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Automated high throughput ADME assays for metabolic stability and cytochrome P450 inhibition profiling of combinatorial libraries

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Abstract

Early determinations of pharmaceutical properties can serve as predictors of a compound's likely development success. Our laboratory has implemented high throughput in vitro absorption, distribution, metabolism and excretion (ADME) assays which address absorption, metabolism, and physico-chemical properties in an effort to identify potential development liabilities early, thereby minimizing discovery to market attrition. In response to the throughput demands of parallel synthesis, we have incorporated a SAGIAN™ core robotics system for the determination of both metabolic stability in human liver microsomes (HLMs) and cytochrome P450 (CYP450) inhibition. This automated solution has led to an increase in capacity, throughput and reliability for both in vitro assays.

The SAGIAN™ core robotics system integrates devices such as liquid handlers, plate hotels and incubators through the use of an ORCA® robotic arm. The HLM stability assay utilizes a Multimek™ 96-channel pipettor for liquid handling. The incubation plates are transferred off-line for final semi-quantitative analysis using high throughput parallel LC/MS. The CYP inhibition method combines both liquid handlers and an integrated fluorescence plate reader to perform single concentration percent inhibition assays for 88 compounds. Cytochrome P450 inhibition is measured for both CYP3A4 and CYP2D6 isozymes.

This system represents a fully integrated approach to high throughput ADME evaluation in support of drug discovery. The core system concept creates a plug-and-play approach, which combines a series of modular stations to build a robotic platform, which is flexible, upgradable, and easily reconfigured when assays change or are newly developed. The application of these strategies as a means of assessing metabolic stability and CYP inhibition of synthetic libraries is discussed.

Keywords: Automated ADME assays; CYP inhibition; HLM metabolism; Parallel LC/MS

1. Introduction

The need for novel therapies has led to a rapid increase in the development of discovery technologies within the pharmaceutical industry. Parallel synthesis

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and high throughput screening have given rise to an increasing number of drug discovery leads requiring optimization in order to progress into pre-clinical development. However, it has been estimated that two-thirds of compounds entering clinical development fail due to poor absorption, distribution, metabolism and excretion (ADME) or efficacy [1]. In response, pharmaceutical companies are shifting drug metabolism and pharmacokinetic (DMPK) profiling from its late stage position to early drug discovery [2–5].

One of the major difficulties with this transition is that many of the methods employed by drug metabolism and pharmacokinetic groups have been developed to generate high quality data for a limited number of compounds, whereas drug discovery departments in most pharmaceutical companies are capable of generating a few hundred compounds per day. Thus, there is a need for innovative high throughput methods providing equally high quality data. The paradigm shift from late stage optimization of ADME properties to a strategy of identifying liabilities early in the discovery process is an area of rapid advancement. In response to this change, the industry has recognized the need for high throughput ADME assays [6–8]. Fortunately, many of these challenges are being met and a large proportion of ADME assays can now be run in a more high throughput fashion, due principally to the wide-spread incorporation of LC/MS. The sensitivity and selectivity advantages of LC/MS have improved standard techniques for analyzing probe substrates in cytochrome inhibition assays [9–12], improved sensitivity in permeability assays such as Caco-2 [13] and provided much more rapid assessment of metabolism [14–16]. Cytochrome P450 (CYP450) inhibition assays have also been positively impacted by the development of fluorometric substrates that have reduced the analysis of microtiter plates to under a minute [17,18].

While a vast amount of development has been focused on addressing methods to speed analysis times, only a limited number of groups have provided details concerning technologies enabling the automation of front-end sample preparation which is often rate-limiting. Automating sample preparation has many inherent advantages, including throughput, reproducibility, and release of resources for additional method development. Automation has recently been applied to metabolic stability studies [19] and the assessment of cytochrome P450 inhibition [20]. These automated systems, while quite useful, employ complex liquid handling instrumentation and custom software development. Additionally, these systems are often developed specifically for one assay type and thus are not easily modified or extended to new methods or protocols.

The integration of automation in the drug discovery environment has typically occurred by one of three main approaches. The first employs developing complex integrated systems that are very specialized for particular tasks. This approach is often seen in biological high throughput screening labs and relies heavily on complex automated systems, robotics and engineering specialists resulting in significant capital investment [21]. The second involves the use of individual workstations, an approach that has been benchmarked in current DMPK departments [22,23]. This approach, while effective, is highly dependent on user intervention for the transfer of materials and reagents. The third principal approach in laboratory automation involves the implementation of a core robotics system, which may be dedicated to a particular assay or configured to support multiple assays [24]. It is this latter approach that we have taken in our laboratory for the implementation of high throughput ADME assays that address absorption, metabolism, and physico-chemical properties.

Sample preparation was identified early on as a key bottleneck in our process. The preparation of assay plates is often labor and resource intensive thereby adversely impacting capacity and throughput. In response to these sample preparation limitations and the need to meet the throughput demands of parallel synthesis, we have established a core robotics system for automated sample preparation, data analysis, and management of results generated from in vitro ADME assays. Two of our frontline ADME assays include cytochrome P450 inhibition and metabolic stability, for which sample preparation has been addressed by employing automation on a SAGIAN^{TM} core robotics system (Fig. 1). In addition, we have developed a three tiered approach, integrating automated sample preparation, high throughput analytical analysis and both custom and commercial software implementation for data analysis and archive.

Fig. 1. SAGIAN™ core robotics system supporting in vitro ADME assays.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Dimethylsulfoxide (DMSO) was obtained from EM Science (Gibbstown, NJ, USA). Formic acid, trichloroacetic acid (TCA), sodium phosphate monobasic, potassium phosphate dibasic, potassium phosphate monobasic, Tris base, sodium hydroxide (NaOH), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), atenolol, buspirone hydrochloride, imipramine hydrochloride, lidocaine hydrochloride, methoxyverapamil hydrochloride, DL-propranolol hydrochloride, (±)-sulpiride, (±)-verapamil hydrochloride, ketoconazole, quinidine, and Eosin Y were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant cytochromes were purchased from Gentest (CYP 3A4 + OR Supersomes® or CYP 2D6 Supersomes®) and stored at −80 °C prior to use.

2.2. Microsomal stability reagents

Pooled human liver microsomes (HLMs) containing 20 mg/ml of total protein were obtained from Gentest Corp. (Woburn, MA, USA). Sodium phosphate buffer was prepared as a 50 mM stock solution in deionized water and adjusted with 1 M NaOH solution to pH 7.4. The stock reference solutions (atenolol, buspirone, imipramine, lidocaine, methoxyverapamil, propranolol, sulpiride, and verapamil) were prepared at 10 mM concentration in DMSO, then diluted to 1 mM with ACN prior to use. NADPH solution was prepared at 4 mM concentration in phosphate buffer prior to use. TCA solution was prepared at 0.3 M in deionized water.

2.3. Cytochrome P450 inhibition reagents

Potassium phosphate monobasic and dibasic buffers were prepared at 0.5 M in deionized water. Assay buffer was prepared by mixing potassium phosphate dibasic and monobasic buffers (7:1.5) and adjusting the pH to 7.4. After dilution, the final assay concentration of the assay buffer was 0.1 M. The stop solution used to quench the cytochrome P450 reactions was composed of 80% acetonitrile/20% 0.5 M Tris base. The NADPH solution contained the substrate for each of the CYP reactions. The substrates were 7-benzyloxy-4(trifluoromethyl)-coumarin (BFC) and 3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) for CYP 3A4 and CYP 2D6, respectively. BFC and AMMC were reconstituted in acetonitrile to obtain a 10 mM solution. AMMC was further diluted in NADPH/assay buffer solution to give a final reagent concentration of 3 μM while BFC was diluted to a working concentration of 150 μM. NADPH was prepared in deionized water at a final concentration of 10 mM. Supersomes® were thawed rapidly at 37 °C and diluted to 60 nmol/μl in ice-cold assay buffer. Final assay concentrations for CYP 3A4 incubations were 1 mM NADPH, 50 μM BFC, and 1 pmol reconstituted microsomal protein.

CYP 2D6 final assay concentrations were 50 μM NADPH, 1 μM AMMC, and 1 pmol reconstituted microsomal protein.

2.4. Plate replication process

Test compounds were archived as 10 mM DMSO stock solutions in microtiter plate format and then evaporated to dryness in a GENEVAC HT-12 (Genevac Technologies, Valley Cottage, NY, USA). The archive plate was then transferred to a Tecan Genesis RSP 150 liquid handler (Tecan US, Durham, NC, USA) for reformatting where 1 ml of acetonitrile was added to each well and the plate sealed and placed on an orbital shaker for 20 min and then transferred to a FS9 sonicator bath (Fisher Scientific, Pittsburg, PA, USA) for 5 min to aid in the resolubilization of the test compounds. The archive plate (100 μM) was then returned to the deck of the liquid handler for the preparation of assay plates. Human liver microsome stability assay plates were prepared from the archive plate by transferring 40 μl to each of two 1.2 ml metabolic stability substrate plates. A second dilution plate was prepared from the archive plate by transferring 75 μl to a second 2.2 ml plate and diluting the plate with acetonitrile to a final concentration of 7.5 μM. The cytochrome P450 inhibition plates were prepared from this dilution plate by transferring 10 and 100 μl to each of four 200 μl microplates for 2D6 and 3A4, respectively. The HLM and CYP substrate plates were then evaporated to dryness and stored at 4 °C until they were assayed.

2.5. Manual metabolic stability sample preparation

The following procedure was applied to test compounds submitted for metabolic stability assessment in human liver microsomes, as schematically represented in Fig. 2. Eight reference compounds (atenolol, buspirone, imipramine, lidocaine, methoxyverapamil, propranolol, sulpiride, and verapamil) were incubated along with 88 test compounds in 96-well format. Liquid transfer steps were performed with a PP-550 MS 96-channel Personal Pipettor (Apricot Designs, Inc., Monrovia, CA, USA) under computer control. The substrate plate was prepared by addition of NADPH solution (500 μl, 4 mM) to each well of a 1.2 ml deep-well microtiter plate. Test compounds were diluted to 1 mM in acetonitrile from a 10 mM DMSO stock then dispensed (4 nmol each), by aid of an eight-channel Finnipipette® multipipettor (Thermo Labsystems, Helsinki, Finland) into 88 wells of the 1.2 ml deep-well substrate plate. Compounds were arrayed in the substrate microtiter plate in such a way as to leave the first column (eight wells) empty for reference compounds. Solutions of eight reference compounds (4 μl, 1 mM) were added into the first column of the microtiter plate. The entire plate was then mixed five times using

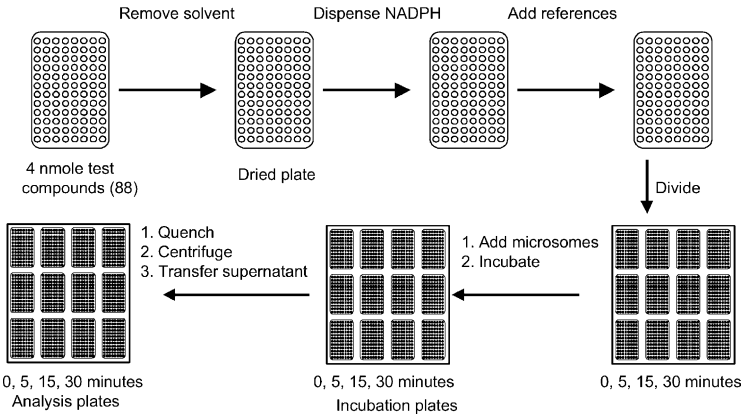

Fig. 2. Representation of manual human liver microsomal (HLM) incubation plates. Each plate of test compounds ($n = 88$) generates 12 assay plates for LC/MS analysis when the experiment is conducted in triplicate with four time points (0, 5, 15, and 30 min).

the 96-channel pipettor and then agitated on a plate shaker for 10 min. The solutions in the plate were then transferred into twelve 200 μl low-volume incubation plates (30 μl each well) and pre-incubated at 37 °C for 5 min in a VWR incubator (VWR Scientific products, Cornelius, OR, USA). Pre-warmed (37 °C, 5 min) microsomal solution (2 mg/ml) was then added into all wells of the incubation plates (30 μl/well). The final incubation solutions contained 4 μM test compound or reference compound, 2 mM NADPH, 1 mg/ml (total protein) microsomes, and 50 mM phosphate (pH 7.4). The incubation plates were then incubated at 37 °C for 0, 5, 15, and 30 min, respectively. At each time point, three incubation plates were removed from the incubator and quenched immediately with an equal volume of ice-cold 0.3 M TCA. Quenched plates were centrifuged for 15 min at 420 rad/s in an Eppendorf 5804R centrifuge (Brinkman Instruments, Westbury, NY, USA) and the supernatants transferred to new 200 μl low-volume analytical plates for high throughput parallel LC/MS analysis.

2.6. Manual cytochrome P450 inhibition sample preparation

Compounds were tested at concentrations of 5 and 0.5 μM against CYP 3A4 and 2D6, respectively. The cytochrome P450 assays were performed in 96-well black round bottom microtiter plates (Costar, Corning, NY, USA). Test compounds previously added to assay plates were re-suspended by the addition of 50 μl of 0.1 M assay buffer. The plates were then sonicated for 5 min to ensure resolubilization of the test compounds. Plates were prepared with blanks, controls and positive control standards (ketoconazole or quinidine). NADPH/substrate (50 μl) solution was then added to each well of the microtiter plate using a Finnipipette® multichannel pipettor. The reactions were then initiated by the addition of 50 μl of Supersomes® to all wells except the blanks. Sample blanks were prepared by addition of 50 μl of assay buffer to wells D1–F1. The plates were then incubated at 37 °C for 30 min in a VWR incubator (VWR Scientific products, Cornelius, OR, USA), quenched upon addition of stop solution and read on a SPECTRAmax® Gemini fluorescence plate reader (409 excitation (ex), 530 emission (em), 3A4, 390 ex, 460 em, 2D6).

2.7. SAGIAN™ core robotics system

The automated assays were developed on a SAGIAN™ core robotics system (Beckman Coulter, Fullerton CA, USA) which incorporated an Orca® robotic arm (3 m rail length) and the following devices: Multimek™ 96-channel liquid hander, Biomek® 2000 multichannel liquid handler, 140 position eight hotel microplate carousel, eight position humidified temperature controlled incubator, microtiter plate barcode reader and a SPECTRAmax™ Gemini fluorescence plate reader (Molecular Devices, Sunnyvale CA, USA). Pipetting procedures on the Multimek™ involved automated tip rinsing using the integrated tip wash station and air gaps where appropriate based on the volume being transferred. The Biomek® liquid handler deck incorporated an integrated thermo-regulated reservoir maintained at 4 °C to preserve enzymatic activity of the reagents. This station also included a filtration manifold, gripper tool and both single and eight-channel pippetting tools. Manufacturer recommended tips were used on both liquid handlers and tips were used only once on the Biomek® liquid handler since it did not contain an integrated tip wash station.

2.8. Routine validation dye test

To evaluate the performance of the pipetting heads and tools incorporated in the SAGIAN™ core robotics system, a simplified dye test was developed and performed on a weekly basis to assess system performance. The validation test could be run on either liquid handler and involved the transfer of 30 μl of Eosin Y dye (0.5 mM) into a 96-well microtiter plate to which 100 μl of water had been previously added. The plate was then read on a SPECTRAmax™ Gemini fluorescent plate reader (485 ex, 538 em) and analyzed via a custom Excel macro. The liquid handlers performed very well overall and typically good pipetting procedures led to enhanced lifetime between cleanings and maintenance. Routine maintenance was not performed beyond the manufacturer recommended schedule. Occasionally, the pipettors would display CVs greater than 5% for a few of the tips. This was typically seen in the Multimek™ head and was due to aggregation of buffer salts and proteinaceous material in the mandrels. This could be remedied through a simple

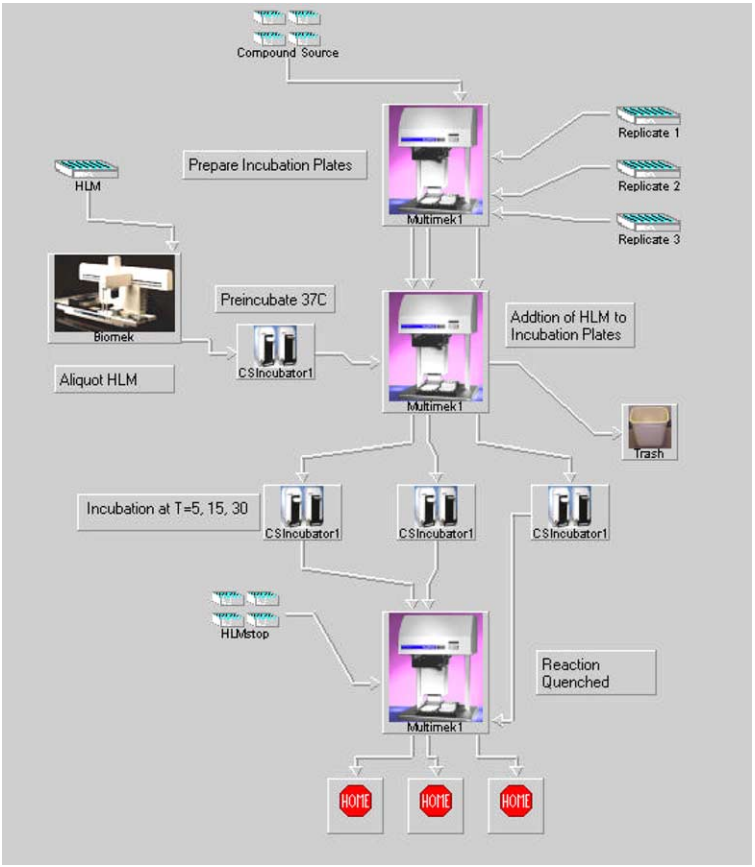

Fig. 3. SAMI NT method schematic for the automated human liver microsome (HLM) stability assay. Plates are run in triplicate at $T = 0$, 5, 15, and 30 min. The zero time point is not shown due to the slight differences in the method (stop solution is added before the addition of enzyme). The assay uses a combination of Multimek™ and Biomek® liquid handlers and is analyzed via high throughput parallel LC/MS analysis. The filtration step is not shown as it is run as a separate automated method.

cleaning procedure, recommended by the manufacturer, which took less than 5 min to complete.

2.9. Automated metabolic stability sample preparation

The following automated procedure was applied to 96-well microtiter plate microsomal incubations, schematically represented in Fig. 3. The substrate plates were prepared according to the plate replication process described earlier and then transferred to the plate carousel along with 200 μl volume assay plates. Liquid transfer steps were performed on either a Multimek™ 96-channel or Biomek® multichannel liquid handler. Assay plates were prepared by transferring 30 μl of the NADPH substrate solution into each of 12 low-volume (200 μl) assay plates. Microsomes were stored on a refrigerated reservoir (4 °C) on the deck of the Biomek® liquid handler and dispensed into a 200 μl microplate, using an eight-channel pipetting tool, before being pre-incubated at 37 °C for 5 min in a Beckman Coulter eight position incubator. The reactions were initiated through the addition of the pre-warmed 2 mg/ml microsome solution to all wells of the assay plates (30 μl/well) using the Multimek™ liquid handler. To ensure rapid oxygen transfer and provide a homogenous solution, the entire volume was mixed extensively (five times). The final incubation solutions contained 4 μM test compound or reference compound, 2 mM NADPH, 1 mg/ml (total

protein) microsomes, and 50 mM sodium phosphate (pH 7.4). The incubation plates were then incubated at 37 °C for 0, 5, 15, and 30 min before being removed from the incubator and quenched immediately with equal volumes of 0.3 M TCA using the Multimek™ 96-channel liquid handler. The quenched plates were filtered through a Unifilter® PKP 0.2 μm filter plate (Whatman, Cliffton, NJ, USA) and transferred to the high throughput parallel LC/MS system for quantitative analysis.

2.10. High throughput parallel LC/MS

While the details of our high throughput parallel LC/MS approach have been previously reported [15], the system is briefly summarized for completeness. The system consists of two Shimadzu LC10AD*vp* Solvent Delivery Modules (pumps) and an SCL-10A*vp* System Controller (Shimadzu Corp. Columbia, MD, USA), a Gilson 215 multiple probe autosampler (eight-channel) (Gilson Inc., Madison, WI, USA), a Valco switching valve (Valco Instruments Co. Inc., Houston, TX, USA), and an Applied Biosystems/MDS-SCIEX API165 single quadrupole mass spectrometer equipped with a TurboIonSpray ion source (AB/MDS-SCIEX, Foster City, CA, USA). The autosampler has eight needles spaced to fit eight wells of a standard 96-well microtiter plate. Eight samples are injected through the eight injection ports simultaneously onto eight separate microbore columns (10 mm × 1 mm i.d., 3 μm, HQ-C18, Peeke Scientific, Redwood City, CA, USA). The volume of the sample loops is 20 μl and full loop injections were used for all experiments. A total mobile phase flow rate of 2.0 ml/min was used and split into eight equivalent streams using a Valco manifold before entering the multiple probe autosampler. The outlets of the columns were recombined using a second Valco manifold. The flow was then passed through a Valco flow divert valve before entering the mass spectrometer. The ion source was maintained at 350 °C to ensure sufficient desolvation of the liquid droplets at the total 2 ml/min flow rate. The mobile phase solvents used in this study were: (A) 0.04% formic acid in water; (B) 0.04% formic acid in acetonitrile. The following mobile phase gradient was applied: 1% (B) hold for 0.3 min; 1–25% (B) in 0.05 min; hold at 25% (B) for 0.1 min; 25–70% (B) in 0.4 min; hold at 70% (B) for 0.4 min. The switching valve was set to divert flow to waste for the first 0.25 min of the gradient. The columns were re-equilibrated at starting conditions while the next set of samples was injected. The mass spectrometer was configured to acquire data in selected ion monitoring (SIM) mode, allowing eight ions to be monitored during each scan (dwell time, 50 ms for each ion). All data were acquired in the positive ionization mode.

2.11. Automated cytochrome P450 inhibition sample preparation

Compounds were tested at concentrations of 5 and 0.5 μM against CYP 3A4 and 2D6, respectively. The cytochrome P450 assays were performed in 96-well black round bottom microtiter plates (Costar, Corning, NY, USA) that were loaded onto the SAGIAN™ system plate carousel. Test compounds or reference standards (ketoconazole or quinidine) previously added to assay plates were re-suspended by the addition of 50 μl of 0.1 M assay buffer using a Multimek™ liquid handler and mixed repeatedly to aid in the resolubilization of the compounds. The plate layout was identical to that used in the manual cytochrome inhibition assay. The plates were prepared as replicate pairs and additional plates, up to 16 analysis plates, were considered as cyclical families of the same method. A diagrammatic representation of the automated assay is shown in Fig. 4. NADPH/substrate (50 μl) solution was added to each well of the microtiter plate using the Multimek™ 96-channel liquid handler. The ORCA® robotic arm then transferred the plates to the deck of the Biomek® liquid handler where the reactions were initiated by the addition of 50 μl of Supersomes® to all wells except the blanks. Sample blanks were prepared by addition of 50 μl of assay buffer to wells D1–F1. The enzyme solution was maintained at 4 °C on the deck of the Biomek® liquid handler in order to maintain enzyme activity at peak levels. The plates were then incubated at 37 °C for 30 min in a Beckman–Coulter eight position incubator. After the appropriate incubation time, the plates were again transferred via the robotic arm to the deck of the Multimek™ where the reactions were quenched by the addition of stop solution. The final transfer relocated the plates to a SPECTRAmax™ Gemini fluorescence plate reader where the plates were analyzed

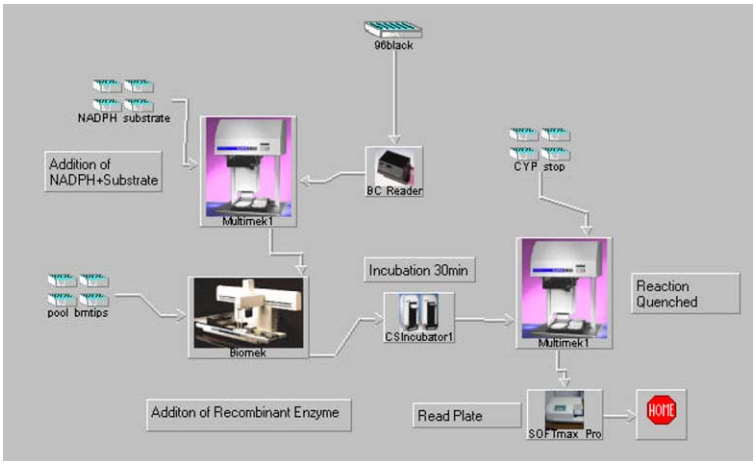

Fig. 4. SAMI NT method for the automated cytochrome P450 inhibition assay. Plates are analyzed in duplicate and liquid handling is performed on a combination of Multimek™ and Biomek® workstations. Plates are analyzed on a SPECTRAmax® Gemini fluorescent plate reader.

(409 ex, 530 em, 3A4; 390 ex, 460 em, 2D6) and then automatically transferred to a trash receptacle.

2.12. Data analysis and visualization

Several custom AppleScripts, Microsoft® Excel macros and templates were developed to facilitate both pre- and post-analysis processing in order to take full advantage of this system for high throughput metabolic stability assays. For data processing, an "ADME 8 SIM Data Processing Script" was developed. The script launches MultiView, opens each data file, extracts and integrates all SIM chromatograms in each file and writes the integration results to an output file that is imported into ActivityBase® for data verification and archive. Due to the volume of data that is generated in our in vitro ADME assays, we have integrated Spotfire® DecisionSite (Spotfire, Cambridge, MA, USA) as our data visualization tool.

3. Results

In order to address validation and robustness of the two automated methods discussed, a series of simple experiments was performed, including the profiling of a 10K member synthetic library for both metabolic stability and cytochrome P450 inhibition data. In addition to the standard validation protocols provided by SAIGIAN™, the reliability of the pipetting protocols was addressed through a series of cytochrome P450 inhibition experiments. Initially, we performed an inhibition experiment where all wells contained positive control standards. Quinidine was tested at 0.5 μM and the average inhibition was 96.6% with CVs less than 5% (average CV = 3.6%, $n = 88$). In order to validate the automated protocol in relationship to our manual method, a series of 88 compounds were analyzed by both methods in duplicate. We defined our acceptance criteria for the coefficient of determination (r^2), determined from linear correlation, in relationship to the degree to which the two methods appropriately rank order compounds. Therefore, values of $r^2 > 0.9$ indicated good–excellent correlation while values of $0.7 < r^2 < 0.9$ indicate adequate correlation for the purpose of rank ordering compounds. Linear correlations that produced values below 0.7 indicate poor correlation and likely indicate errors in the experimental design. The 3A4 percent inhibition for each compound in the manual assay was plotted versus its corresponding automated data point (Fig. 5). The linear correlation coefficient of 0.81 clearly shows a satisfactory relationship between the automated and manual methods. Finally, the average values for positive control standards for both 2D6 and 3A4 were charted for the analysis of >50 plates. The relative interplate CVs were less

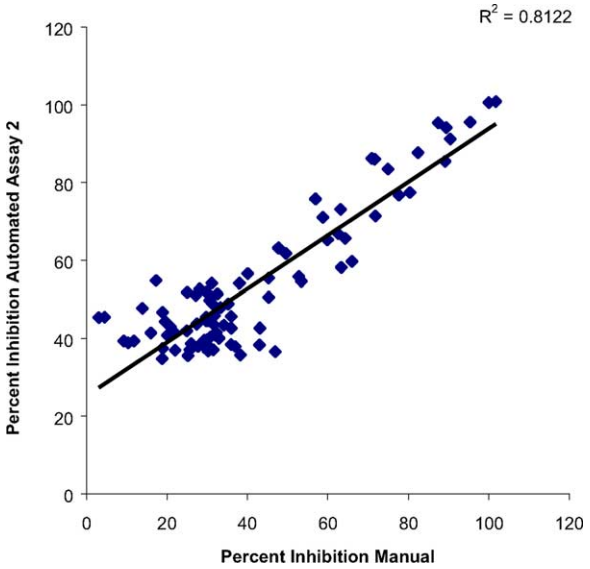

Fig. 5. Comparison of manual and automated CYP 3A4 inhibition methods. Data represents a microtiter plate of 88 library compounds tested at a concentration of 5 μM. Correlation coefficient of 0.81 indicates that the methods correspond satisfactorily ($n = 88$).

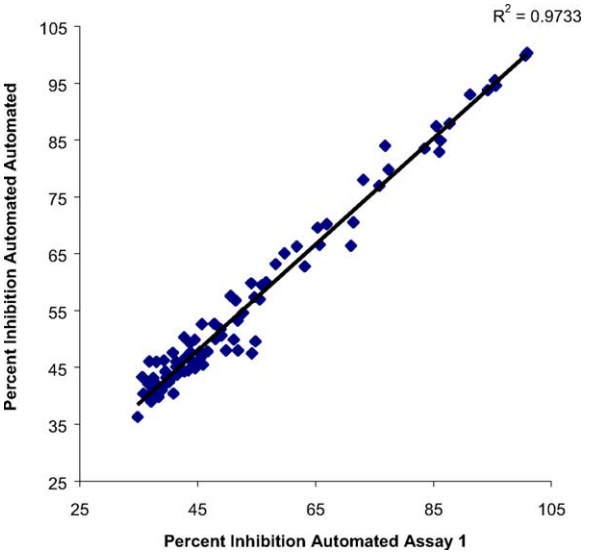

Fig. 6. Reproducibility of the automated CYP inhibition assay developed on the SAGIAN™ core system. Data represents a microtiter plate of 88 library compounds tested at a concentration of 5 μM against CYP 3A4. Correlation coefficient of 0.97 indicates that the method is highly reproducible.

than 5% ($n = 100$) for both quinidine (average CV = 3.3%) and ketoconazole (average CV = 0.5%). To address the reproducibility of the automated method, we examined the linear correlation between pairs of automated CYP 3A4 assays performed on identical sets of synthetic compounds (Fig. 6). The linear correlation coefficient of 0.97 ($n = 88$) clearly illustrates that the assay is highly reproducible.

Additional experiments were performed to address the degree to which assay preparation times could be extended in order to address throughput. The stability of each enzyme (2D6 and 3A4) on the deck of the Biomek® at 4 °C was evaluated by performing assays at 0, 1, 2, 3, 4, 5 and 6 h and the relative signal to noise (S/N) for control samples (complete conversion to product) was determined. The results of these experiments are shown in Table 1 and indicate that CYP 2D6 has a much longer lifetime at 4 °C as evidenced by the long-term stability of its signal to noise ratio for control samples. However, while the signal to noise for CYP 3A4 decreased with time, the discrimination is substantial even after 6 h (S/N = 5).

Since enzyme activity can vary from lot to lot with HLMs, initial tests to validate the automated HLM stability assay involved single plates run with consistent reagents. The automated HLM assay was validated by performing a series of experiments to address assay precision and reproducibility. First, the automated method was evaluated by testing our eight reference compounds in each of the 12 columns across a microplate. This experiment was designed to address variability across the entire incubation plate. The

Table 1
Signal to noise (S/N) calculations determined for time comparison experiments with CYP isozymes 3A4 and 2D6

Time (h)	S/N	
	CYP 2D6	CYP 3A4
0	3	96
1	2	53
2	2	35
3	2	21
4	2	12
5	2	6
6	2	5

S/N values for CYP 2D6 do not decrease significantly over the 6 h test while CYP 3A4 exhibits some degree of enzyme activity degradation as indicate by the decreasing S/N ratios over the experiment time.

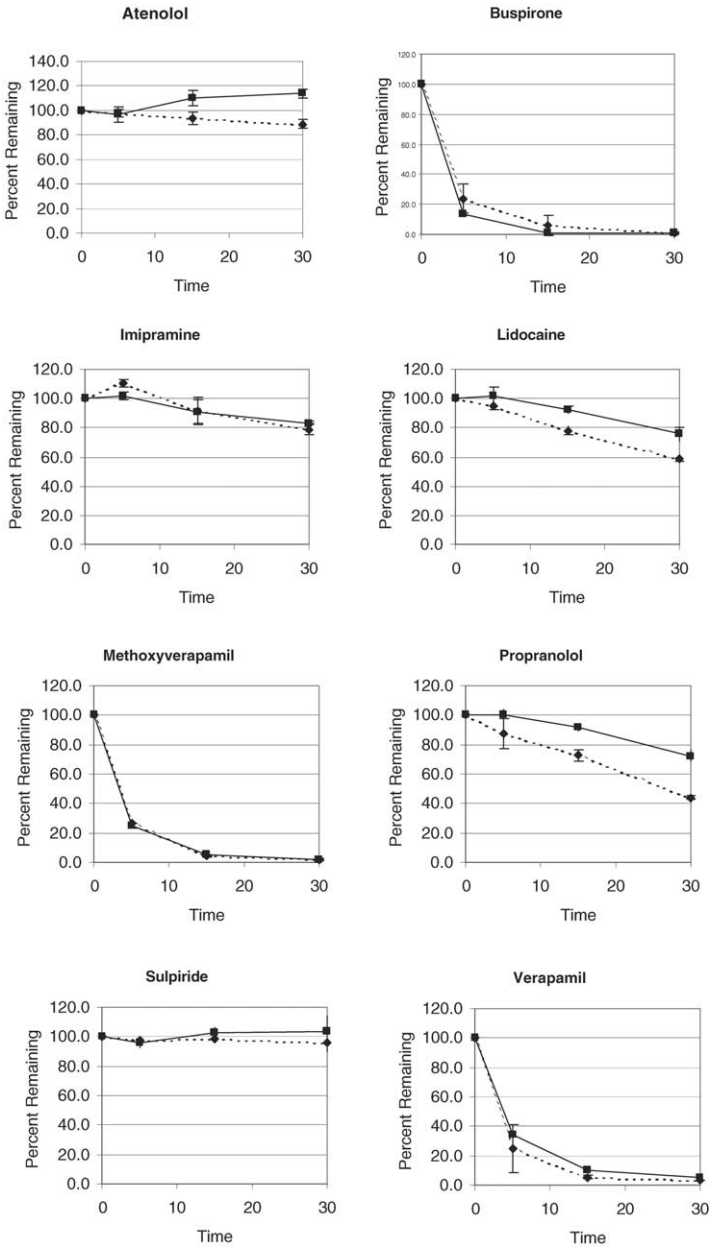

Fig. 7. Comparison of metabolic stability trends for reference compounds (atenolol, buspirone, imipramine, lidocaine, methoxyverapamil, propranolol, sulpiride, and verapamil) tested in both the manual and automated HLM stability assay. Dashed lines represent data from the manual assay and solid lines represent the automated assay on the SAGIAN^{TM} core system. The trends correspond very well and can easily be placed into three categories (rapidly metabolized, moderately metabolized, and stable).

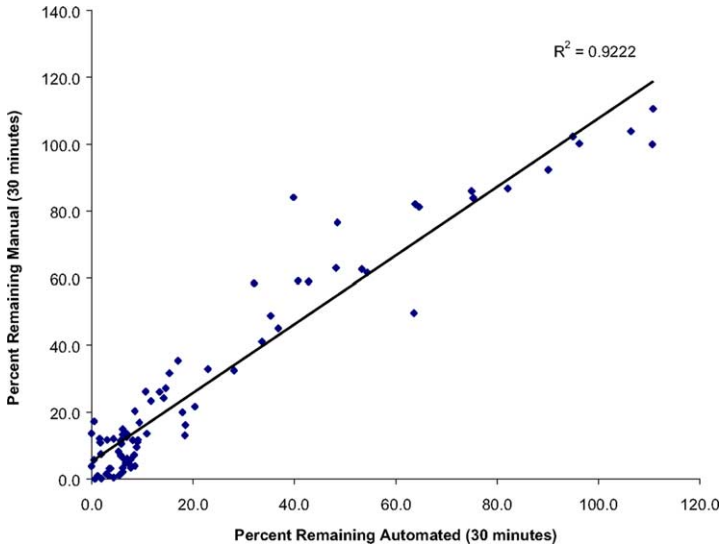

Fig. 8. Comparison of automated and manual HLM stability methods for 88 library compounds tested at 4 μM concentration. Data points represent the percent remaining at $T = 30$ min for both automated and manual assays. The automated method correlates very well with the manual method ($r^2 = 0.92$, $n = 88$).

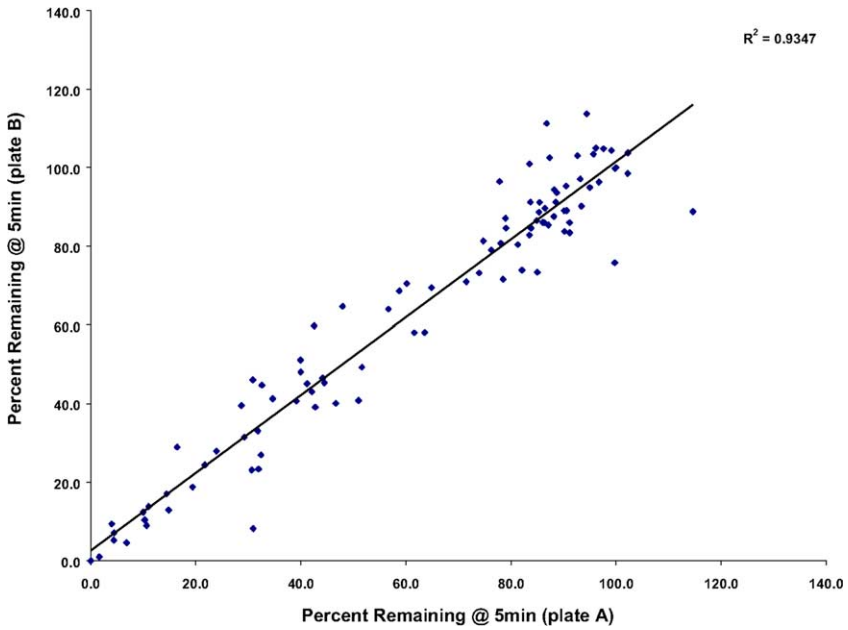

Fig. 9. Reproducibility of the automated HLM stability method determined for 88 library compounds tested at a concentration of 4 μM. Data points represent the percent remaining at $T = 5$ min (most variable data point) for a pair of automated assays run on the same 88 compounds. Assay is highly reproducible as evidenced by the correlation coefficient of 0.93.

qualitative trends generated for each of the compounds fit well within the metabolic stability ranges normally observed (i.e., rapidly metabolized, moderately metabolized and stable). Representative time course stability plots for both the manual and automated methods are shown in Fig. 7. In addition, assay variability was examined and we observed average CVs of less than 5% (Fig. 7). In an effort to address the precision of the assay, manual and automated assays were performed on a series of 88 test compounds in triplicate. The linear correlation between the two methods was examined and summarized in Fig. 8. The correlation coefficient ($r^2 = 0.92$) illustrates that the two methods correlate well. The reproducibility of the automated assay was addressed by examining the correlation of automated methods run on identical sets of test compounds, indicating that the automated method is very reproducible with a correlation coefficient of 0.93 (Fig. 9).

The automated methods for CYP inhibition and HLM stability were applied to a synthetic screening library of 10K compounds directed against G-protein coupled receptors (GPCRs). A representative assay result for CYP 3A4 inhibition is shown in Fig. 10. This library was also profiled for metabolic stability and a small representative subset is shown in Fig. 11 which illustrates the variety of stability trends that are observed and also depicts compounds that have been flagged by our intelligent data processing macro that identifies compounds with bad trends, low intensity and bad peak shape. Metabolic liabilities were identified in over half of the compounds with 56% of the compounds analyzed being rapidly metabolized (<40% of parent remaining after 30 min). Cytochrome P450 inhibition was less prevalent with only 4 and 1.7% showing liabilities (inhibition >75%) for CYP 3A4 and CYP 2D6, respectively. The anticipated throughput for each of the assays was estimated to be 352 compounds per week per 0.5 full-time employee (FTE). In practice, we have been able to achieve a throughput of 317 compounds per week per 0.5 FTE for HLM stability and 390 compounds per week per 0.5 FTE for cytochrome P450 inhibition.

4. Discussion

The aim of this work was to integrate a flexible robotics platform to support automated sample preparation solutions for in vitro ADME assays that are integral to establishing lead candidate selection and optimization criteria. It is clear that the transfer of in vitro ADME assays from late stage development programs to a position earlier in the discovery cycle has led to increased pressures on ADME groups to develop new approaches which address bottlenecks in sample handling, analysis and data management. Our laboratory

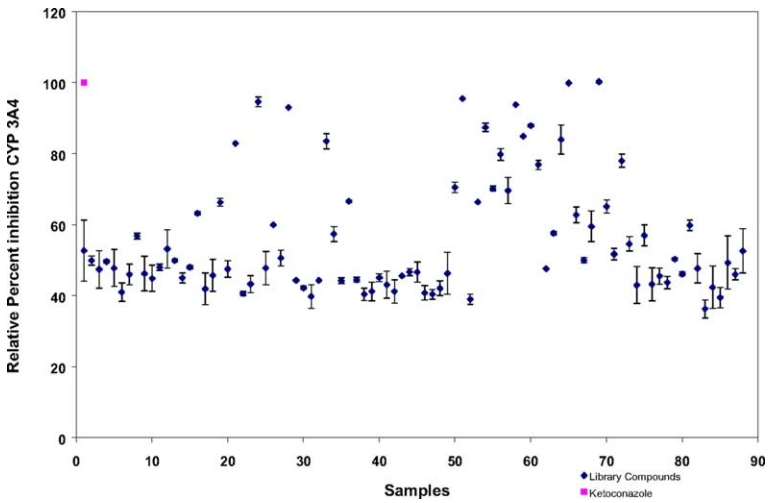

Fig. 10. Data representing the rapid profiling of 88 library compounds through the CYP 3A4 inhibition screen. Compounds were tested in duplicate at a concentration of 5 μM.

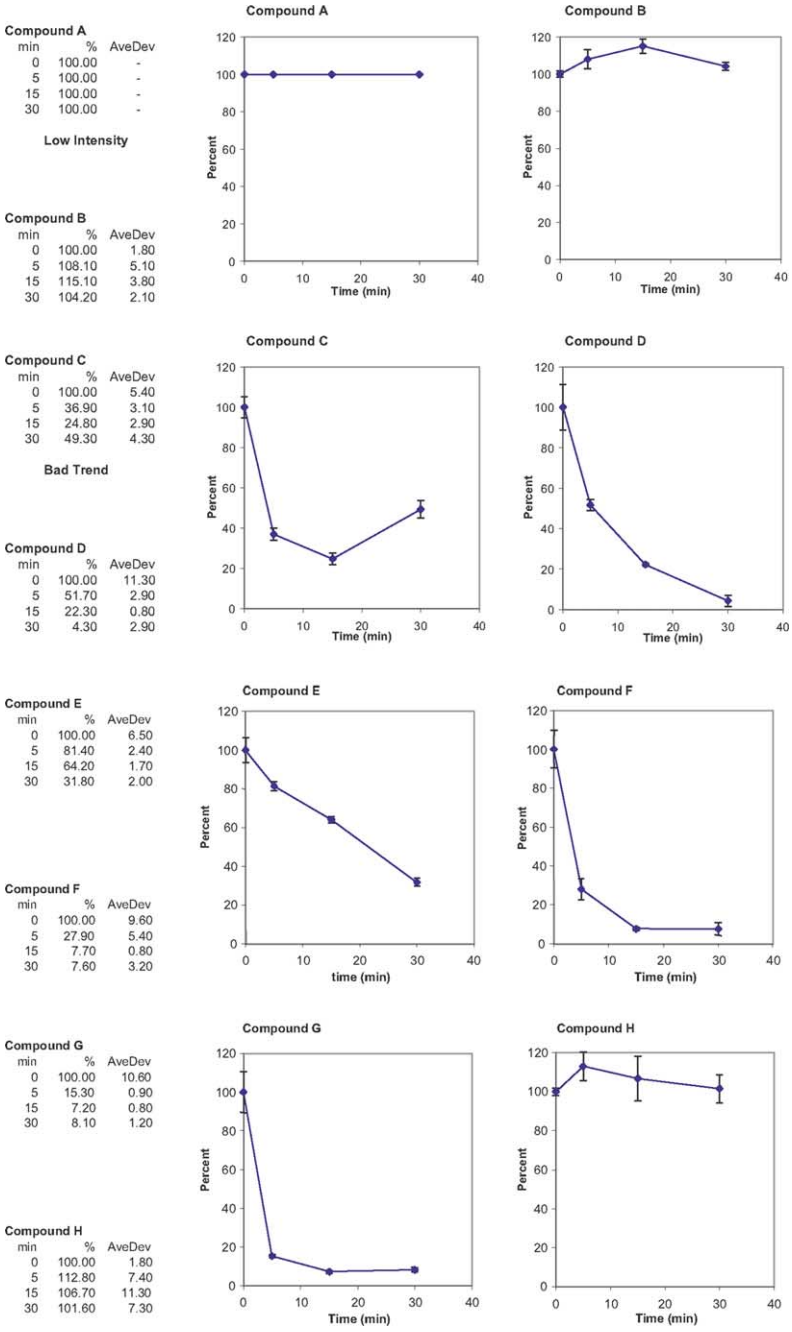

Fig. 11. Representative data report for the rapid profiling of HLM stability. Compounds D, F, and G showed fast turnover; compound E intermediate; compounds B and H slow. As identified by the "intelligent validation macro" and noted next to the plots, compound A did not yield a profile due to low ion intensity and compound C showed a poor stability trend.

has successfully implemented a SAGIAN™ core robotics system for the automated sample preparation of in vitro human liver microsomal (HLM) stability and cytochrome P450 inhibition. Overall, we have established a multi-disciplinary approach that leverages technology to improve throughput in the area of sample preparation, analytical analysis, and data management.

The implementation of an automated solution for in vitro ADME has significantly improved throughput and reliability for two assays. The manual HLM assay was estimated to have a throughput of 880 compounds per week per full-time employee. Through the incorporation of automation, we have had an indirect impact on throughput by removing much of the sample preparation burden from the scientist, and thus nearly 50% of their time has been redirected to project specific method development. Since the LC/MS used for analysis is the rate-limiting step, we have only been able to acquire data on 176 compounds per day per parallel LC/MS system. Our initial commitment involved 0.5 FTE to screen the GPCR library resulting in an expected throughput of 352 compounds per week. Our realized throughput of approximately 300 compounds per week for the GPCR 10K member library missed our anticipated mark only slightly. We attribute the small loss in throughput to a number of factors, including LC/MS system failures and troubleshooting combined with inconsistent delivery of compounds early in the program. We are currently considering additional improvements to the assay that would enable a further increase in throughput. Capacity on the Gilson 215 multiple probe autosampler is another bottleneck in the process. Currently, we are able to analyze one set of assay plates (12 microtiter plates) per 4 h of analytical time. The enhancements, while simple, could include dropping one set of the triplicate data points as well as removing the 5 min incubation, thus enabling us to double our analysis effort by loading two sets of HLM stability experiments onto the deck of the Gilson 215 multiple probe autosampler.

Throughput was also improved for the CYP inhibition assays and relieved the scientist of routine sample preparation tasks in order to spend more time performing method development. Typically, the manual assay would allow one plate of 88 compounds to be analyzed per hour. The automated CYP inhibition assay has improved this by a factor of eight with reductions in both sample preparation and data analysis time. This assay has the potential to have the greatest gains in throughput since the analysis is performed on a fluorescence plate reader that can read a plate of 96 compounds in less than 1 min. Our realized throughput of 390 compounds per week per 0.5 FTE exceeded our expectations of 352 compounds per week per 0.5 FTE. This indicates a capacity that is able to accommodate a substantially higher number of compounds for screening on a weekly basis.

One of the greatest advantages in the automation of sample preparation is the reduction in human errors and thus, a reduction in the variation seen in any particular assay. This has clearly been one of the enhancements that we have observed in the automation of these assays. The CYP inhibition assays have seen a reduction in CVs between replicate samples from greater than 20% for manual assays to less than 5% for the automated methods. Similarly, we have seen both a qualitative and quantitative improvement in our HLM stability assay by incorporating automation and robotics. We had previously noticed that the data generated in our HLM assay would vary quite substantially according to the technician that performed the incubations. This is no longer a concern since automated liquid handlers perform the assays in an identical fashion each time they prepare samples. We have also seen a reduction in CVs between the triplicate analyses of 30% ($n = 352$). Another crucial improvement that comes from the implementation of automation and robotics is throughput. In many cases, the automation is simply better able to track samples and thus the experiment time can be shortened. This is particularly true of processes that can be done in parallel. Also, while simply improving throughput is often considered one of the most important reasons for implementing robotics, there are other less obvious advantages, including employee retention, release of resources for other tasks, and reduced training requirements for new employees. These enhancements while less quantitative and measurable should not be overlooked as they contribute to the success of an organization.

The incorporation of ActivityBase® into our automated ADME approach allowed us to significantly simplify our data analysis process. This was accomplished by incorporating the features of a number of our standalone Microsoft® Excel macros with

integrated data processing within ActivityBase®. This has made the process of analyzing data much more streamlined and represents significant savings in resources. ActivityBase® also incorporates features that allow it to directly upload data from plate reader output files through an automated protocol, increasing overall throughput. The incorporation of these features is planned as part of future improvements in our automated ADME process.

Efficient extraction, visualization, and interpretation of data are often overlooked as crucial components of the early ADME screening paradigm. We choose to use Spotfire® DecisionSite (Spotfire, Cambridge, MA, USA) as our data visualization tool since it is ideally suited to support the rapid and efficient analysis of large quantities of data. Spotfire® is able to directly interface with databases such as ActivityBase® enabling discovery project teams to view interactive data visualizations as part of their decision making process. For example, Fig. 12 demonstrates the utility of visualizing multiple property data such as metabolic stability and cytochrome P450 inhibition for a specific chemical series. Chemical structure can also be imported through Spotfire® allowing the medicinal chemist to design SAR (structure activity relationship) tables for compound optimization. While the volume of data that is generated in early ADME screening can be daunting, tools such as Spotfire® allow the discovery scientist to spend more of their time interpreting the data, looking for correlations and trends that help them to optimize the ADME and physicochemical properties of their chemical matter.

We detected significant CYP inhibition liabilities in less than 5% of our compounds (4 and 1.7% in 3A4 and 2D6, respectively; 75% threshold for activity). Those compounds that have been flagged with a potential liability are then subjected to an automated IC_{50} assay based on an 11-point dilution series. While these assays are a good first-pass indicator of CYP substrate competition, there are a number of other factors that must be considered in CYP optimization. Perhaps most important is the large degree of variability that is observed for CYP 3A4 substrates, one would be prudent to run all CYP 3A4 inhibitory compounds through a counterscreen which incorporates multiple substrates including non-fluorescent probes. Fortunately, a number of groups have recognized this need resulting in several methods that address this technique [10,25,26]. Additional considerations could include identification of the inhibition mechanism, evaluation of other CYP isozymes and potential inhibition by metabolites.

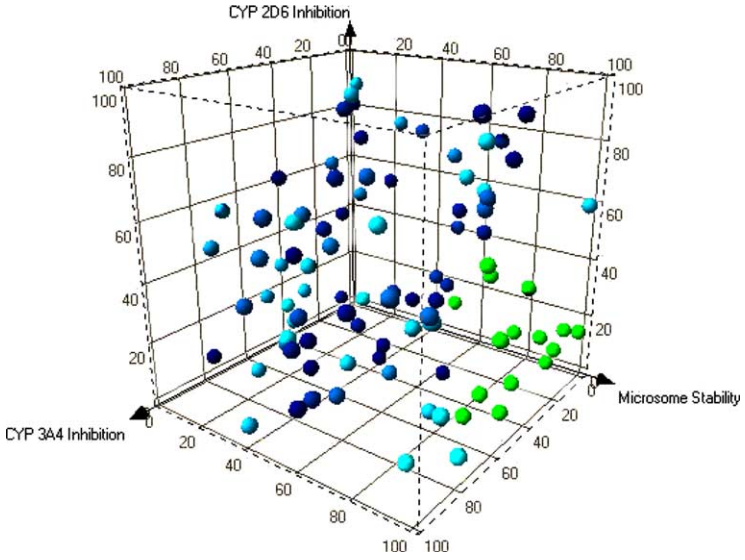

Fig. 12. Representative Spotfire® visualization showing data analysis for cytochrome P450 inhibition (2D6 and 3A4) and metabolic stability. The best compounds from this series are those in the back right corner (indicated in green) since they exhibit good metabolic stability and low CYP inhibition potential.

In vitro HLM stability assays are a useful first-pass assessment of potential metabolic liabilities. However, additional detail about the location of metabolic soft-spots is particularly useful in identifying whether observed liabilities are specific to a molecule's core or introduced as part of a monomer in parallel synthesis. This has clear implications for the degree to which the liability can be engineered out of a chemical series since this assay only measures Phase I metabolism and does not encompass aspects of Phase II liabilities which can be assessed by performing S9 or hepatocyte incubations [27]. While in vitro methods can provide good approximations of metabolic liabilities and may serve to rank order compounds according to their metabolic stability, the degree to which in vitro data correlates to clearance is less clear. However, to better assess the existence and utility of such a correlation, many groups are now establishing an infrastructure to support early ADME profiling [4,5].

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