An Automated High-Throughput Metabolic Stability Assay Using an Integrated High-Resolution Accurate Mass Method and Automated Data Analysis Software


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ABSTRACT

Advancement of in silico tools would be enabled by the availability of data for metabolic reaction rates and intrinsic clearance (CLint) of a diverse compound structure data set by specific metabolic enzymes. Our goal is to measure CLint for a large set of compounds with each major human cytochrome P450 (P450) isozyme. To achieve our goal, it is of utmost importance to develop an automated, robust, sensitive, high-throughput metabolic stability assay that can efficiently handle a large volume of compound sets. The substrate depletion method [in vitro half-life (t1/2) method] was chosen to determine CLint. The assay (384-well format) consisted of three parts: 1) a robotic system for incubation and sample cleanup; 2) two different integrated, ultrasensitive methods for the detection of the percent remaining of parent compound, and 3) an automated data analysis system. The CYP3A4 assay was evaluated using two long t1/2 compounds, carbamazepine and antipyrine (t1/2 > 30 minutes); one moderate t1/2 compound, ketoconazole (10 < t1/2 < 30 minutes); and two short t1/2 compounds, loperamide and buspirone (t1/2 < 10 minutes). Interday and intraday precision and accuracy of the assay were within acceptable range (∼12%) for the linear range observed. Using this assay, CYP3A4 CLint and t1/2 values for more than 3000 compounds were measured. This high-throughput, automated, and robust assay allows for rapid metabolic stability screening of large compound sets and enables advanced computational modeling for individual human P450 isozymes.

Introduction

Hepatic intrinsic clearance (CLint) is an important parameter for a drug candidate as it influences oral bioavailability as well as systemic exposure. Thus, it is common to determine this property early in drug discovery so that it can act as a compound selection criterion as well as aid medicinal chemists in drug design. The translation of drug leads into clinical candidates could be improved by the further development of in silico tools for reliable prediction of human clearance. The major enzymatic system responsible for metabolism of xenobiotics is the cytochrome P450 family (Nebert and Russell, 2002). P450s are responsible for more than 75% of drug metabolism, and the major isozymes among these enzymes are CYP3A4, which is responsible for the metabolism of ~50% of known xenobiotics in humans. Other important isozymes include CYP2C9, CYP2D6 and CYP1A2 (Guenigerich, 1999, 2008). A joint team comprising of members from the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ Consortium) and the National Institutes of Health (NIH) National Center for Advancing Translational Sciences (NCATS) established a goal to measure CLint for a large set of compounds, with each major human cytochrome isozyme, beginning with CYP3A4, from which in silico prediction tools can be developed. Such an extensive data base has not been previously published, and the resulting in silico tools are expected to enhance drug discovery by aiding lead selection and structure optimization.

In vitro metabolic stability assays for drugs and drug candidates have been reported over the past 20 years (Houston, 1994; Linget and du Vignaud, 1999). These assays use microsomes, S9, and cytosol fractions from various species and have been extended to stability assessment in living hepatocytes (Obach et al., 1997; Niro et al., 2003; Ito and Houston, 2004). These assays are regularly used by pharmaceutical companies to provide valuable insights for drug design during discovery research to optimize pharmacokinetic profiles of chemical series (MacKenzie et al., 2002; Korfmacher, 2003; Nassar et al., 2004). In silico tools for the prediction of metabolic stability have been developed using various matrices, with number of compounds ranging from a handful few up to thousands (Bursi et al., 2001; Shen et al., 2003; Sakijama et al., 2008). For this project, we chose to test 5000 compounds with multiple major cytochrome isozymes. Using a multiple time point assay, this testing would require >30,000 measurements per isozyme (5000 test compounds + additional control samples × six time points). Thus, it was of utmost importance to use an automated, robust, sensitive, high-resolution accurate mass detection method and automated data analysis system. The CYP3A4 assay was evaluated using two long t1/2 compounds, carbamazepine and antipyrine (t1/2 > 30 minutes); one moderate t1/2 compound, ketoconazole (10 < t1/2 < 30 minutes); and two short t1/2 compounds, loperamide and buspirone (t1/2 < 10 minutes). Interday and intraday precision and accuracy of the assay were within acceptable range (∼12%) for the linear range observed. Using this assay, CYP3A4 CLint and t1/2 values for more than 3000 compounds were measured. This high-throughput, automated, and robust assay allows for rapid metabolic stability screening of large compound sets and enables advanced computational modeling for individual human P450 isozymes.

ABBREVIATIONS: ACN, acetonitrile; CLint, intrinsic clearance; DMSO, dimethylsulfoxide; IS, internal standard; MRM, multiple reaction monitoring; MS, mass spectrometry; P450, cytochrome P450; t1/2, half-life; NCATS, National Center for Advancing Translational Sciences; NIH, National Institutes of Health; QC, quality control; UPLC, ultraperformance liquid chromatography.
A high-throughput metabolic stability method that could rapidly handle this large volume of samples.

Attempts have been made to automate microsomal stability assays; however, these attempts have reported modest success. These assays typically use 96-well technologies for incubations and sample preparation and ultraperformance liquid chromatography/mass spectrometry (UPLC/MS) for data acquisition (Korfmancher et al., 1999; Di et al., 2003), which would require more than 50 separate analyses for each enzyme. More recently, high-resolution mass spectrometers have been used for analysis; however, data extraction and data analysis remain cumbersome (O’Connor et al., 2006; Shui et al., 2011). These existing methods, although useful, are semi-automated at best and have their limitations. A higher-throughput analytical method was needed for this project.

In this article, we discuss the development of: 1) a fully automated procedure for microsomal incubation and sample cleanup; 2) two separate automated UPLC/MS methods for screening of large sample sets, and 3) an automated software that extracts data and performs regression analysis using different combinations of data points from which the analyst can choose the most pertinent combination. This method can benefit drug research and possibly be used to measure metabolic lability in diverse matrices (e.g., microsomes, S9 fractions, cytosol fractions).

Materials and Methods

Materials

Albendazole, antipyrine, buspirone, ketoconazole, loperamide, and propranolol were purchased from Sigma-Aldrich (St. Louis, MO). Water, acetonitrile (ACN), and formic acid, all UPLC/MS grade, were purchased from Thermo Fisher (Waltham, MA). Human CYP3A4 supersomes and NADPH Solution A/B were purchased from BD Gentest (Woburn, MA). Test compounds were provided by NCATS Compound Management after verification of identity and purity. Unless specified, all other materials were purchased from Sigma-Aldrich.

Incubation Method

The substrate depletion method (in vitro t1/2 method) to determine CLint was chosen. Disappearance of the parent compound over time was measured with amount of drug at time zero as the reference.

Incubation and liquid handling were carried out using a Tecan EVO 200 robotic system equipped with a 96-channel head, EVOware software (version 3.2), a shaking Inheco heating block, and an Inheco cooling block (Inheco, Munich, Germany) (Fig. 1). The heating block was calibrated beforehand using a thermocouple inserted in incubation matrix solution, and a setting of 45°C produced a solution temperature of 37°C. Pipette tips (50 μl and 200 μl) were purchased from Tecan (Morrisville, NC) and reservoirs (low-profile; RES-SW384-LP and high-profile; RES-SW384-HP) for the incubation were purchased from Tecan (Morrisville, NC) and reservoirs (low-profile; RES-SW384-H and high-profile; RES-SW384-HP) for the incubation were purchased from Tecan (Morrisville, NC) and reservoirs (low-profile; RES-SW384-H and high-profile; RES-SW384-HP) for the incubation were purchased from Tecan (Morrisville, NC). Supersomes and NADPH solution A/B were diluted in 100 mM potassium phosphate buffer (pH 7.4). A solution of albendazole (internal standard, IS) in ACN was prepared by adding 20.0 μl of 10 mM albendazole in dimethylsulfoxide (DMSO) to 722 ml of ACN and 7.5 ml of chilled ACN/IS were aspirated in a fresh, time 0 plate (i.e., T0 plate). The substrates were added to the test plate and an equal volume of ACN/IS was added to the control plate, henceforth is called ACN/IS.

The 384-well plate received from NCATS Compound Management included control (duplicates) and test compounds at a 10 mM concentration in DMSO. These compounds were diluted to 50 μM in ACN using the robot in a secondary plate. In the first step, 82.73 μl of diluted supersomes (3 pmol) was transferred to the incubation plate (384-well, 250 μl) and 40 μl of NADPH solution A/B was added to the incubation plate. The final concentration of the test compound was 1 μM. Two minutes before each subsequent time point, 40 μl of the substrate depletion method (in vitro t1/2 method) to determine CLint was employed.

TABLE 1
Reproducibility assessment for control samples

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Molecular Formula/Exact Mass ([M + H]+)</th>
<th>Linearity Range</th>
<th>No. of Repeats</th>
<th>t1/2 (Mean ± S.D.)/Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buspirone</td>
<td>C20H12N2O2/386.2550</td>
<td>1–1000</td>
<td>18</td>
<td>2.5 ± 0.45 min/low</td>
</tr>
<tr>
<td>Loperamide</td>
<td>C16H12CN.O4/477.2303</td>
<td>2–1000</td>
<td>18</td>
<td>3.5 ± 0.59 min/low</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>C26H32ClN6O4/531.1560</td>
<td>4–1000</td>
<td>12</td>
<td>21 ± 3.6 min/moderate</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>C11H12N2O/189.1022</td>
<td>5–1000</td>
<td>18</td>
<td>&gt;&gt;30 min/high</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>C13H16N2O2/237.1022</td>
<td>2–1000</td>
<td>18</td>
<td>&gt;&gt;30 min/high</td>
</tr>
</tbody>
</table>

*Initial three experiments did not include ketoconazole as a control.

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chilled ACN/IS was added to a fresh 100 µl plate. An aliquot of 9.92 µl of the incubation mixture was sampled at 5, 10, 15, 30, and 60 minutes and added to the respective plates containing chilled ACN/IS. After each time point, the plates were heat-sealed with foil plate sheets (Thermo Fisher) and centrifuged for 20 minutes at 3000 rpm (6°C).

Each automated run produced six 384-well plates, with six time points for each of the 384 compounds. This would mean that the sample acquisition time would be very long, even with a short UPLC method. To reduce data acquisition time, adjacent wells were pooled, thus combining six plates into three to cut the acquisition time by half.

Data Acquisition. Two separate data acquisition methods were developed: one using a triple quadrupole MS and the other using a high-resolution MS. They were both validated for data quality, operation time, and ease of acquiring data. The rationale behind developing two methods was to offer alternatives for various laboratory setups.

**Method 1: Triple Quadrupole MS.**

**UPLC method.** The Waters Acquity UPLC system consisted of a Waters Acquity Binary Solvent Manager, Column Manager and 2777 autosampler along with QuanOptimize software. Chromatography used a Waters Acquity UPLC BEH Shield RP18 column (1.7 µm, 2.1 x 50 mm) equipped with a Waters Acquity UPLC BEH Shield RP18 VanGuard precolumn (1.7 µm, 2.1 x 5 mm). The mobile phases were (A) water with 0.1% formic acid and (B) ACN with 0.1% formic acid. The flow rate was 0.6 ml/min, with a gradient of 99% A: 1% B isocratic for 0.1 minutes, to 80% A: 20% B over 0.3 minutes, to 1% A: 99% B over 0.5 minutes, and held at 1% A: 99% B for 0.7 minutes. The column re-equilibration time was 0.4 minutes. The cycle time was 2.0 minutes from injection to injection. Sample plates were held at 7°C in the 2777 autosampler until injected.

**Triple-quadrupole MS method.** MS data were acquired on a Waters Xevo TQ-S triple quadrupole mass spectrometer equipped with MassLynx version 4.1 software. Multiple reaction monitoring (MRM) methods were automatically developed by the instrument for each compound using the QuanOptimize application (described later herein). The samples were injected in the following order: 60 minutes, 30 minutes, 15 minutes, 10 minutes, 5 minutes, and 0 minute to minimize carryover effects.

**QuanOptimize.** An aliquot of 3.0 µl (containing 50 µM drug) from the secondary plate was diluted into 75 µl of 1:2 ACN/H2O to get the QuanOptimize plate. The QuanOptimize plate was covered with a heat seal and transferred to the UPLC/MS/MS. An aliquot of 2 µl of solution, prepared for QuanOptimize, was injected twice in a loop injection without a UPLC column. The flowrate for QuanOptimize was 0.3 ml/min of 50% A: 50% B. The first injection determined the optimum ion source cone voltage for the MH⁺ precursor ion, and the second injection determined the optimum collision voltage and product ion. QuanOptimize then built an MRM analytical method for the compound and the IS for each compound set and applied these MRM conditions to the respective samples in the sample list.

**Sample analysis.** For each pooled sample, 2.0 µl was injected onto the BEH Shield column with BEH Shield precolumn using the 2777 autosampler. One precursor-product ion pair, with a dwell time of 0.030 seconds, was used for each compound. The retention times of the test compounds were determined by reinjecting 2 µl of the QuanOptimize solution for analysis under the same UPLC chromatography as the samples, using MS2 scanning analysis at mass-to-charge (m/z) 50–1300 at a scan rate of 0.25 seconds per scan. The retention times of each analyte were determined by manual evaluation of the chromatograms. The peak area under the respective MRM signal for each test compound in the respective pooled samples was integrated at its retention time using Waters TargetLynx. The integration of every pooled sample component was manually checked and, in some cases, reintegrated after evaluation.

The output TargetLynx comma delimited text data file was input to the Validator software (Bioinformatics, NCATS). The Validator then produced plots of percent remaining versus time, and Ln (response) versus time and calculated $t_{1/2}$ and $Cl_{int}$ using equations 1 and 2 (Obach et al., 1997):

$$t_{1/2} = \frac{0.693}{k} \quad \text{(1)}$$

$$Cl_{int} = \left( \frac{0.693}{t_{1/2}} \right) \times \left( \frac{\text{mL incubation/mg microsomes}}{\text{mg microsomes/gm liver}} \right) \times \left( \text{gm liver/kg b.w.} \right) \quad \text{(2)}$$

**Method 2: High-Resolution MS.**

**UPLC method.** The Thermo Ultimate 3000 UPLC comprised an HPG-3400 binary rapid separation pump and the WPS-3000 autosampler. The column was an Acquity UPLC BEH C18, 2.1 x 50 mm, particle size 1.7 µm. The mobile phases were (A) water with 0.1% formic acid and (B) ACN with 0.1% formic acid at a flow rate of 0.7 ml/min. The UPLC conditions were 5%B at 0-0.2 minutes, a linear gradient from 5%–95%B from 0.2 to 1.7 minutes, followed by 95%B for 0.4 minutes. The column re-equilibration time was 0.4 minutes. The column effluent was directed to the high-resolution mass spectrometer.

**High-resolution MS method.** MS data were acquired on a benchtop QExactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The instrument was equipped with a heated electrospray ionization source, and the analysis was performed in positive ionization mode. The operating parameters were as follows: ion transfer tube temperature 400°C, sheath gas 80, auxiliary gas 30, and spray voltage 3.5 kV. A full-scan MS method with mass ranging from 50 to 1000 m/z and resolution of 35,000 was used. The instrument was calibrated.
using the positive ion calibration solution, which comprised a mixture of caffeine, MRFA peptide, Ultramark 1621, and n-butylamine in an ACN-methanol-acetic acid solution. This calibration was performed before acquiring data for each 384-compound batch; the same external calibration was applied throughout each batch. The samples were injected in the following order: 60 minutes, 30 minutes, 15 minutes, 10 minutes, 5 minutes, and 0 minute, to minimize carryover effects.

Data analysis by TraceFinder 3.2. The TraceFinder method developed contained all the necessary information to run the instruments data acquisition as well as the parameters required for processing, data review, and reporting as an automated workflow.

Before each acquisition, parent molecular formulae for the entire batch of compounds were imported into TraceFinder. The software automatically calculated the exact \( m/z \) of the \([M + H]^+\) ion. Parent compounds were identified by their \( m/z \) values with a mass precision of 5 ppm. The signal-to-noise ratio was set above 10 to eliminate interference peaks. TraceFinder automatically detected and integrated peaks from each raw file and provided an Excel output file, which included IS response, target compounds response, retention times, chromatograms, and sample details. For each batch, 1152 output Excel files were obtained. The Validator software extracted the response data for each compound and produced the following results: plots of percent remaining versus time, and Ln versus time; regression analysis of various combinations of data points by the utility and ranked by quality of the fit \((r^2, \text{ root mean squared error})\) and calculated \( t_{1/2} \) and CL_{int}.

Validator Software

To facilitate the calculation of CL_{int} from the response data generated by TargetLynx and TraceFinder software, we developed the IQC Validator software.

Fig. 3. Sensitivity of the HRMS instrument: (A) 0-minute chromatogram and (B) 60-minute chromatogram for buspirone (\( >98\% \) turnover) generated from full-scan data acquired with the Thermo QExactive.
to perform automated fitting and ranking of calculated CL\text{int} values. The ranking serves an important function in that it allows the user to quickly validate the fitted data with minimal effort. For a given set of time points (in minutes) \( T = \{0, 5, 10, 15, 30, 60\} \) and the corresponding response values, all \( \sum_{t=0}^{6} t \cdot \beta^2 \) = 57 possible combinations of \( T \) are used to perform Ln linear regression fit. Each fit in turn is evaluated based on the scoring scheme in eq. 3:

\[
S = N r^2 e^{-\alpha} \sum_{i=0}^{N} \frac{S_{max} - S_{norm}}{S_{max} - S_{norm}}
\]

where \( N \) is the number of time points, \( r \in [-1, 1] \) is the Pearson’s correlation, and \( \alpha \) is the root mean square error. The best possible score (i.e., \( N = 6, r = 1, \) and \( \alpha = 0 \)) is \( S_{max} = 6 \left( \frac{1}{2 \cdot 2^3} \right) = 0.1181 \). The score \( S_{norm} = S_i / S_{max} \in [0, 1] \) is the normalized score that is used in the final ranking, with 1 being the best possible fit. This scoring scheme, when sorted in descending order, identified the most likely fit and calculated \( t_{1/2} \) and \( \text{CL}_{\text{int}} \).

The IQC Validator has been implemented in the Java programming language as a desktop client. Figure 8 shows a brief overview of its main user interface. A simple workflow is as follows: the user loads a data file, in either Excel or text format, of time points and response values. For each loaded sample, the user selects the best possible fit by any combination of visual inspection and/or calculated parameters (e.g., \( t_{1/2} \), \( S_{norm} \) score, etc.). The selections made by the user are saved to a relational database management system and can be accessed at a later time. The source code for the IQC Validator software is available at https://spotlit.nih.gov/ncats/iqc.

**Results**

All experiments were performed with both data acquisition methods, and the results were very similar (\( t_{1/2} \) values ±10%). Results for the UPLC/HRMS method are described.

**Method Validation.** Five commercial compounds with different \( t_{1/2} \) values were selected as controls to test the qualitative and quantitative performance of developed method. Calibration curves for these control compounds were prepared, and peak area ratios (compound/IS) versus their nominal concentrations were plotted. The calibration curves were linear over the concentration range of 1 to 5–1000 nM for the control compounds (Table 1). Intraday precision and accuracy were determined by measuring three different quality control (QC) concentrations (10 nM, 50 nM, and 500 nM) three times in one day, and the interday precision and accuracy were determined by measuring concentrations of three QC samples over 5 days. The intraday and interday precision for QC samples were below 7% and 11% for all control compounds. The intraday and interday inaccuracies were below 8% and 13.5% which were within acceptable limits (data not shown).

The robustness of the UPLC/HRMS method was determined by comparing peak responses of the IS across three batches (i.e., three 384-well plates or 3456 sample injections). The response was consistent within the same batch as well as across different batches (Fig. 2). The sensitivity of the HRMS instrument in detecting peaks for test compounds with >98% turnover is shown in Fig. 3. Instrument calibration was performed before each batch analysis and mass accuracy (±2 ppm) was sustained throughout each batch run without need for recalibration or use of an internal reference (Fig. 4). These results indicated that the UPLC/HRMS method developed was reliable, sensitive, and robust.

**Reproducibility.** The reproducibility of the liquid handler system was investigated by comparing the \( t_{1/2} \) values of control compounds, included twice in each 384-well plate across multiple plates. The response over time of the control compounds across three experiments were plotted to demonstrate the reproducibility between and within experiments (Fig. 5). The results show excellent reproducibility within plates and between plates (Fig. 5; Table 1). The percent coefficient of variation for the \( t_{1/2} \) values of buspirone, loperamide, and ketoconazole between experiments was between 15% and 25%, which is significantly below the 2-fold acceptable limits. Since antipyrine and carbamazepine are stable compounds, no SD was reported.

**Automated Assay Workflow and Throughput Speed.** The automated assay workflow for the high-throughput metabolic stability assay is summarized in Fig. 6. The total preparation and incubation time for each 384-well plate experiment was 2 hours. The automated liquid handler system increased efficiency, reduced error, and increased walk-away time for the scientist. Each incubation plate produced six 384-well plates, with six time points (0–60 minutes) for each of the 384 compounds. Adjacent wells were combined from each plate, thus converting six plates into three, which reduced the UPLC/HRMS acquisition time by half and further increased the efficiency of the method without compromising the quality of the data. A typical extracted ion chromatogram for a sample that contains two test compounds and the IS is shown in Fig. 7. The UPLC/HRMS acquisition was allowed to run overnight and the time required for each batch (1152 samples) was 2½ days. Under optimal conditions, two 384-well incubation plates can be assayed in a week by using one robot and one UPLC/MS instrument. Once the acquisition was complete, TraceFinder detected integrated peaks and provided separate output files for each sample. These 1152 files were then imported into the Validator software which automatically extracted data from all samples, generated plots (Fig. 8), and calculated \( t_{1/2} \) and \( \text{CL}_{\text{int}} \). These software tools completely eliminated data extraction time and drastically reduced data analysis time.

**Compound Library.** About 3000 compounds were tested using the newly optimized high-throughput method. Most of these compounds were a part of NCGC (NIH Chemical Genomics Center) pharmaceutical collection, which encompasses publically approved approved and investigational drugs (Huang et al., 2011) and contains more than 2400 compounds that have been approved for clinical use by US, Canadian, Japanese and European health regulatory authorities. The remaining compounds tested were from NCATS annotated collection. Molecular properties of compounds, such as logP, topological polar surface area, molecular weight, and Lipinski rule of 5, were calculated using Chemistry Development Kit descriptors tool (Chemistry Development Kit Chemistry Development Kit download I SourceForge. net https://sourceforge.net/projects/cdk/) implemented in the Konstanz Information Miner (KNIME | Open for Innovation https://www.knime.org/) analytical platform (Warr, 2012). Figure 9 includes plots of the distribution of molecular properties and the CYP3A4 \( t_{1/2} \) of our test
compounds. As seen from the plots, a large portion of compounds have $t_{1/2}$ values greater than 60 minutes, belong in the 251–500 mol. wt. range, and most of them do not violate Lipinski rules. We did not find any direct correlation of calculated $t_{1/2}$ values with the preceding molecular descriptors. Whereas much microsomal metabolic stability data are available in literature, this is, to our knowledge, the first time that such an extensive compound data base is being tested with an individual isozyme. A detailed presentation of the data as well as in silico model development will follow once CYP3A4 CLint values for the remaining ~2000 compounds have been determined.

**Discussion and Conclusion**

A joint team comprised of members from the IQ Consortium and the NIH National Center for Advancing Translational Sciences undertook the task to measure and publish a data base of CLint values for compounds by major metabolic enzymes, for the benefit of advancing drug-design efforts with regard to metabolic stability. Advantages include enabling advanced computational human metabolic models for individual metabolic isozymes; improving hit selection by high-throughput and computational screening; improving computational models for predicting human pharmacokinetics and enhancing lead optimization by guiding structure modification. For such data to be generated, a high-density assay format was required. Therefore, a high-throughput assay using automation, 384-well technology, rapid UPLC separations, high-resolution MS (as well as MS/MS using MRM) quantitation, and an automated data analysis method was developed and successfully applied.

Initial pilot experiments with the automated liquid handler produced highly variable results. The $t_{1/2}$ values of control compounds between runs exhibited more than 4-fold variation. Compounds in the peripheral wells of the plate had $t_{1/2}$ values slightly different than if the

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**Fig. 5.** Drug concentration-time profiles for control samples: Ln response of control samples were plotted against time. Letters a and b in the legend correspond to duplicate samples within the same 384-well test plate.
same compounds were plated somewhere in the middle of the plate, a phenomenon known as the edge effect. This problem was rectified by preheating the incubation plate and enclosing the liquid handler system during the experiment to ensure even heat distribution across the entire plate. Air entrapment in the narrow bottoms of the 384-well plates caused random splashing and mixing in adjacent samples. This issue was completely eliminated by reducing the dispensing speed of the liquid handler. Since DMSO concentration affects enzyme activity

![Automated Incubation Method](image1)
![Automated UPLC-HRMS Method](image2)
![Automated Data Analysis Method](image3)

**Fig. 6.** Workflow schematic for the high-throughput metabolic stability assay.

**Fig. 7.** Extracted ion chromatograms of the parent compounds in a single sample containing ketoconazole, loperamide, and the IS, albendazole, based on accurate mass with mass tolerance of 5 ppm.
(Di et al., 2003), the final concentration of DMSO was kept below 0.1% in the final incubation. The enzyme was purchased in bulk quantity to completely avoid interbatch variability.

Of the 3000 compounds tested, the UPLC/HRMS produced reliable data for 2642 compounds with an 88.1% success rate. There could be several reasons for not obtaining reliable data for the 358 undetected compounds.
Automated High-Throughput Metabolic Stability Assay Method

compounds such as weak signal, inefficient ionization and adduct formation. Some compounds that undergo ionization in the positive mode may form [M + Na]+, [M + K]+, or [M + NH4]+ adduct ions (Ortelli et al., 2000; Li et al., 2002). TraceFinder can be programmed to identify whether any of these adducts are present for the 358 compounds that were not successfully detected. The method described in this article has several advantages over existing published methods: integrating automated incubation, automated data acquisition, and automated data analysis. The high-throughput high resolution MS method also has several advantages, including: 1) 4-fold higher capacity (384-well format) than existing 96-well formats, 2) efficient testing of large number of compounds with minimal labor and supervision, 3) avoiding individual compound optimization as the same generic method can be used to acquire data, and 4) significantly reduced time for data analysis. Additionally, by using HRMS in scanning mode, it is possible to interrogate the data afterward for a preliminary look at metabolite structure information.

In conclusion, we have successfully established and validated an automated high-throughput metabolic stability assay. This system can be used as a rapid assessment tool for initial screening of novel compounds. Future efforts will focus on developing in silico tools and characterizing additional compounds with the system using CYP2C9, CYP2D6, and other major CYP isozymes.

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Authorship Contributions

Participated in research design: Shah, Kerns, Obach, Wang.
Conducted experiments: Shah, Kerns.
Contributed new reagents and analytic tools: Nguyen, Xu.
Performed data analysis: Shah, Kerns, Obach.
Wrote or contributed to the writing of the manuscript: Shah, Kerns, Nguyen, Obach, Wang, Zakharov, McKew, Simeonov, Hop, Xu.

References


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