bioengineered liver model for *in vitro* species comparison studies. Excellent metabolic coverage is obtained during incubations with physiologically relevant compound concentrations, and the formation of metabolites can be correlated with drug clearance in kinetic studies.
- The appearance of major human circulating and excreta metabolites were demonstrated for three compounds in human hepatocytes and in multiple pre-clinical species.
- Metabolites formed using physiologically relevant 1 µM drug concentrations were simultaneously identified and quantified metabolites for low and intermediate turnover compounds.
- SWATH Acquisition on the TripleTOF® 6600 System enables comprehensive metabolite identification in both metabolite ID and clearance studies conducted at the physiologically relevant, 1 µM concentrations.
- Having a complete array of spectra (both MS and MS/MS scans) provides a digital archive of all analytes for samples with restricted availability (*e.g.,* pediatric studies, expensive toxicological studies).
- The ultimate safety net is realized with 100% MS/MS coverage by capturing structural information for both predicted and unpredicted metabolites, such as low-level and geno-toxic products.
- MetabolitePilot™ is an all-in-one integrated software tool that helps rapidly identify and confirm metabolites with structural elucidation capabilities built-in without the need to switch between multiple software packages.

Acknowledgements

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Appendices

Appendix A. Drug metabolites in Figure 8 correlation studies (*identified by bar color*).

- Tolbutamide: parent (*blue*), amide-hydrolysis product (violet), carboxy-tolbutamide (*rust*), and hydroxyl-tolbutamide (*lime*).
- Ziprasidone: parent (*blue*), *S*-methyl ziprasidone (*violet*), oxidized ziprasidone (*fuchsia*), methylated ziprasidone (*orange*), oxidation and glucuronide conjugation (*lime*), loss of chloride/hydrogen ion desaturation (*aqua*).
- Linezolid: PNU-142586 (*blue*), PNU-142300 (*violet*), PNU-142618 (*fuchsia*), PNU-143011 (*rust*), PNU-143131 (*orange*), desaturation (*lime*), loss of fluoride (*Kelly green*), loss of CO (*aqua*).

Appendix B. Human drug metabolites in Figure 10 evaluated over time (and identified by line color):

- Tolbutamide: parent (*blue*), oxidation (*fuchsia*), demethylation to carboxylic acid (*rust*), loss of C_5H_9NO and oxidation (*lime*), demethylation to carboxylic acid (*Kelly green*).
- Ziprasidone: parent (*fuchsia*), loss of chloride, internal hydrolysis and oxidation (*blue*).
- Linezolid: parent (*lime*), PNU-143131 (*rust*), PNU-142586 (*violet*), and PNU-142618 (*blue*).

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