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

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DMD#072017

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DMD#072017

Abstract:

Advancement of *in silico* tools would be enabled by availability of data for metabolic reaction rates and intrinsic clearance (CL_{int}) of a diverse compound structure dataset by specific metabolic enzymes. Our goal is to measure CL_{int} for a large set of compounds with each major human cytochrome P450 (CYP) isozyme. In order to achieve our goal, it is of utmost importance to develop an automated, robust, sensitive, high-throughput metabolic stability assay that can efficiently handle large volume of compound sets. The substrate depletion method (*in vitro* half-life ($t_{1/2}$) method) was chosen to determine CL_{int} . The assay (384-well format) consisted of three parts: a robotic system for incubation and sample clean up; two different, integrated, ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) platforms to determine the percent remaining of parent compound, and an automated data analysis system. The CYP3A4 assay was evaluated using two long- $t_{1/2}$ compounds, carbamazepine and antipyrine ($t_{1/2}>30$ min), one moderate- $t_{1/2}$ compound, ketoconazole ($10<t_{1/2}<30$ min), and two short- $t_{1/2}$ compounds, loperamide and buspirone ($t_{1/2}<10$ min). Inter-day and intra-day precision and accuracy of the assay was within acceptable range (~12%) for the linear range observed. Using this assay, CYP3A4 CL_{int} and $t_{1/2}$ 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 CYP isozymes.

DMD#072017

Introduction:

Hepatic intrinsic clearance (CL_{int}) 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 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 (CYP450) family (Nebert et al., 2002). CYP450s are responsible for more than 75% of drug metabolism and the major isoform amongst these enzymes is CYP3A4 which is responsible for metabolism of ~50% of known xenobiotics in humans. Other important isoforms include CYP2C9, CYP2D6 and CYP1A2 (Guengerich et al., 1999, 2008). A joint team comprised of members from the International Consortium for Innovation and Quality in Pharmaceutical Development (IQ Consortium) and the NIH National Center for Advancing Translational Sciences (NCATS) established a goal to measure CL_{int} for a large set of compounds with each major human CYP isozyme, beginning with CYP3A4, from which *in silico* prediction tools can be developed. Such an extensive database 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 et al., 1994; Linget et al., 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 et al., 2004). These assays are regularly used by pharmaceutical companies to provide valuable insights for drug design during discovery research in order to optimize pharmacokinetic profiles of chemical series (MacKenzie et al., 2002; Korfmacher et al., 2003; Nassar et al., 2004). *In silico* tools for 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; Sakiyama et al., 2008). For this project, we chose to test 5000 compounds with multiple major CYP isozymes. Using a multiple time

DMD#072017

point assay, this would require >30,000 measurements per isozyme (5000 test compounds + additional control samples X 6 time points). Thus, it was of utmost importance to use an automated, robust, sensitive, 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/sample preparation and UPLC/MS (ultra-performance liquid chromatography–mass spectrometry) for data acquisition (Korfmacher et al., 1999; Di et al., 2003), which would require more than fifty 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, while 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 methodology can benefit drug research and possibly be used to measure metabolic lability in diverse matrices (e.g., microsomes, S9 fractions, cytosol fractions).

DMD#072017

Materials & 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 (Catalog# 456202) and NADPH Solution A/B (Catalog# 451220/451200) 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* $t_{1/2}$ method) to determine CL_{int} 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) (Figure 1). The heating block was calibrated beforehand using a thermocouple inserted in incubation matrix solution and a setting of 45° C was found to produce a solution temperature of 37° C. Pipette tips (50 μ L; Catalog# 30038609 & 200 μ L; Catalog# 30038619) were purchased from Tecan (Morrisville, NC) and reservoirs (low profile; RES-SW384-LP & high profile; RES-SW384-HP) for the incubation were purchased from Axygen (Woburn, MA). 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 μ L of 10 mM albendazole in DMSO to 722 mL of ACN and is called “ACN/IS” henceforth.

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 μ M in ACN using

DMD#072017

the robot in a secondary plate. In the first step, 82.73 μL of diluted supersomes (3 pmol) were transferred to the incubation plate (384-well, 250 μL : Waters, Milford, MA) on the Inheco heating block. During this pre-incubation period, 2.43 μL of NADPH solution A/B (1 μM) and 40 μL of chilled ACN/IS were aspirated in a fresh, time 0 plate i.e. T0 (Waters; 100 μL). After 5 min of pre-incubation, 2.27 μL of compound (50 μM in ACN) was added to the incubation plate and 7.5 μL of this mixture was added to the T0 plate. After the T0 plate was prepared, 25 μL of NADPH regenerating solution A/B was added to the incubation plate. The final concentration of the test compound is 1 μM . Two minutes before each subsequent time point, 40 μL of 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 min 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; #AB-1720) and centrifuged for 20 min 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. In order 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 method

UPLC Method:

DMD#072017

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 μ m, 2.1 X 50 mm, Catalog# 186002852) equipped with a Waters Acquity UPLC[®] BEH Shield RP18 VanGuard[™] pre-column (1.7 μ m, 2.1 X 5 mm, Catalog# 186003977). 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 min, to 80% A: 20% B over 0.3 min, to 1% A: 99% B over 0.5 min, and held at 1% A: 99% B for 0.7 min. The column re-equilibration time was 0.4 min. 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 v4.1 software. Multiple reaction monitoring (MRM) methods were automatically developed by the instrument for each compound using the QuanOptimize application (described below). The samples were injected in the following order: 60 min, 30 min, 15 min, 10 min, 5 min and 0 min, to minimize carry-over effects.

QuanOptimize:

An aliquot of 3.0 μ L (containing 50 μ M drug) from the secondary plate was diluted into 75 μ L 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 μ L 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.

DMD#072017

Sample Analysis:

For each pooled sample, 2.0 μL was injected onto the BEH Shield column with BEH Shield pre-column 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 re-injecting 2 μL of the QuanOptimize solution for analysis under the same UPLC chromatography as the samples, using MS2 scanning analysis at m/z 50 to 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, re-integrated 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 the following equations (Obach et al., 1997).

$t_{1/2} = -0.693/k$ ($k =$ - slope of the linear regression from Ln percentage remaining vs incubation time relationship).

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

Data Acquisition: Method 2: High Resolution MS

UPLC method:

The Thermo Ultimate 3000 UPLC comprised a 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 min, a linear gradient from 5%-95%B from 0.2-1.7

DMD#072017

min, followed by 95%B for 0.4 min. The column re-equilibration time was 0.4 min. The column effluent was directed to the high-resolution mass spectrometer.

High resolution MS method:

MS data were acquired on a bench top QExactive mass spectrometer (Thermo Fisher Scientific, San Jose, USA). 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 50-1000 m/z and resolution of 35,000 was employed. The instrument was calibrated using the positive ion calibration solution which comprised of 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 and the same external calibration was applied throughout each batch. The samples were injected in the following order: 60 min, 30 min, 15 min, 10 min, 5 min and 0 min, to minimize carry-over 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 mass-to-charge (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

DMD#072017

results: plots of percent remaining versus time, and Ln (response) versus time; regression analysis of various combinations of data points by the utility and ranked by quality of the fit (r-squared, 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 to perform automated fitting and ranking of calculated CL_{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 $\sum_2^6 \binom{6}{n} = 57$ possible combinations of T are used to perform Ln linear regression fit. Each fit in turn is evaluated based on the following scoring scheme

$$S = Nr^2 e^{-\sigma_e} \sum_{i=0}^5 2^{-i}$$

where N is the number of time points, $r \in [-1,1]$ is the Pearson's correlation, and σ_e is the root mean square error. The best possible score (i.e., $N = 6, r = 1$, and $\sigma_e = 0$) is $S_{max} = 6 \left(1 + \frac{1}{2} + \frac{1}{4} + \frac{1}{8} + \frac{1}{16} + \frac{1}{32} \right) = 11.81$. The score $S_{norm} = S/S_{max} \in [0,1]$ 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 CL_{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 in 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

DMD#072017

(RDBMS) and can be accessed at a later time. The source code for the IQC Validator software is available at <https://spotlite.nih.gov/ncats/iqc>.

DMD#072017

Results:

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

Method Validation:

Five commercial compounds with different half-life 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 1-5 nM – 1000 nM for the control compounds (Table 1). Intra-day precision and accuracy was determined by measuring three different quality control (QC) concentrations (10 nM, 50 nM and 500 nM) three times in one day and the inter-day precision and accuracy were determined by measuring concentrations of three QC samples over five days. The intra- and inter-day precision (CV%) for QC samples were below 7% and 11% for all control compounds. The intra- and inter-day 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 internal standard 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 (Figure 2). The sensitivity of the HRMS instrument in detecting peaks for test compounds with >98% turnover is shown in Figure 3. Instrument calibration was performed before each batch analysis and mass accuracy (~2 ppm) was sustained throughout each batch run without need for re-calibration or use of an internal reference (Figure 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 half-life values of control compounds, included twice in each 384-well plate across multiple plates. Ln response over time of the

DMD#072017

control compounds across three experiments were plotted to demonstrate the reproducibility between and within experiments (Figure 5). The results show excellent reproducibility within plates and between plates (Table 1; Figure 5). The percent coefficient of variation (%CV) for the half-life values of buspirone, loperamide and ketoconazole, between experiments was between 15%-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 Figure 6. The total preparation and incubation time for each 384-well plate experiment was 2 h. 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 min) for each of the 384 compounds. Adjacent wells were combined from each plate, thus converting six plates into three. This 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 which contains two test compounds and the internal standard is shown in Figure 7. The UPLC/HRMS acquisition was allowed to run overnight and the time required for each batch (1152 samples) was two and a half days. Under optimal conditions, two 384-well incubation plates can be assayed in a week 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 (Figure 8) and calculated $t_{1/2}$ and CL_{int} . These software tools completely eliminated data extraction time and drastically reduced data analysis time.

Compound library:

~3000 compounds were tested using the newly optimized high-throughput method. The majority of these compounds were a part of NCGC (NIH Chemical Genomics Center) pharmaceutical collection (NPC). The NPC library encompasses publically-available approved and investigational drugs (Huang et al., 2011).

DMD#072017

The NPC contains than 2400 compounds which 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, Lipinski rule of 5, were calculated using CDK descriptors tool (The Chemistry Development Kit download, 2016) implemented in the KNIME (KNIME, 2016) analytical platform (Warr et al., 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 half-life values greater than 60 minutes, belong in the 251-500 MW range and majority of them do not violate Lipinski rules. We did not find any direct correlation of calculated $t_{1/2}$ values with the above molecular descriptors. While a lot of microsomal metabolic stability data are available in literature, this is, to our knowledge, the first time that such an extensive compound database is being tested with an individual isozyme. A detailed presentation of the data as well as *in silico* model development will follow once CYP3A4 CL_{int} values for the remaining ~2000 compounds have been determined.

DMD#072017

Discussion/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 database of CL_{int} 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. Half-life values of control compounds between runs exhibited more than 4-fold variation. Compounds in the peripheral wells of the plate had half-life values slightly different than if the same compounds were plated somewhere in the middle of the plate, a phenomenon known as the edge effect. This problem was rectified by pre-heating 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 has been shown to effect enzyme activity (Di et al., 2002), the final concentration of DMSO was kept below 0.1% in the final incubation. The enzyme was purchased in bulk quantity to completely avoid inter-batch variability.

Out 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 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 $[M + NH_4]^+$ adduct ions (Li et

DMD#072017

al., 2002; Ortelli et al., 2000). 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; (4) significantly reduced time for data analysis. Additionally, by using HRMS in scanning mode, it is possible to interrogate the data afterwards 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.

DMD#072017

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DMD#072017

Authorship Contributions:

Participated in research design: Shah, Kerns, Wang & Obach

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, Xu, Wang, Simeonov,
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DMD#072017

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DMD#072017

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DMD#072017

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DMD#072017

Figure Legends:

Figure 1: Tecan liquid handler deck layout for the high-throughput metabolic stability assay.

Figure 2: Peak response for albendazole: Peak area of the internal standard, albendazole was plotted (A) across 3 batches i.e. three 384-well test plates. The mean and SD for the 3 batches are described in (B).

Figure 3: Sensitivity of the HRMS instrument: (A) 0 min chromatogram and (B) 60 min chromatogram for buspirone (>98% turnover) generated from full-scan data acquired with the Thermo QExactive.

Figure 4: Mass accuracy of the QExactive: Mass deviation of the internal standard, albendazole across nine test plates was measured by comparing the theoretical mass-to-charge ratio (m/z) value to the observed m/z value. The mass deviation was measured twice, once at the beginning of the batch with the first sample and once at the end of the batch with the last sample i.e. sample 1152.

Figure 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.

Figure 6: Workflow schematic for the high-throughput metabolic stability assay.

Figure 7: Extracted ion chromatograms of the parent compounds in a single sample containing ketoconazole, loperamide and the internal standard, albendazole, based on accurate mass with mass tolerance of 5 ppm.

Figure 8: Overview of user interface of the Validator software. For each sample, the analyst has the option of selecting the most appropriate regression fit with the help of Ln response or % remaining vs time curves as well as fitted and calculated parameters. Once regression fit is assigned, the data can be saved and exported for further analysis/modeling and simulation.

Figure 9: Distributions of molecular weight, experimental $t_{1/2}$, logP, topological polar surface area and rule-of-five (RO5) violations of the metabolic stability dataset generated using KNIME analytical platform.

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Tables:

Table 1: Reproducibility assessment for control samples:

Compound name	Molecular formula (parent)/Exact mass [M+H] ⁺	Linearity range	Number of repeats	t _{1/2} (mean ± S.D)/category
Buspirone	C ₂₁ H ₃₁ N ₅ O ₂ /386.2550	1 – 1000 nM	18	2.5±0.45 min/Low
Loperamide	C ₂₉ H ₃₃ ClN ₂ O ₂ /477.2303	2 – 1000 nM	18	3.5±0.59 min/Low
Ketoconazole	C ₂₆ H ₂₈ Cl ₂ N ₄ O ₄ /531.1560	4 – 1000 nM	12*	21±3.6 min/Moderate
Antipyrine	C ₁₁ H ₁₂ N ₂ O/189.1022	5 – 1000 nM	18	>>30 [^] min/High
Carbamazepine	C ₁₅ H ₁₂ N ₂ O/237.1022	2 – 1000 nM	18	>>30 [^] min/High

Half-life values of the control samples across nine 384-well plates based metabolic stability assays were measured by UHPLC/HRMS. Half-life categories: <10 min: Low; >10<30 min: Moderate; >30 min: High.

**Initial 3 experiments did not include ketoconazole as a control*

^Half-lives of antipyrine and carbamazepine ranged from 150-400 min. Hence no S.D depicted.

Figure 1

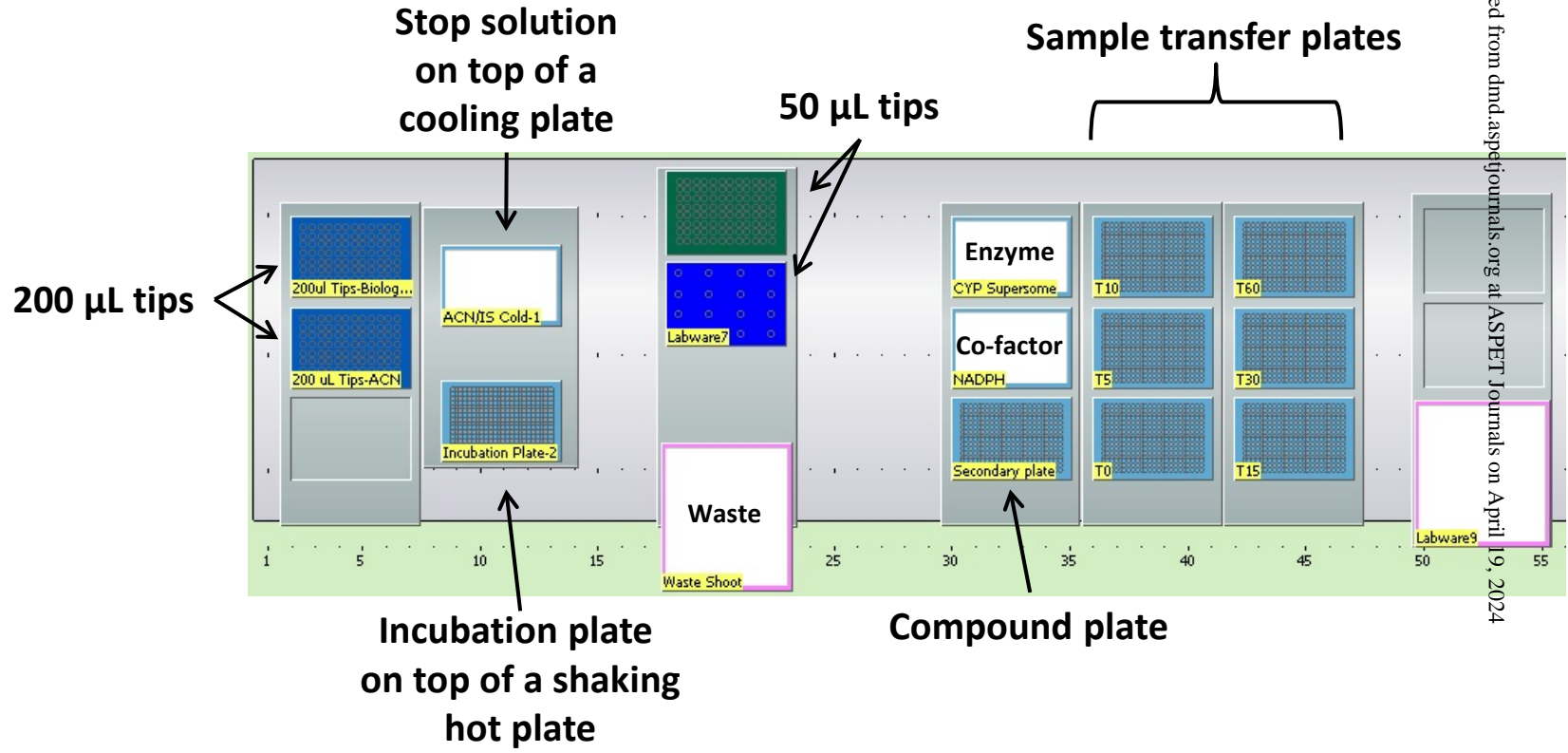
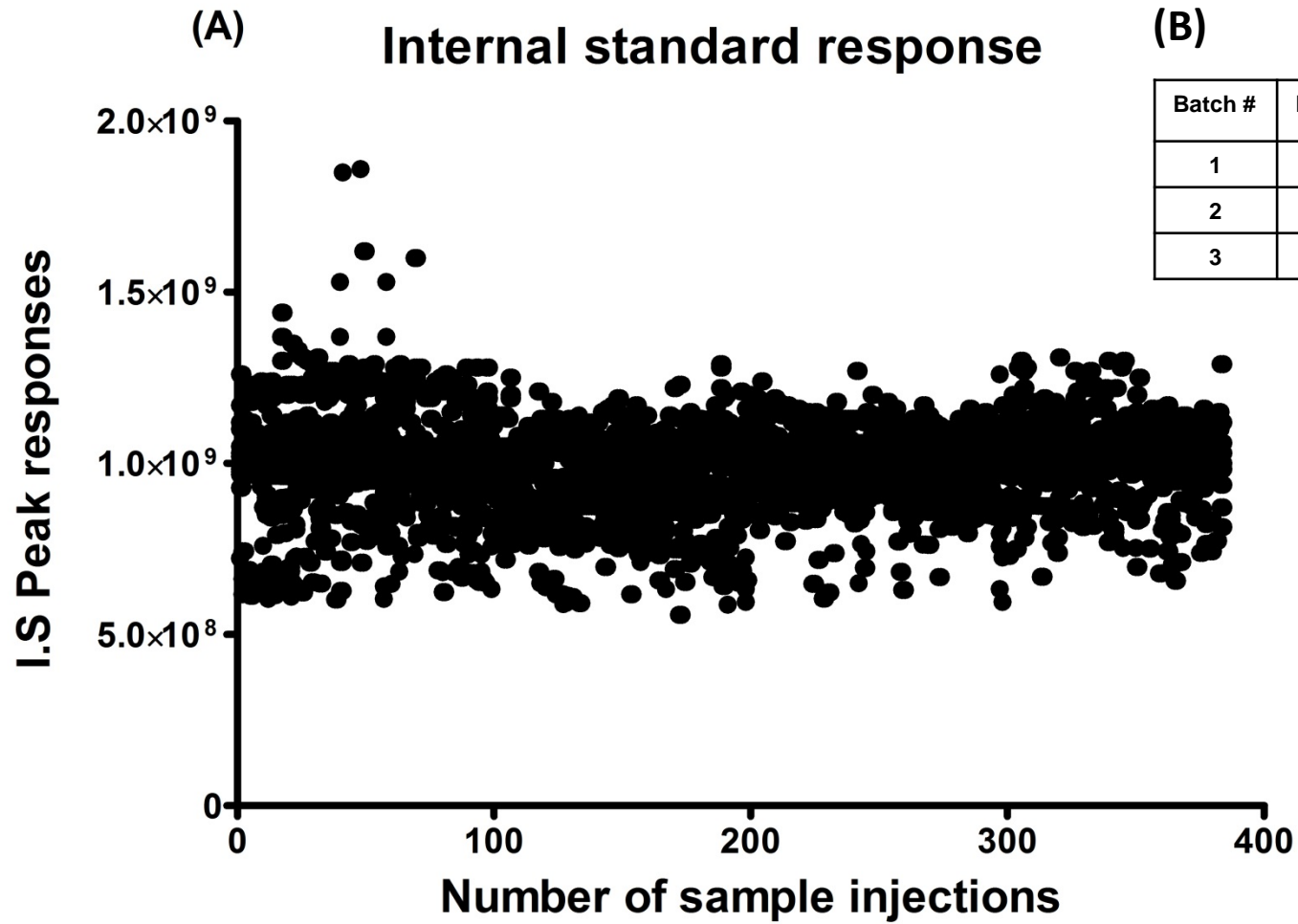


Figure 2



(B)

Batch #	Peak area (Mean \pm S.D)
1	9.4E+08 \pm 1.4E+08
2	7.95E+08 \pm 1.9E+08
3	9.11E+08 \pm 1.67E+08

Figure 3

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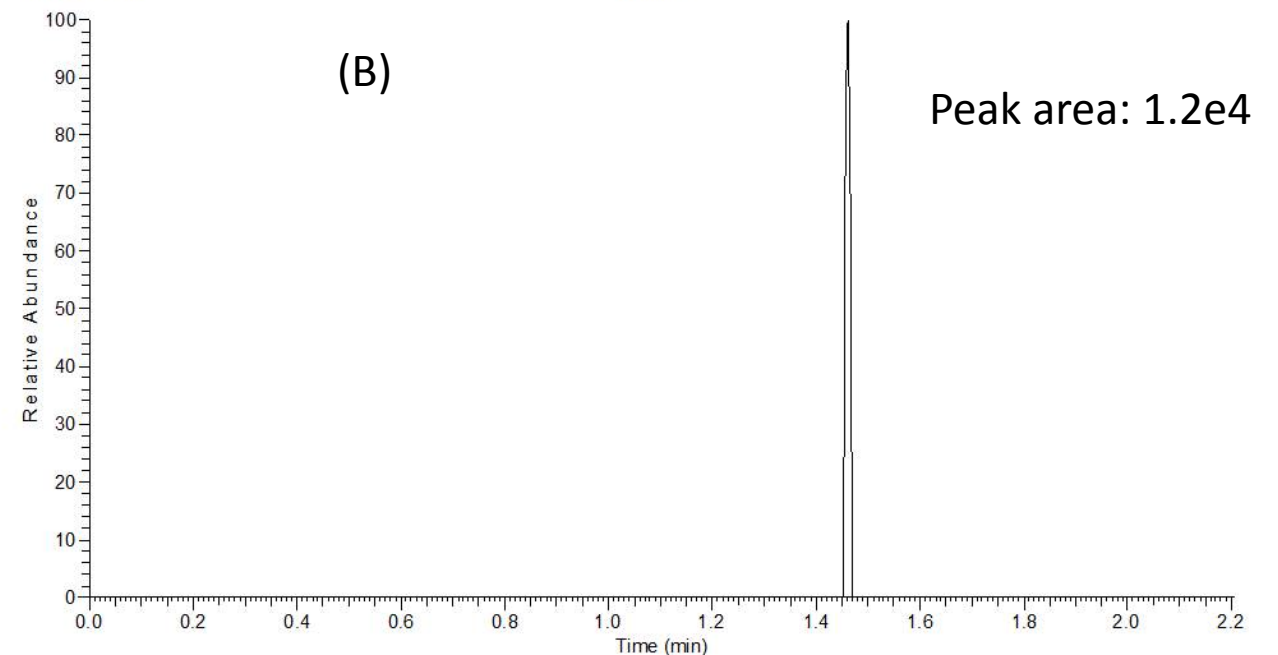
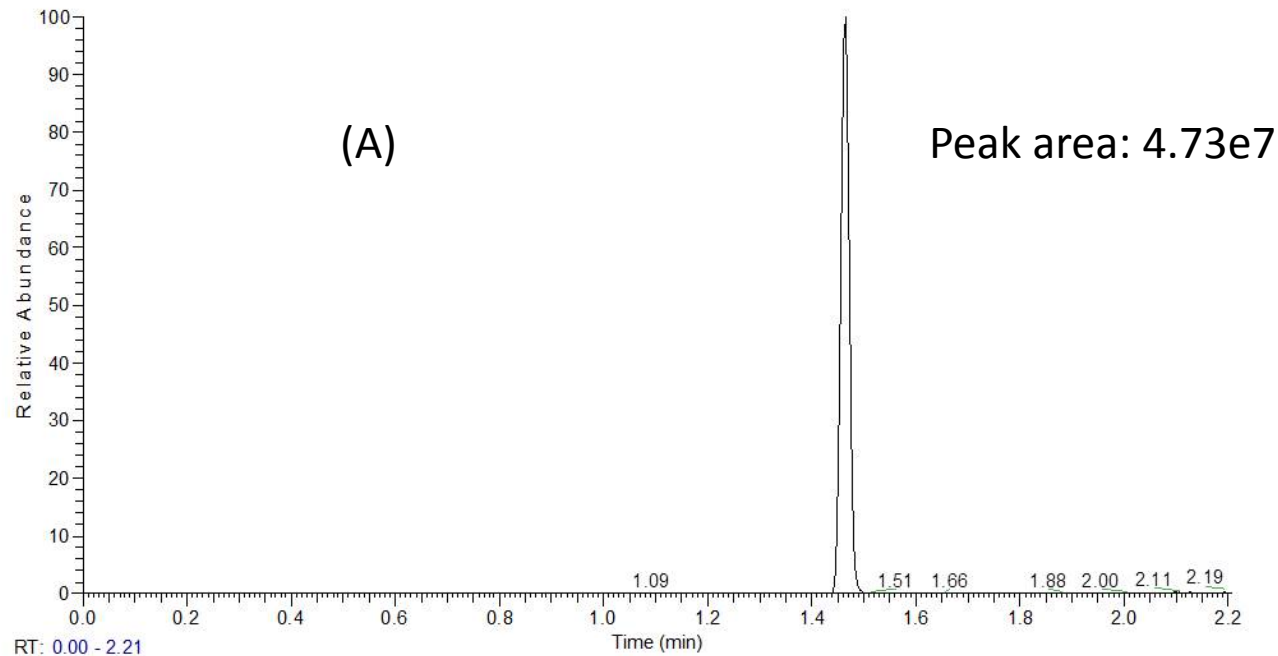


Figure 4

Albendazole mass accuracy through & across runs

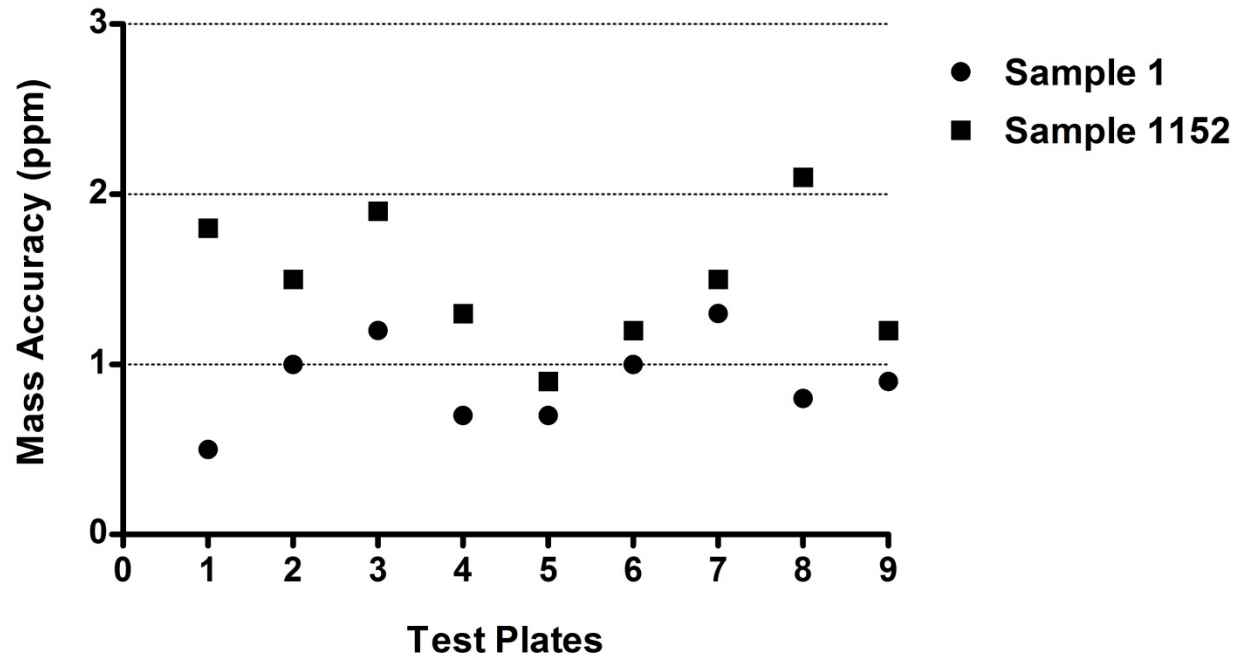
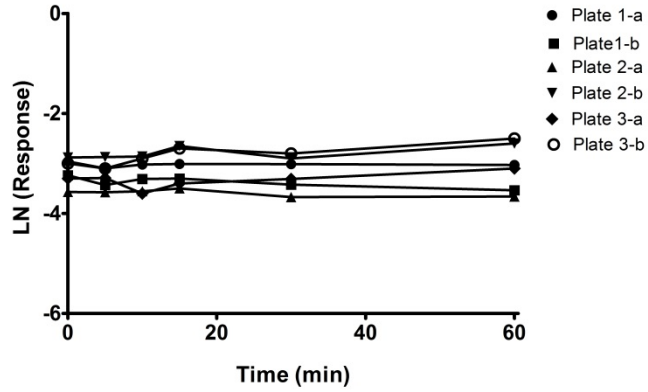
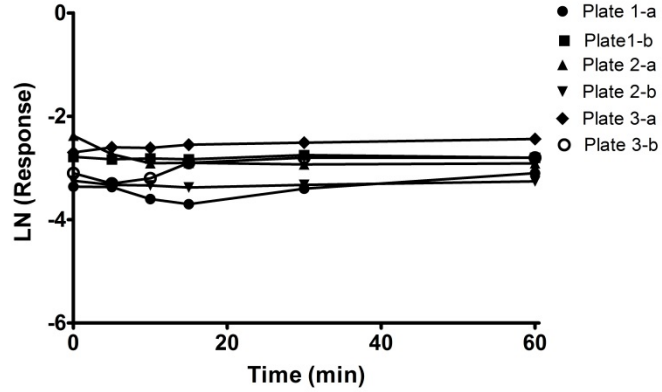


Figure 5

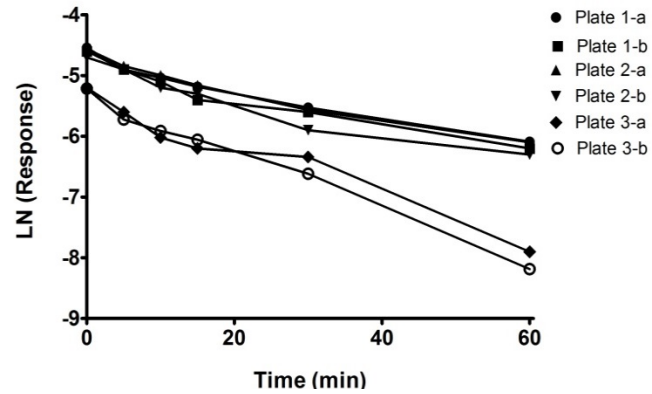
Carbamazepine



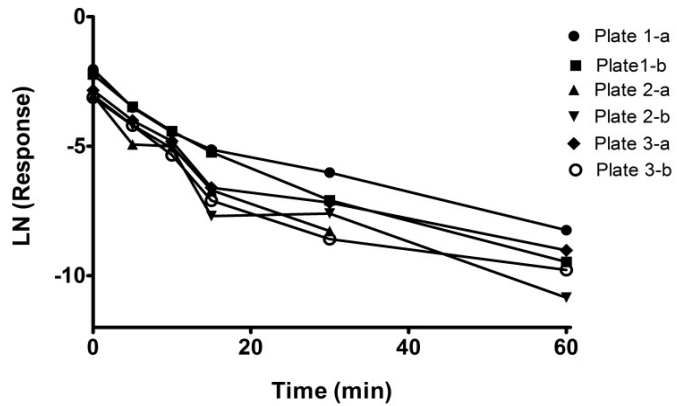
Antipyrine



Ketoconazole



Loperamide



Buspirone

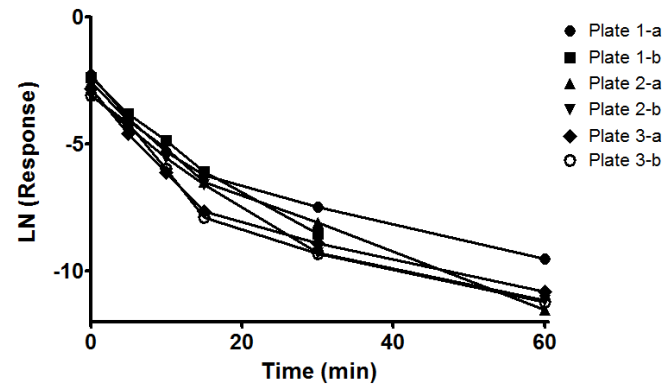


Figure 6

Automated Incubation Method

10 mM compound plate from compound management diluted to 50 μ M using ACN. 1 μ M (final conc) compound added to incubation plate

Supersomes/NADPH cofactor



37°C with shaking

10 μ L incubation mixture in 40 μ L cold ACN + Internal Standard at 0, 5, 10, 15, 30 and 60 mins

Centrifuge the plates for 20 mins at 4°C

Combine adjacent wells to convert 6 plates into 3 plates using the Tecan. This reduces number of sample analysis from 2304 to 1152.

Automated UHPLC-HRMS Method

Import compound names and molecular formulae into TraceFinder 3.2 software

Analyze using a pre-developed UHPLC-Full Scan MS method

TraceFinder 3.2 will detect and integrate peaks based on exact mass and automatically generate 1152 output reports/batch

Automated Data Analysis Method

Import all reports into Validator software

Validator will extract data from the TraceFinder 3.2 output reports.

Validator will perform regression analysis of various combinations of data points and rank them by quality of the fit & calculate $t_{1/2}$ and CL_{int} .

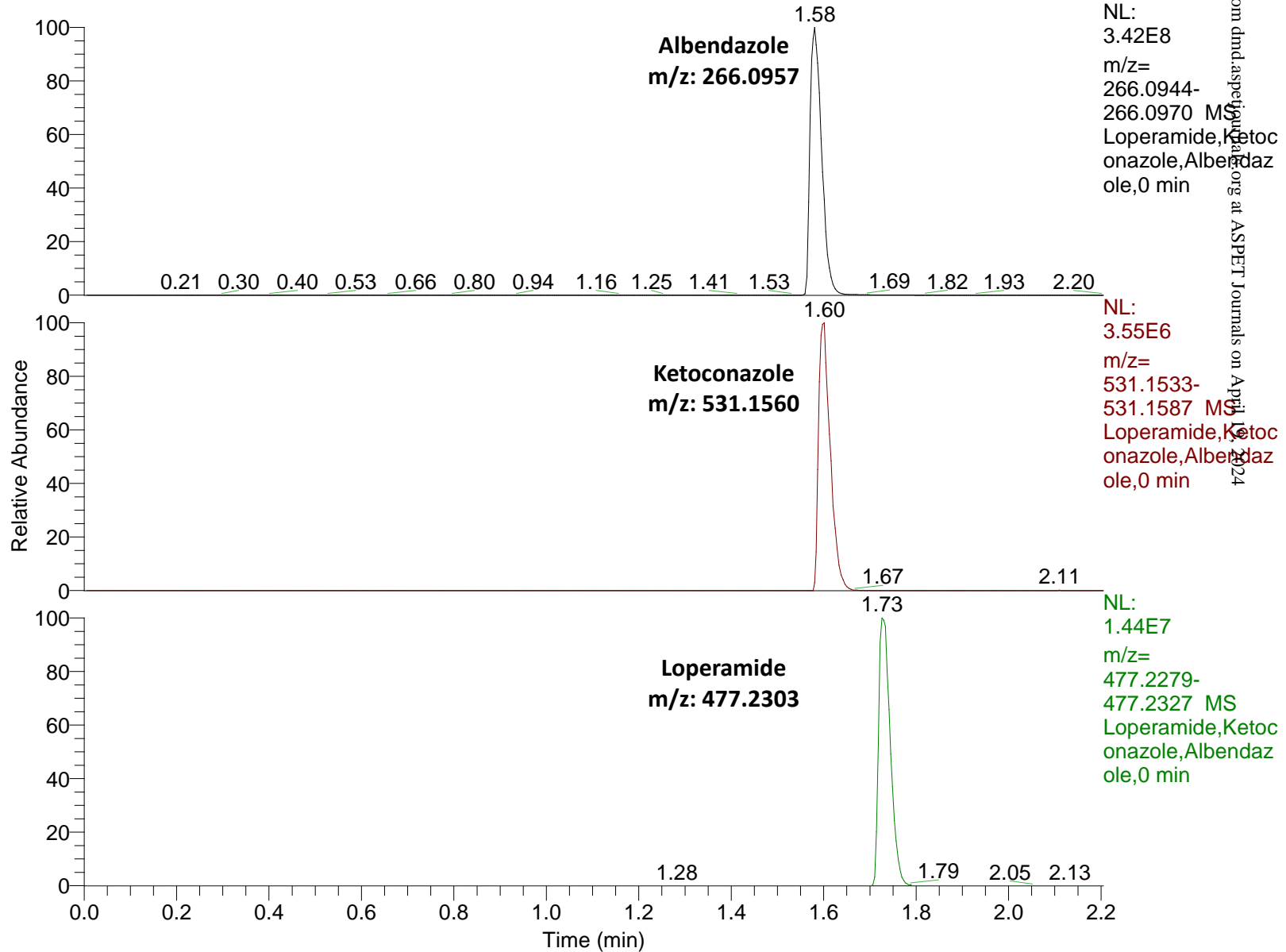
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Figure 7

Loperamide,Ketoconazole,Albendazole,0...

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Figure 8

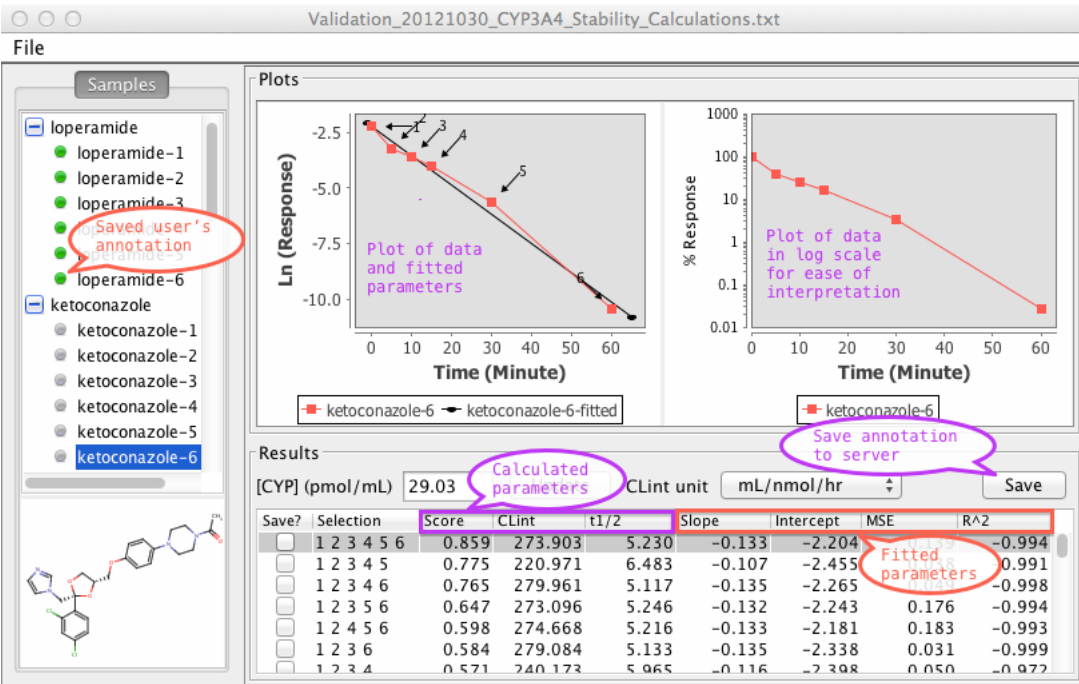


Figure 9

