An Integrated Hepatocyte Stability Assay for Simultaneous Metabolic Stability Assessment and Metabolite Profiling

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Abstract

The determination of metabolic stability is critical for drug discovery programs, allowing for the optimization of chemical entities and compound prioritization. As such, it is common to perform high-volume in vitro metabolic stability experiments early in the lead optimization process to understand metabolic liabilities. Additional metabolite identification experiments are subsequently performed for a more comprehensive understanding of the metabolic clearance routes to aid medicinal chemists in the structural design of compounds. Collectively, these experiments require extensive sample preparation and a substantial amount of time and resources. To overcome the challenges, a high-throughput integrated assay for simultaneous hepatocyte metabolic stability assessment and metabolite profiling was developed. This assay platform consists of four parts: 1) an automated liquid-handling system for sample preparation and incubation; 2) a liquid chromatography and high-resolution mass spectrometry-based system to simultaneously monitor the parent compound depletion and metabolite formation; 3) an automated data analysis and report system for hepatic clearance assessment; and 4) a streamlined auto-batch processing for software-based metabolite profiling. The assay platform was evaluated using eight control compounds with various metabolic rates and biotransformation routes in hepatocytes across three species. Multiple sample preparation and data analysis steps were evaluated and validated for accuracy, repeatability, and metabolite coverage. The combined utility of an automated liquid-handling instrument, a high-resolution mass spectrometer, and multiple streamlined data processing software improves the process of these highly demanding screening assays, and allows for simultaneous determination of metabolic stability and metabolite profiles for more efficient lead optimization during early drug discovery.
Significance Statement

Metabolic stability assessment and metabolite profiling are pivotal in drug discovery to fully comprehend metabolic liabilities for chemical entity optimization and lead selection. The process of these assays can be repetitive, time-consuming, and resource demanding. Here, we developed an integrated hepatocyte stability assay that combines automation, HRMS and batch-processing softwares, to improve and combine the workflow of these assays. The integrated approach allows simultaneous metabolic stability assessment and metabolite profiling, significantly accelerating screening and lead optimization in a resource-effective manner.
**Introduction**

Clearance is an essential pharmacokinetic parameter necessary to understand how quickly a drug gets metabolized and eliminated from the body. The liver is a major metabolic organ that accounts for approximately 60% of marketed drug clearance via the major cytochrome P450 enzyme CYP3A4 (Eijk et al., 2019; Cai and Shalan, 2021). Therefore, hepatic metabolic stability assays evaluating hepatic clearance are critical in the early stage of drug discovery as they provide an efficient route for metabolic liability screening and allow for the extrapolation of in vivo clearance data from in vitro results (Cai and Shalan, 2021). Metabolic stability assays are normally performed by incubating the compound in liver subcellular fractions and monitoring the parent compound depletion over time. Suspended cryopreserved hepatocytes are commonly used among various hepatic subcellular in vitro systems (Houston, 1994; Ito and Houston, 2004). The advantages of hepatocyte suspension include encompassing a full spectrum of drug-metabolizing enzymes and the absence of a requirement for cofactors to initiate metabolic physiological reactions (Kulkarni et al., 2021). To monitor parent compound depletion over time, a liquid chromatography (LC) system coupled with a triple quad mass spectrometer (QqQ) is normally used with the high sensitivity and selectivity of multiple reaction monitoring (MRM) methods. However, high-resolution mass spectrometers (HRMS), including orbitrap and time-of-flight instruments, have gained popularity over QqQ because less method development is required and the MS data can concurrently be used for quantitation and structure elucidation (Shou and Zhang, 2012; Cai and Yan, 2021).

Besides clearance, understanding the major metabolic clearance mechanisms and identifying potential metabolic liabilities of compounds can facilitate the structural design of
more stable drugs. In addition, identification of bioactive or toxic metabolites is important to
gain a better understanding of drug liabilities, efficacy, toxicity, and drug-drug interactions (Wu
et al., 2021). Therefore, metabolite profiling and identification (MetID) is performed routinely
during lead optimization and development stages. Experimentally, MetID in the discovery stage
is also completed by incubating the test compound in hepatocyte suspensions with the goal of
identifying potential metabolic soft spots. In this assay, metabolite formation and parent
depletion are monitored over time using LC-HRMS/MS, and the acquired MS² fragmentation
spectra are used to propose more specific metabolite structures that can inform regions of
metabolic instability. In the early phases of drug discovery, to maximize the use of resources and
obtain an initial and quicker understanding of compound metabolic liabilities, metabolite
profiling is ideally carried out using readily available MS data. A caveat of using MS data to
estimate metabolite abundance is that it could be misleading due to metabolites potentially
having different MS responses from the parent compound (Hatsis et al., 2017; Blanz et al., 2017).
As compounds advance beyond the screening stage, the need for higher throughput metabolite
profiling lessens, and more comprehensive and quantitative metabolite profiling using
customized analytical methods and reference standards can be undertaken.

Both hepatocyte metabolic stability and MetID assays are critical in the progression of
molecules in drug discovery programs, but they come with several logistical challenges. These
challenges include, but are not limited to: labor-intensive and repetitive experimental procedures,
demanding use of animal cell resources, lengthy analytical runtimes, time-consuming analytical
optimization and troubleshooting, tedious data processing, quality control (QC), and report
generation. Leveraging recent advancements in liquid handling technology, LC-HRMS
analytical methods, and HRMS data processing platforms, we have developed an optimized high-throughput protocol to streamline and integrate the workflow of these two assays. The integrated hepatocyte metabolic stability assay we devised could significantly simplify and expedite compound screening, while providing a convenient way to optimize lead compounds in a high throughput resource-effective manner.

In this article, we discuss the development of an automated protocol for the hepatocyte stability experimental procedure; a streamlined LC-HRMS data acquisition workflow; and streamlined processes for simultaneous metabolic stability assessment and metabolite identification investigation. This optimized and cost-effective protocol simplifies the workflow of essential, large-capacity in vitro assays and allows for efficient, informative, and high-quality data generation.

**Materials and Methods**

**Chemicals, Reagents, and Instruments**

Zoniporide, imipramine, diazepam, testosterone, raloxifene, midazolam, diclofenac, bupropion, and tolbutamide were purchased from Sigma-Aldrich (St. Louis, MO). Pooled (n = 10) human, Sprague-Dawley rat, and ICR/CD-1 mouse cryopreserved hepatocytes were purchased from BioIVT (Westbury, NY); HRMS-grade water, acetonitrile (ACN), formic acid, and methanol were obtained from ThermoFisher Scientific Inc. (Rockford, IL). Dulbecco’s Modified Eagle Medium (DMEM) was from ThermoFisher Scientific Inc. INVITROGRO HT medium was purchased from BioIVT. All other chemicals and reagents were of analytical grade and were acquired from Sigma-Aldrich unless otherwise specified. Tecan Fluent liquid handling
system and incubator were purchased from Tecan (Männedorf, Switzerland). The automation-compatible incubator and automation-compatible centrifuge were obtained from ThermoFisher Scientific Inc. and Agilent Technologies (Santa Clara, CA), respectively. On-deck incubators were acquired from INHECO (Martinsried, Germany).

Automated Liquid Handling Method with Tecan Fluent

Parent compound depletion was measured by comparing the amount of compound at different time points (60, 120, 180, and 240 min) to a reference amount at the initial starting time (0 min). Metabolic hepatocyte stability studies were performed using cryopreserved primary human, rat, and mouse hepatocytes. Incubations were performed at 1 µM compound concentration and 0.5 million cells mL\(^{-1}\) at 37°C with 5% CO\(_2\). Hepatocytes were prepared by thawing in INVITROGRO HT media and diluted to 1 million cells mL\(^{-1}\) with DMEM buffer. Compound dilution, incubation, and various liquid handling procedures were carried out using a Tecan Fluent liquid handling system.

The automated assay was composed of two separately coded protocols: compound dilution and incubation (**Figure 1**). First, test compounds were diluted from stock vials and aliquoted into incubation plates (**Figure 1A**). Then, hepatocytes were added to the plates and incubated before quenching at their respective time points, thus producing the final plates for LC-MS analysis (**Figure 1B**).

More specifically, for the compound dilution protocol, 1 mM test compounds in DMSO were diluted 500-fold in a two-step serial dilution using DMEM. The compounds were first diluted to 10 µM by adding 5 µL of the test compound to 495 µL DMEM and mixing via
aspiration. Subsequently, 200 µL of the 10 µM compound was added to 800 µL DMEM and mixed to reach the target of 2 µM. 50 µL of 2 µM compound was added in triplicate columns to 5 separate round bottom 96-well plates using the Fluent Flexible Channel Arm multi-dispense. One plate was allocated for each time point (i.e., 0 min, 60 min, 120 min, 180 min, 240 min) over the incubation period.

In the incubation protocol, 50 µL of 1 million cells mL\(^{-1}\) was added to each well of each plate using wide-bore tips (Tecan Pure, Männedorf, Switzerland). The hepatocytes were then placed on an INHECO Thermoshake RM on-deck shaking incubator set at 37°C and 650 rpm to maintain cell suspension until they were added to each plate. Immediately after the addition of the cells, the plates were lidded and placed in the attached Thermo Cytomat 2 incubator set at 37°C with 5% CO\(_2\). These plates were removed from the incubator and quenched with 200 µL of ACN containing 0.1 µM propranolol as internal standard (IS) at their respective times. The T0 minute plate was immediately quenched after hepatocyte addition. The ACN quench solution was kept on an identical on-deck incubator set at 4°C to mitigate evaporation over the course of the experiment. Upon quenching, samples were centrifuged at 3700 rpm for 10 minutes using a below-deck Agilent vSpin centrifuge, and 150 µL of supernatant was transferred to a separate final plate. The final plate was heat-sealed and stored at 4°C prior to LC-MS analysis. Standard 200 µL Tecan Multiple Channel Arm tips were assigned per set to aliquot the ACN quench solution and supernatant. The pipette tips were rinsed in methanol between each use.

**Manual Hepatocyte Stability Assay**

The same hepatocyte metabolic stability assay conditions were applied to manually run experiments as previously described with minor modifications (Cai and Shalan, 2021). Briefly,
test compounds were diluted to 2 µM in DMEM buffer, and 50 µL was added in triplicate to each 96-well plate—one plate per time point. 50 µL of 1 million cells mL⁻¹ was added to each well of each plate using wide-bore tips. Immediately after the addition of the cells, the plates were lidded and placed in an incubator at 37°C with 5% CO₂. These plates were removed from the incubator and quenched with 200 µL of ACN containing 0.1 µM propranolol as an internal standard (IS) at their respective time points. Upon quenching, samples were centrifuged at 3700 rpm for 10 minutes, then 150 µL of supernatant was transferred to a separate final plate. The final plate was heat-sealed and stored at 4°C prior to LC-MS analysis.

**HRMS Data Acquisition and Processing**

HRMS data was acquired with a Shimadzu Nexera x2 ultra-performance liquid chromatography (UPLC) (Kyoto, Japan) using a Kinetex C18 column (2.6 µm, 100Å, 100 x 2.1 mm). UPLC was coupled with a ThermoFisher Orbitrap Exploris 480 mass spectrometer (Thermo, Waltham, MA) equipped with a Heated Electrospray Ionization (H-ESI) Probe. The informative LC-MS method is as previously described, with modifications (Cai and Yan, 2021). Briefly, the LC condition was set at a flow rate of 0.4 mL/min. The starting gradient was 5% organic (Solvent B: 0.1% formic acid in ACN) and 95% aqueous (Solvent A: 0.1% formic acid in water) for 0.5 min. Then, Solvent B was ramped up to 75% until 3 min before increasing to 95% until 3.8 min, and holding until 4.3 min. B was subsequently decreased to 5% until 4.5 min and held until 5.3 min. The MS acquisition was conducted in positive mode with a spray voltage of 3.2 KV, sheath gas flow rate at 60 psi, and an ion transfer tube temperature of 320 °C. A Full scan-data dependent MS² mode (FS-ddMS²) was used, in which the full scan covered the 100-1000 m/z range at a resolution of 15000. Following each FS, ddMS² was triggered under a
stepped normalized collision energy of 25, 45, and 60 eV during a 0.8-second cycle time with a resolution of 7500.

The acquired data was processed with Gubbs Mass Spec Utilities (GMSU) software (Shou and Zhang, 2012; Shou, 2020). The GMSU data processing workflow is illustrated in Figure 2. The data processing workflow consists of three steps: sequence generation, data acquisition, and GMSU data processing and report generation. First, a sequence generator was utilized (macro-based Excel template; see Figure S1) to generate the sequence by inputting the data path, instrument method, sequence name, replicates, injection volume, injection position, compound name and formula, time point, and species. As the assay is routinely run using a fixed plate map, species, time course, and method, the variables that needed to be regularly changed were simply the new compound name and formula. Several sequences were generated with the sequence generator. Xcalibur.sld and Tracefinder.csv were used for data acquisition using the operational software of choice (Xcalibur or Tracefinder). Xcalibur was used in the current study. Another sequence, GMSU.sld, was applied in data processing in GMSU.

Data processing and report generation with GMSU software occurred after HRMS raw files were acquired. At first, the GMSU was configured to include all the analytical and experimental parameters needed for MS data processing and hepatic clearance calculations. For MS data processing, the parent compound was detected by the exact m/z of the ionized compound [M+H]⁺ (by default) with a mass tolerance window of 5 ppm. The peak noise factor was set to 10 to eliminate baseline interference. The smoothing factor was set to 1 by default to avoid significant manipulation of the original peak shape. For clearance parameter calculations,
the species (human, rat, mouse), matrix (hepatocyte), time point (0, 60, 120, 180, 240 min), and
species-specific constants (Liver weight/Body weight, [Cells]/Incubation volume, [Cells]/Liver
weight, Hepatic Blood Flow) were input into the GMSU configuration utility. The detailed
species-specific scaling factors have been listed in Table S1 (Houston, 1994). The hepatic
clearance calculations were based on the well-stirred model, and species-dependent scaling
factors were used for extrapolation. In the following equations, “k” is the slope of the log-
transformed regression of the percent of compound remaining over time, $T_{1/2}$ is the half-life, $Q_H$
is the hepatic blood flow of a particular species, $CL_{int}$ is the intrinsic clearance (mL/min/kg), and
$CL_{hep}$ is the hepatic clearance (mL/min/kg):

**Eq 1)** $\text{In vitro } T_{1/2} = - \frac{0.693}{k}$

**Eq 2)** $CL_{int} = k \times \frac{\text{Number of cells} \times 10^6}{\text{Incubation vol} \ (mL)} \times \frac{\text{Cells} \times 10^6}{\text{Liver wt} \ (g)} \times \frac{\text{Liver wt} \ (g)}{\text{Body wt} \ (kg)}$

**Eq 3)** $CL_{hep} = \frac{Q_H \times CL_{int}}{Q_H + CL_{int}}$

After configuration, the GMSU sequence was loaded into the Hepatic Clearance Calculator for
auto-processing, clearance calculating, data review, and QC (Figure S2). Then, the customizable
GMSU report function was used to generate a final clearance report consisting of the following
summarized clearance parameters: % parent remaining, $T_{1/2}$, $CL_{int}$, scaled $CL_{hep}$, and time course
vs. % remaining plots.

Automated Hepatocyte Stability Assay Validation
Automation validation was performed on the Tecan Fluent to evaluate uniformity in various steps of the protocol, including the compound dilution, the addition of the crash solution, and hepatocyte cell viability assessment. To test the uniformity of the compound dilution protocol, the automated assay protocol was run without hepatocytes, using tolbutamide as a reference control. For this validation, DMEM buffer was used in place of hepatocytes. Tolbutamide was serially diluted from 1 mM to 2 µM and aliquoted into each plate using the dilution protocol. 50 µL of DMEM buffer was added to the wells of each plate, then crashed immediately with 200 µL of ACN containing IS. Peak area ratios of tolbutamide to the IS for each well from each plate were compared to each other. To test the uniformity of the quenching step where the crash solution is added, the entire automated protocol was run without the addition of the compound, and the IS peak areas were compared. Hepatocyte cell viability was evaluated by comparing the viable hepatocytes between automated and manual aliquoting. Cell viability was measured using a Nexcelom Bioscience Cellometer (Lawrence, MA). Percentage difference between the two data sets were calculated using the following formula:

$$\text{Eq 4) Relative percentage difference} = \frac{\text{automated data} - \text{manual data}}{\text{manual data}} \times 100$$

After automation protocol validation, the assay accuracy and robustness were validated using eight commercial compounds. These compounds were selected based on their range of clearance values and various enzymatic mechanisms of metabolism. Accuracy was validated by comparing clearance data between the automated assay and manually run experiments.
Assay robustness was validated by intraday and interday variability. Intraday assay variability was assessed by including multiple replicates (n = 4) of the same control compound in a given experiment. Interday assay variability was assessed by comparing control hepatic clearance data (CL_{hep}) from 50 independent experimental runs, where one experiment was run per day.

**Metabolite Identification Assay Conditions and Auto-batch Processing with MassMetasite**

The same hepatocyte stability samples were re-injected on the LC-MS using a longer LC method routinely used in traditional MetID assays (Wang et al., 2021). This allowed for more definitive metabolite profiling of the dataset compared to using the automated hepatocyte stability assay data acquired with a much shorter method. The instrumentation, column, and LC-MS acquisition parameters are described above, and the alternative LC gradient is as follows: The column was initially held at 2% B for 1 min, increased to 50% B until 8.5 min, further increased to 95% B until 12.5 min, held at 95% B until 13 min, decreased to 2% B until 13.5 min, and then held at 2% B until 16 min.

An auto-batch processing method was configured in MassMetaSite to enable automatic experiment creation and processing of data files for multiple compounds, species, and time points based on the systematic naming of the input hepatocyte stability raw data files. To do this, a WebMetabase protocol in ONIRO was first defined to specify the species (human, rat, mouse), matrix (hepatocyte), and time points (0, 60, 120, 180, 240 min) of experimental data to be analyzed for metabolite identification (Figure S3). Within MassMetaSite, processing settings for MetID were specified, and key processing parameters included filtering for structures with scores > 199, mass error < 5 ppm, and relative abundance of MS peak area > 1% (Figure S4).
configure the auto-batch processing in MassMetaSite, additional inputs such as file paths to the raw data and structure files folders, naming conventions for identifying blank and substrate data files, and descriptors for incubation and blank files to associate them to the appropriate experimental variables were defined (Figure S5). With the setup of this auto-batch processor workflow, MassMetaSite processing of the LC-MS raw data followed by automatic upload to ONIRO commences immediately upon the addition of raw data and structure files to the specified folders.

For metabolite profiling, LC-MS raw data was processed using MassMetaSite (version 4.2.6-1) and analyzed in ONIRO for metabolite identification and relative quantification (Bonn et al., 2010; Zamora et al., 2013). Data acquired from both chromatography methods (LC gradient for the automated hepatocyte stability assay and LC gradient for the traditional metabolite identification assay) were independently processed by MassMetaSite and uploaded into ONIRO as separate experiments. Within ONIRO, the proposed metabolite structures were refined or confirmed through additional manual analysis of the corresponding MS² data, and in some cases, retention time information and literature reports were used to propose more specific metabolite assignments.

**Results**

*Automated hepatocyte stability assay workflow and throughput*

The workflow of the automated assay is summarized in Figure 3. The total run time of the automated assay was 4 hours and 30 minutes. Compound dilution and sample preparation required thirty minutes while 4 hours were dedicated for the time course of the stability assay.
Cell preparation simultaneously took place during the dilution and aliquot steps of the automated protocol to reduce experimentation time. The Tecan Fluent liquid handling system improved assay efficiency, reduced assay variability, and allowed for more hands-off time compared to a manually run assay. At full capacity, a total of 60 compounds can be assessed in three species of hepatocytes, resulting in ten 96-well plates generated for HRMS analysis at the end of each experiment. Analysis of these ten plates (totaling 1000 samples, including blanks) required around 90 hours of LC-MS analysis using the informative LC-MS method (5.3 minutes per run).

GMSU was used for hepatic clearance data analysis and report generation while MassMetaSite was used for early metabolite identification. The automation and software tools significantly simplified the workflow and reduced the time spent on experimental procedures, data processing, analysis, and reporting.

Automated protocol validation

The reproducibility of the Tecan Fluent was investigated at multiple steps of the automation protocol. First, the compound dilution and aliquot protocol was assessed by comparing the peak area ratios of the compound to IS dispensed into DMEM buffer. Tolbutamide was used as the test compound for this assessment due to ease of access. Data from a total of 480 injections were summarized and plotted in Figure 4A. The overall peak area ratio for each well over five total plates was $1.12 \pm 0.07$ with a low coefficient of variation (% CV) of 5.8. The individual plate average peak area ratios ranged from 1.10-1.13 with a standard deviation range of 0.06-0.07 and low % CV values ranging from 5.5-6.0. Those data demonstrate that the compound dilution and aliquoting within the same plate and across multiple plates were very consistent.
The quenching step in the incubation protocol was then investigated by comparing the IS peak areas within and across each plate. The automated protocol was run by quenching each incubation plate with a crash solution of ACN containing IS, and the peak area responses of the IS are shown in Figure 4B. Data analysis is separated by species to account for any matrix effect of the IS peaks during injection. The IS peak areas were fairly consistent across all plates, supported by the low %CV values of 9.4, 8.5, and 8.8 in human, rat, and mouse hepatocytes, respectively. The IS consistency additionally confirms negligible carryover between quenching steps. Regardless of the matrix differences, the overall % CV across all species is still fairly low at 14.1, further demonstrating the uniformity of the quenching step of the incubation protocol.

Hepatocyte viability was also investigated to ensure the liquid handling system maintained adequate cell viability compared to manual sample preparation. Data comparing cell viability between manual and automated sample dispensing are shown in Table 2. Five replicates for the cell viability of each species under both automated and manual conditions were measured. Hepatocyte cell viability was comparable for all species between both sample preparation methods, indicated by low relative percentage differences of 1.6%, 4.9%, and 5.0% for human, rat, and mouse averaged cell viability data, respectively. The average % cell viability of all species was slightly higher after automated dispensing compared to manual dispensing.

**Hepatocyte stability data accuracy and robustness validation**

After validating the automation protocol, the entire automated hepatocyte stability assay was run with eight physicochemically and metabolically distinct commercial compounds. Hepatic clearance data was generated using the HRMS and GMSU workflows. To validate the accuracy of the data generated from the automated assay, we compared in-house historical
hepatic clearance data from manually run experiments to the hepatic clearance data from the automated experiments. Because low clearance measurements are known to be inaccurate and inconsistent during short term incubation (Di and Obach, 2015; Bowman and Benet, 2019), we have excluded two low clearance compounds in human hepatocytes (zoniporide and diazepam) from our analysis. When comparing the average CL\text{hep} values between the manual and automated assays in Figure 5, the $R^2$ value of 0.9597 suggested good data correlation. The clearance values of all eight test compounds in all three species are within a 20% difference error, with a relative percentage difference values ranging between 0.2%-19.4%. Over 86.3% of the compared data sets (19/22) were within 10% difference. The clearance classification of the test compounds did not significantly change regardless of using automated or manual methods. In general, the automation clearance data was more consistent on a run-to-run basis, indicated by the smaller error bars in comparison to the manual clearance data. Standard deviations of the automated assay ranged from 0.3 - 5.3, while the manual clearance data ranged from 0.9 - 13.4 (Table S2). The tighter consistency in the hepatic clearance data for the automated assay could be attributed to its proven uniformity, resulting in less potential for assay variability. Those data clearly demonstrate that the automated assay was as reliable as a manually run assay, with additional benefits such as being less labor-intensive, higher throughput, and more consistent.

In addition to accuracy, the robustness of the automated assay was also evaluated by comparing intraday and interday clearance data. The intraday data displayed in Figure 6 is composed of four replicates from one experiment. The range of standard deviations across all test compounds and species was 0.2-2.2. The % CV values ranged from 0.9 to 36. The greatest variation was derived from two low-clearance compounds, diazepam and zoniporide. Measured
clearance values showed greater variation for low clearance compounds, which is consistent with literature (Di and Obach, 2015; Bowman and Benet, 2019). Therefore, rather than reporting a low-confidence exact value, an assay cutoff value is recommended (Sodhi and Benet, 2021). When excluding those two low-clearance compounds in human hepatocytes, the % CV values ranged from 0.9 to 6.9. These low % CV values indicated that the intraday clearance values for the same compound in the same run were consistent with each other. Interday assay robustness was also tested by running the automated assay once per day over 50 times. Clearance data obtained from 50 automated assays are shown in Figure 7. The range of standard deviations across all test compounds and species was 0.3 - 5.3. The % CV values ranged from 1.4 to 57.1. When excluding %CV values from two low clearance compounds diazepam and zoniporide in human hepatocytes, the %CV values ranged from 1.4 to 13.3. As with the intraday clearance values, the interday values were also highly comparable. In summary, all of the data suggested successful generation of repeatable data within the same experiment or different experiments run over multiple days using the highly robust automated assay.

**MetID Investigation using Automated Hepatocyte Stability Data**

To further expand the utility of the automated hepatocyte stability assay, an integrated workflow was developed that enabled the raw data files acquired from the stability assay to also be used directly for metabolite identification investigation (Figure 3). Using this workflow, metabolite identification was performed on the automated hepatocyte stability data for eight commercial control compounds. Following manual confirmation and revision of the metabolite structures proposed within ONIRO based on MS2 fragmentation data, retention time information, and literature reports, the resulting proposed metabolic pathways are shown in Figure S6. As traditional metabolite identification assays use a much longer (16 min. vs. 5.3 min) LC-MS
method, we ran the same hepatocyte stability incubation samples using this longer analytical method and processed them with MassMetaSite/ONIRO to evaluate how closely the metabolite identification results from the two methods aligned.

A comparison of the identified metabolites for all eight test compounds is shown in Table 1. For simplicity, only the metabolites and estimated relative abundances observed in human hepatocytes at 4 hours are displayed here, though all species and time points are included in the MetID analysis auto-batch processing setup for MassMetaSite (Figure S3). As metabolite standards are generally unavailable during the early stage of drug discovery, relative abundances were estimated using MS peak area but it should be cautioned that MS responses can vary significantly and unpredictably between parent and metabolites, so these relative values may not reflect true metabolite abundances (Hatsis et al. 2017; Blanz et al. 2017). Given this caveat, for the five compounds tested with high human CL_{hep} (testosterone, raloxifene, midazolam, diclofenac, and bupropion), 3-6 metabolites with a relative MS area abundance >1% were identified for each compound from the data acquired using the traditional MetID LC-MS method (Table 1). Of these 21 metabolites identified using the traditional MetID LC-MS method, 18 were identified through metabolite profiling of the automated hepatocyte stability data, resulting in >85% coverage of metabolites for labile compounds using this integrated workflow. For the three unidentified metabolites in the hepatocyte stability data (M5 for testosterone, M2 for midazolam, and M3 for bupropion), we determined that this was due to isomers of the metabolites (M4 for testosterone and M3 for bupropion), higher abundance metabolites (M1 for midazolam) co-eluting under the conditions of the shorter chromatographic method, or both (Figure 8). Because these metabolites were relatively low in abundance, representing less than ~5% of the relative MS peak area, and in some cases, because their metabolic pathway was
already represented by the more abundant isomer identified, no critical differences between using the traditional MetID method and the alternative integrated workflow were identified. Importantly, the most abundant 1-3 metabolites for each compound, representing at least ~10% of the relative MS area, were determined to be the same and at comparable levels with both methods. These major metabolites include M6, M3, and M4 for testosterone; M3 and M1 for raloxifene; M1 and M3 for midazolam; M2 and M4 for diclofenac; and M4 and M2 for bupropion (Table 1 and Figure S6). In the illustrative case of midazolam, its two major metabolites, M1 and M3, were clearly detected at similar relative abundances using both analytical methods in Figure 8. Additionally, the major metabolic pathways in human hepatocytes identified for this set of labile compounds were found to be consistent with previous reports in the literature. Specifically, oxidation (-2), glucuronidation (+176), and reduction + glucuronidation (+178) have been reported for testosterone (Fabregat et al., 2015) (Di et al., 2012; Cai and Yan, 2021). Glucuronidation (+176) has been reported for raloxifene (Sun et al., 2013; Cai and Yan, 2021); oxidation + glucuronidation (+192), and oxidation (+16) has been reported for midazolam (Kronbach et al., 1989; Seo et al., 2010; Cai and Yan, 2021); oxidation + glucuronidation (+192) and oxidation (+16) has been reported for diclofenac (Bort et al., 1999; Kenny et al., 2004; Cai and Yan, 2021); and ketoreduction (+2) and oxidation (+16) have been reported for bupropion (Connarn et al., 2015, 2016).

For the three moderate and low clearance compounds tested in this set, six metabolites were identified for imipramine, two metabolites were identified for zoniporide, and one metabolite was identified for diazepam when using the traditional MetID method (Table 1). The nine total metabolites were also identified at comparable relative abundances when using the automated hepatocyte stability assay data. This high coverage supports the consistency of the
metabolite identification results across the traditional MetID method and the integrated workflow, even for moderately stable to stable compounds that may have fewer and lower abundance metabolites. The most abundant metabolites identified for these compounds were M3 and M5 for imipramine, M2 for zoniporide, and M1 for diazepam, and they were the same across both methods. These metabolites were likewise consistent with previous observations noting oxidation (+16) and/or \( \text{N} \)-demethylation (-14) for imipramine (Lemoine et al., 1993; Cai and Yan, 2021), zoniporide (Dalvie et al., 2010; Cai and Yan, 2021), and diazepam (Seddon et al., 1989).

**Discussion and Conclusion**

In vitro screening assays including hepatocyte stability and metabolite profiling are run routinely in drug discovery programs to rank order compounds, optimize compound stability, and move lead molecules forward into drug development. Rarely acknowledged are the time-consuming and resource-intensive efforts necessary to perform these experiments. Advancements in automation, analytical and computational tools provide ample opportunity to optimize assay workflows. An automated liquid handling system, LC-HRMS, and streamlined data analysis platforms were utilized to develop an integrated hepatic metabolic stability and MetID assay.

Utilization of an automated liquid handling system enables a much higher throughput assay by being able to perform simultaneous sample preparation steps in a fraction of the time it would take compared to manual sample preparation. Manually run hepatic metabolic stability experiments are repetitive, lengthy, and susceptible to interpersonal variation. Additionally, manual procedures limit the lab scientist's flexibility because physical presence is required
continuously for repetitive sample quenching, sealing, and transferring. The liquid handler enables many samples to be processed without additional manual intervention, lab time, and stress on the scientist handling the assay. At full capacity, the liquid handler handles 60 compounds and three species of hepatocytes within 4 hours and 30 minutes; while a manual assay at the same capacity may take 8 hours. Utilization of automation also provides an assurance of validated consistency, which is particularly important as updates or modifications to the stability assay protocol are requested or required due to novel chemical spaces. Additionally, automation allows accommodations to be made within the same experiment without sacrificing assay efficiency or data quality.

Several automation optimizations were implemented to achieve the consistency, robustness, cost-efficiency, and sustainability of the assay. For instance, a reverse pipetting yielded a more consistent aliquot across replicates while keeping the risk of cross-contamination of sample compounds negligible and tip usage low. Uniform peak area ratios of tolbutamide and IS from each plate support that consistent pipetting when using the automated protocol. Additional time can be saved by preparing cells simultaneously while this dilution and aliquot portion of the automation occurs. It is critical to ensure they remained viable when using the automated liquid handler. For the transfer of suspended hepatocytes, wide-bore tips and a slow aspiration and dispense speed were utilized to reduce cell shearing. The low cell viability differences (1.6%-5.0%) between sample preparation methods demonstrated that automation dispensed cells are comparably viable. Regardless of the sample preparation method used, ensuring hepatocytes are not settled to the bottom of the reservoir is critical for the uniform dispensing of cells. Gentle back-and-forth agitation in a manual assay and the utility of an on-
deck thermoshaker in the automated assay allow the cells to remain in suspension prior to dispensing. Another opportunity to reduce plastic waste came in the form of washing the tips used for the addition of the IS quench solution to each time point plate to limit carryover of both compound and IS for tip reuse. We incorporated a wash step to rinse the tips in methanol between usages. If carry over were to occur between subsequent quenching of timepoints, an increase in IS would be observed overtime during our assessment of the quenching step. This is not observed in our data as we saw very consistent IS peak area ratios across species (Figure 4B).

After validating the automated protocol performs sample preparation steps similarly to a manual preparation, it was necessary to ensure data quality was also satisfactory. Both intraday and interday clearance data yielded similar values using the automated procedure. Automated and manual sample preparations also yielded comparable clearance data. No significant change in the clearance classification of each compound was observed and the same conclusions were reached regardless of sample preparation method. Any differences between sample preparations are inconsequential and within the expected experimental variability. There is less inherent experiment variability when utilizing the automated protocol due to fewer manual sample preparation steps (Figure 5, Table S2). This similar clearance data between both sample preparations also provides further support that hepatocytes are viable and behave similarly regardless of the procedure.

The application of an HRMS analytical platform further promoted the development of an integrated assay for both confident metabolic stability measurement and informative MetID investigation (Cai and Yan, 2021). The integrated assay enables greater confidence in metabolic
stability assessment, as true enzymatic degradation rather than non-metabolic instability (e.g.,
non-specific binding) can be confirmed by the detection of metabolites. This consideration is
particularly important for high-lipophilicity compounds, which are prone to non-specific binding
and may show non-enzymatic disappearance from the test matrix. Performing metabolite
profiling in parallel allows confirmation that metabolism is truly taking place, thus improving
data quality and confidence in the metabolic stability results for such challenging compounds. As
discovery compounds are increasingly being characterized as highly lipophilic and analytically
challenging (Agarwal et al., 2022), confirmation of metabolic stability results with metabolite
identification is incrementally more important. Additionally, the integrated assay allows
simultaneous metabolite profiling and identification in a high-throughput and efficient fashion,
providing early metabolic soft spot information to medicinal chemists for timely liability
reduction and structure optimization.

Importantly, the metabolite profiling results from the automated hepatocyte stability data
aligned well with the results from the HRMS data acquired with the longer LC method
traditionally used for MetID assays. Based on the test set of eight diverse compounds
representing various levels of clearance and metabolic pathways, we were able to achieve
approximately 90% coverage of metabolites with >1% relative mass spectrometry abundance
when using the hepatocyte stability assay data as compared to the traditional MetID method.
This high level of coverage was applicable to the range of low, moderate, and high clearance
compounds tested, indicating that despite the reduced chromatographic separation of metabolites,
performing metabolite profiling with the hepatocyte stability assay data is reliable.
However, given the high-throughput nature of the automated hepatocyte stability assay, there are a few situations where MetID analysis of this data may be less informative than the traditional MetID method data. For example, because of the short chromatography method used to assess hepatocyte stability, some isobaric metabolites may be missed due to their lack of separation. In addition, the lack of chromatographic separation may also make it more difficult to reliably identify low abundance metabolites. Despite these shortcomings, this workflow is still useful to obtain a quick and initial understanding of the major metabolic soft spots and metabolic pathways to enable improved structural drug design during early stage drug discovery. To this end, we confirmed that all major metabolites identified using the traditional MetID method had similar relative abundances when using the hepatocyte stability assay data. Moreover, the major metabolic pathways for all of the test compounds were consistent with what has previously been reported in the literature. We also found that the MS$^2$ fragmentation data from the hepatocyte stability assay was sufficient to give comparable structural information to the traditional MetID method, further supporting that no critical metabolite information is sacrificed with this higher-throughput integrated workflow. Nevertheless, for compounds that warrant more thorough metabolite profiling, follow-up studies that are specifically optimized for a particular compound’s separation, ionization, and fragmentation could be performed on the same hepatocyte stability incubation samples to potentially achieve more extensive metabolite identification information.

Preliminary metabolite profiling using automated hepatocyte stability data allows for conserving resources and time needed for additional incubations and sample analysis while also providing timely qualitative insights about potential metabolic liabilities. However, the acquired
MS data may not be reliable to quantitate metabolites. Metabolite abundances estimated based on relative MS peak areas can be inaccurate if parent compounds and metabolites have significantly different mass spectrometry response factors (Hatsis et al. 2017; Blanz et al. 2017). This can result in either underestimating or overestimating metabolites that ionize much worse or better than the parent, respectively, with conjugative metabolites such as glucuronides and sulfonates being particularly prone to underestimation. It is therefore still crucial to rely on the expertise of MetID scientists and chemists to interpret and provide context to any semi-quantitative data as structural, logD, and pKa knowledge of compounds and metabolites are needed for appropriate use of the data (Kiontke et al., 2016). Likewise, to achieve good detection and quantification for the wide array of parent compounds seen in early discovery, positive ionization mode is used for the automated hepatocyte stability assay as is general practice in pharmaceutical research (Loos et al., 2015; Liigand et al., 2017). Consequently, metabolites that ionize better or only under negative ion mode can be underestimated or altogether missed. In situations where compounds show high clearance but appear to have a low abundance of metabolites due to potential ionization differences, closer inspection of UV signals and a dedicated MetID study to further investigate the results and optimize method development could be triggered. Although the challenges of using MS response to estimate metabolite abundance make drawing quantitative conclusions from early-stage metabolite profiling difficult, metabolite profiling using readily available MS data can still efficiently provide valuable information about potential metabolic liabilities.

The integrated workflow described here is primarily geared towards early stages of drug discovery, and while the use of a standardized LC-MS method for both parent quantitation and
metabolite profiling is beneficial for increased throughput, there are some limitations to this approach. In particular, the use of a single ionization mode, a fixed set of fragmentation energies, a short gradient, and a single type of column to analyze a large number and diversity of compounds and their metabolites can run the risk of missing or inaccurately assessing the abundance of metabolites. As compounds or scaffolds of interest advance towards later discovery and early development, lower throughput approaches such as running experiments with multiple ionization modes, longer gradients, and higher compound concentrations to monitor UV signals should be taken to obtain a more thorough, quantitative understanding of metabolism. Additionally, available metabolite standards could be used to supplement preliminary metabolite profiling data by enabling confirmation and more definitive quantification of observed metabolites.

The configuration and combination of several computational tools, such as the sample sequence generator, GMSU, and the metabolite identification software, MassMetaSite, facilitate streamlined data acquisition and analysis workflows. The sequence generator is used to quickly produce an Xcalibur software-compatible sequence for HRMS data acquisition. GMSU further improves the workflow through automated MS data processing and hepatic clearance calculations. The GMSU interface allows for enhanced data review through peak visualization, time course illustration, and integration parameter optimization. After data review, GMSU generates a custom clearance report that summarizes all the clearance data. Likewise, MassMetaSite with an auto-batch processing method is used to automatically process and upload the HRMS data into ONIRO, allowing for easy and timely MetID analysis. In the current process, the manual refinement of metabolite structure proposals is a time-consuming step, so hepatocyte
stability data may be used to prioritize the set of compounds for traditional MetID analysis. Further advancements in metabolite profiling software that lessen the need for extensive manual review of metabolite predictions have the potential to enhance the overall efficiency of this workflow.

Looking forward, the development of such an integrated workflow can serve as a flexible blueprint for creating other in vitro stability assays. Other common in vitro matrices, such as liver microsomes, S9, plasma, etc., can easily be incorporated into this protocol’s design. Alternative formats such as the 384-well plate format are also compatible with liquid handling systems, which could further boost assay throughput. For fit-for-purpose study design, additional modifications to parameters such as time points, quenching solution, initial compound concentration, and incubation shaking speed can be readily made to the existing automated protocol.

In summary, the integrated hepatocyte stability assay for metabolic stability and MetID encompasses a robust, automated assay, an HRMS platform, and streamlined data processing software, all of which collectively facilitate high-throughput and time-efficient determinations of hepatic clearance. Additionally, this workflow enables the simultaneous identification of metabolites, offering substantial potential to enhance timely compound screening and lead optimization with efficient resource utilization during early phase of drug discovery.

**Acknowledgments**

The authors thank Pasquale Carione, Li Ma, Susan Wong, and Jing Wang for their input in experimental design. They also thank Teresa Mulder, Matt Baumgardner, and Lionel Cheruzel
for their preliminary investigations and discussions. Lastly, the authors would like to thank Jane Kenny, Cyrus Khojasteh, and Cornelis E. C. A. Hop for their support.

**Data Availability Statement**

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

**Authorship Contributions**

Participated in research design: Cai, Leung, Cunico, Liu, Johnson, Yan

Conducted experiments: Leung, Cunico, Cai

Performed data analysis: Leung, Cai, Liu

Wrote or contributed to the writing of the manuscript: Leung, Cai, Liu, Cunico, Yan, Johnson

**Reference**


Footnotes:

This work received no external funding.

No author has an actual or perceived conflict of interest with the contents of this article.

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Figure Legends

**Figure 1.** Automation Workflow for the Two Method Steps. The first method (A) diluted compound and aliquoted into 5 incubation plates. The second method (B) added hepatocyte into the incubation plates, incubated the plates for a 4-hour time course, quenched the incubation with ACN at the respective time points, spun down and transferred the supernatant to final plates for HRMS analysis.

**Figure 2.** GMSU Workflow. The GMSU data processing workflow consists of three steps: 1) Sequence generation with sequence generator with pre-defined experimental conditions and plate layout, 2) Data acquisition with HRMS using sequence generated in Step 1; and 3) Data processing with GMSU software, and report generation with the clearance report template.

**Figure 3.** High-Throughput Assay Workflow. Workflow schematic for the high-throughput metabolic stability assay. The assay is run on the Tecan Fluent, data is acquired by UPLC-HRMS, and data analysis is performed using GMSU and MassMetaSite.

**Figure 4.** Automation Protocol Uniformity Validation. (A) Peak area ratio of tolbutamide to the IS, propranolol, was plotted across an entire set of the automated assay (i.e., five 96-well test plates, 480 test injections) along with individual plate and overall mean, standard deviation, and %CV (B) Peak area response for propranolol. Peak area of the IS, propranolol, was plotted across an entire set of the automated assay along with species-specific and overall mean, S.D. and %CV.
Figure 5. Average of Automated Clearance Data vs. Manual Clearance Data. Calculated average hepatic clearance data (ml/min/kg) from automated assays are plotted against manually run assays (n=50) for human, rat and Mouse. Low clearance compounds zoniporide and diazepam in human were excluded from this. S.D. is expressed as error bars. Relative percent changes between automated and manual clearance were calculated for each test compound.

Figure 6. Intrady Hepatic Clearance Data. Overall average hepatic clearance data (ml/min/kg) of human, rat, and mouse were calculated from four replicates within an automated assay. Data includes S.D. and %CV.

Figure 7. Robustness of the Hepatic Clearance Data Generated from Automated Metabolic Stability Assay. Overall calculated hepatic clearance data (ml/min/kg), SD and %CV were obtained from 50 automation assays (one run per day for 50 different days).

Figure 8. Metabolite Profiling Chromatograms and Relative Metabolite Abundances for Midazolam in Human Hepatocytes at 4 hr. Metabolites were identified for midazolam using LC-HRMS/MS data acquired from the automated hepatocyte stability assay as well as data acquired with the longer, traditional MetID analytical method for comparison.
Table 1. Comparison of MetID Results for Eight Test Compounds Obtained Using the Traditional MetID Method and the Automated Hepatocyte Stability Assay Data.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human CL&lt;sub&gt;hep&lt;/sub&gt;</th>
<th>Metabolites identified in Human hep after 4 hr</th>
<th>Traditional MetID</th>
<th>Integrated Workflow MetID</th>
</tr>
</thead>
<tbody>
<tr>
<td>imipramine</td>
<td>Moderate</td>
<td>Parent, M1 (oxidation &amp; glucuronidation, +192)</td>
<td>60.8</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2 (oxidation &amp; N-demethylation, +2)</td>
<td>3.3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M3 (oxidation, +16)</td>
<td>4.7</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M4 (glucuronidation, +176)</td>
<td>17.6</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M5 (N-demethylation, -14)</td>
<td>11.3</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M6 (oxidation, +16)</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>diazepam</td>
<td>Low</td>
<td>Parent, M1 (N-demethylation, -14)</td>
<td>97.3</td>
<td>96.1</td>
</tr>
<tr>
<td>testosterone</td>
<td>High</td>
<td>Parent, M1 (oxidation &amp; glucuronidation, +190)</td>
<td>3.1</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2 (oxidation, +14)</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M3 (glucuronidation, +176)</td>
<td>5.4</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M4 (reduction &amp; glucuronidation, +178)</td>
<td>17.5</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M5 (reduction &amp; glucuronidation, +178)</td>
<td>10.4</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M6 (oxidation, -2)</td>
<td>4.9</td>
<td>ND</td>
</tr>
<tr>
<td>raloxifene</td>
<td>High</td>
<td>Parent, M1 (glucuronidation, +176)</td>
<td>55.8</td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1 (glucuronidation, +176)</td>
<td>9.4</td>
<td>11.3</td>
</tr>
<tr>
<td>Compound</td>
<td>Metabolite Details</td>
<td>% Metabolite</td>
<td>% Parent</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------</td>
<td>-------------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>midazolam</td>
<td>M2 (GSH conjugation, +305)</td>
<td>3.1</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3 (glucuronidation, +176)</td>
<td>28.1</td>
<td>41.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4 (sulfonation, +80)</td>
<td>3.6</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>midazolam High</td>
<td>Parent</td>
<td>46.3</td>
<td>40.7</td>
</tr>
<tr>
<td></td>
<td>M1 (oxidation &amp; glucuronidation, +192)</td>
<td>34.3</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 (glucuronidation, +176)</td>
<td>1.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3 (oxidation, +16)</td>
<td>17.9</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td>diclofenac</td>
<td>M1 (oxidation, +16)</td>
<td>5.1</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 (oxidation &amp; glucuronidation, +192)</td>
<td>33.9</td>
<td>38.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3 (glucuronidation, +176)</td>
<td>8.7</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4 (oxidation, +16)</td>
<td>29.5</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>zoniporide</td>
<td>zoniporide Moderate-Low</td>
<td>Parent</td>
<td>70.2</td>
<td>71.4</td>
</tr>
<tr>
<td></td>
<td>M1 (oxidation, +16)</td>
<td>4.2</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 (oxidation, +16)</td>
<td>25.6</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>bupropion</td>
<td>M2 (oxidation, +16)</td>
<td>16.3</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M1 (oxidation, +36)</td>
<td>2.7</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2 (oxidation, +16)</td>
<td>16.6</td>
<td>26.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M3 (ketoreduction, +2)</td>
<td>4.5</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4 (ketoreduction, +2)</td>
<td>60.0</td>
<td>56.6</td>
<td></td>
</tr>
</tbody>
</table>

Hep = hepatocyte; ND = not detected.
Table 2. Average hepatocyte cell viability data after automated liquid handling and manual dispensing of cells. Data includes AVG, S.D., %CV, and relative percentage difference for human, rat and mouse (n=5)

<table>
<thead>
<tr>
<th>Species</th>
<th>Automation Cell Viability (%)</th>
<th>Manual Cell Viability (%)</th>
<th>Relative % Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AVG ± SD</td>
<td>% CV</td>
<td>AVG ± SD</td>
</tr>
<tr>
<td>Human</td>
<td>70.2 ± 5.0</td>
<td>7.1</td>
<td>69.1 ± 8.9</td>
</tr>
<tr>
<td>Rat</td>
<td>80.4 ± 3.9</td>
<td>4.8</td>
<td>76.7 ± 5.3</td>
</tr>
<tr>
<td>Mouse</td>
<td>69.9 ± 6.8</td>
<td>9.7</td>
<td>66.6 ± 5.9</td>
</tr>
</tbody>
</table>
Dilute 1 mM test compound to 2µM in DMEM buffer

Aliquot 2µM compounds in triplicate to each time point plate

Step 1
50µL cells

Step 2
Incubate at 37°C for a given time (60, 120, 180, 240 min)

Step 3
200µL ACN
Stop reaction with ACN at each time point

Step 4
Centrifuge at 3700 rpm for 10 min

Step 5
150µL
Aliquot supernatant into plate to be analyzed by MS
GMSU Work Flow

**Sequence Generator**
- Data Path
- Methods
- Replicates
- Injection volume
- Compounds
- Cassette
- Species
- Time points

**Data Acquisition**
- Xcalibur.sld
- Tracefinder.csv
- GMSU.sld

**GMSU**

**Data. Raw**
- Xcalibur
- TraceFinder

**GMSU Sequence**
- Hepatic Clearance Calculator
- Hepatic Clearance Report Template

**Data Processing and Review**
- Clearance Report (uploadable)
Figure 3

**Tecan Fluent Automation Method**
- Load 1 mL compound plate from compound management, DMEM buffer, 1mL deep 96-well plates, 96-well plates, and tips onto Tecan Fluent.
- Run the Dilution Protocol: compounds diluted to 2 mM in DMEM buffer and 50 μL of compound added into 6 incubation plates in triplicate.
- Load MeOH Ep wash, ACN - internal standard crash solution, and hepatocytes (diluted 1 million cells per mL in DMEM) onto Tecan Fluent.
- Run the Incubation Protocol: 50 μL cells are added to incubation plates, plates are placed in incubator* for 0 min, 60 min, 120 min, 180 min, and 240 min, 200 μL of ACN - IS added at respective time points, plates centrifuged for 10 mins, 150 μL supernatant transferred to separate 96-well plate for LC-MS analysis.

* incubation conditions: 37°C ± 5% CO2, 50K cells/mL, cell concentration. 1 mM compound concentration.

**UPLC-HRMS Data Acquisition Method**
- Import compound names and molecular formulas into sequence generator.
- Generate Sequence to be used on Xcalibur software.
- Analyze samples using a pre-developed UPLC-Full Scan MS-MS method.

**Automated Data Analysis Method**
- Gobbio Mass Spec Utilities (GMSU) software will detect and integrate parent peaks based on exact mass. Metabolites peaks can also be detected based on exact mass.
- User performs quality control on the clearance data.
- Hepatocyte metabolic stability reports and hepatocyte metabolite identification reports are generated.
- Import compound structure files and HRMS data files to dedicated folder.
- MassMetaSite processes files for metabolite identification and uploads experiment into ONIRO.
- User performs metabolite identification and structural elucidation analysis in ONIRO, and hepatocyte metabolite identification reports are generated.
Figure 4

A

Tolbutamide Peak Area Ratios

<table>
<thead>
<tr>
<th>Plate #</th>
<th>Average Peak Area Ratios ± Standard Deviation</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.10 ± 0.07</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>1.13 ± 0.06</td>
<td>5.7</td>
</tr>
<tr>
<td>3</td>
<td>1.13 ± 0.07</td>
<td>5.9</td>
</tr>
<tr>
<td>4</td>
<td>1.13 ± 0.06</td>
<td>5.5</td>
</tr>
<tr>
<td>5</td>
<td>1.13 ± 0.07</td>
<td>6.0</td>
</tr>
<tr>
<td>All</td>
<td>1.12 ± 0.07</td>
<td>5.8</td>
</tr>
</tbody>
</table>

B

Internal Standard Peak Response

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak Area (Mean ± S.D.)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>6.61E+08 ± 6.22E+07</td>
<td>9.4</td>
</tr>
<tr>
<td>Rat</td>
<td>5.93E+08 ± 5.05E+07</td>
<td>8.5</td>
</tr>
<tr>
<td>Mouse</td>
<td>5.20E+08 ± 4.60E+07</td>
<td>8.8</td>
</tr>
<tr>
<td>All</td>
<td>5.69E+08 ± 8.28E+07</td>
<td>14.1</td>
</tr>
</tbody>
</table>
Figure 5

Average Clearance Data Automation vs Manual

Relative % Difference between automated and manual clearance data

<table>
<thead>
<tr>
<th>Drug</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zonisamide</td>
<td>-</td>
<td>8.8%</td>
<td>19.4%</td>
</tr>
<tr>
<td>Imipramine</td>
<td>4.3%</td>
<td>3.0%</td>
<td>4.2%</td>
</tr>
<tr>
<td>Diazepam</td>
<td>-</td>
<td>7.5%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4.1%</td>
<td>5.9%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>6.2%</td>
<td>0.3%</td>
<td>10.8%</td>
</tr>
<tr>
<td>Midazolam</td>
<td>5.4%</td>
<td>3.8%</td>
<td>3.2%</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>5.3%</td>
<td>0.3%</td>
<td>10.8%</td>
</tr>
<tr>
<td>Bupropion</td>
<td>0.2%</td>
<td>2.4%</td>
<td>5.3%</td>
</tr>
</tbody>
</table>
Figure 6

Average Intraday CL_{hep} Values (mL/min/kg) ± Standard Deviation (n=4)  

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Intraday %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>13.6 ± 0.6</td>
<td>42.1 ± 0.8</td>
<td>71.8 ± 1.4</td>
<td>Human</td>
</tr>
<tr>
<td>Diazepam</td>
<td>4.5 ± 0.8</td>
<td>38.5 ± 1.7</td>
<td>70.0 ± 1.5</td>
<td>4.4</td>
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<tr>
<td>Testosterone</td>
<td>18.5 ± 0.5</td>
<td>46.8 ± 0.8</td>
<td>69.4 ± 0.7</td>
<td>18.2</td>
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<tr>
<td>Raloxifene</td>
<td>15.9 ± 0.8</td>
<td>32.8 ± 1.2</td>
<td>55.9 ± 1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Midazolam</td>
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<td>42.1 ± 1.1</td>
<td>71.3 ± 0.9</td>
<td>3.6</td>
</tr>
<tr>
<td>Diclofenac</td>
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<td>34.1 ± 1.0</td>
<td>31.6 ± 2.2</td>
<td>4.3</td>
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<tr>
<td>Zoniporide</td>
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<td>35.8 ± 0.9</td>
<td>47.1 ± 1.9</td>
<td>36.0</td>
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<tr>
<td>Bupropion</td>
<td>16.8 ± 0.2</td>
<td>37.8 ± 0.4</td>
<td>71.2 ± 1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Average Interday CLhep Values
(mL/min/kg) ± Standard Deviation (n=50)

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
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</thead>
<tbody>
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<td>Imipramine</td>
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<td>43.8 ± 1.3</td>
<td>73.7 ± 2.1</td>
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<td>67.6 ± 3.1</td>
<td>57.1</td>
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</tr>
<tr>
<td>Testosterone</td>
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<td>72.2 ± 2.6</td>
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<td>3.6</td>
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<td>56.9 ± 2.7</td>
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</tr>
<tr>
<td>Midazolam</td>
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<td>41.6 ± 1.4</td>
<td>67.7 ± 3.2</td>
<td>7.1</td>
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<td>4.7</td>
</tr>
<tr>
<td>Diclofenac</td>
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<td>33.4 ± 3.8</td>
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<td>Zonisamide</td>
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</tr>
<tr>
<td>Bupropion</td>
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<td>38.8 ± 1.6</td>
<td>67.2 ± 5.3</td>
<td>4.0</td>
<td>4.2</td>
<td>7.9</td>
</tr>
</tbody>
</table>
Figure 8

Traditional MetID

M2 +178
m/z = 502.1176

M3 +18
m/z = 342.0904

M1 +152
m/z = 518.1125

Integrated Workflow MetID

Highest value: 1.04E08

Human hep, 4 hr

Relative MS Peak Area %

M3, 17.9%
M2, 1.5%
M1, 34.1%
Parent, 46.3%

Relative MS Peak Area %

M3, 25.2%
M2, 1.5%
M1, 34.1%
Parent, 40.7%