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High throughput metabolic stability screen for lead optimization in drug discovery

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Abstract

A high throughput approach for the determination of in vitro metabolic stability and metabolic profiles of drug candidates has been developed. This approach comprises the combination of a Biomek FX liquid handling system with 96-channel pipetting capability and a custom-designed 96-well format on-line incubator with efficient thermal conductivity. This combination facilitates automated reagent preparation, sample incubation, and sample purification for microsome stability studies. The overall process is both fast and accurate and meets the challenges of high throughput screening for drug discovery. A custom designed, user-friendly computer program has been incorporated for large-scale data processing and report generation. Several applications are discussed that implement this strategy for rapid selection of compounds in early drug discovery.
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Keywords: High throughput; Metabolic stability; Automation

1. Introduction

The drug discovery paradigm has changed significantly over the last 10 years. Obtaining early information on potency, pharmacological activity, pharmacokinetic properties and toxicity of lead compounds in drug discovery have been recognized as the key for success [1]. In the past decade, new technologies, especially new state-of-the-art analytical techniques, have supported this paradigm change by rapidly providing a tremendous amount of relevant data on newly screened and synthesized compounds. Rapid, early pharmacokinetic screening has proven to be essential to build an in vivo profile for shortening the time between identifying lead molecules and selecting drug development candidates. In addition, obtaining qualitative measures of ADME/toxicity properties for drug candidates in the same time frame is becoming increasingly important [2–6]. In this new trend, structure–activity relationships (SAR) developed by medicinal chemists are not only based on in vitro binding activity, but in vivo pharmacokinetic parameters as well as pharmacodynamic behavior in animal models.

In the early stage of drug discovery, all in vivo research data are limited to preclinical animal species. Finding a way to successfully predict human PK profiles will give information on how much and how frequently a drug can be given for disease treatment, which is of great interest and poses a significant challenge in drug discovery research. Among various in vitro approaches, liver microsomes, which retain key enzyme activities involved in drug metabolism, can provide broad metabolic information and thus are widely used in the drug discovery research [7–12]. However, the successful prediction of in vivo pharmacokinetic parameters from in vitro metabolic stability presents many hurdles. Much research has shown that in vitro liver microsome metabolic stability of a compound is related to the metabolic clearance and half-life in vivo only if Phase I hepatic metabolic clearance is the major clearance mechanism [20–23]. Thus, the ability to evaluate in vitro metabolic stability in human liver microsomes can provide useful information to predict clearance and half-life in humans for new chemical entities if they are known to be cleared predominantly through hepatic oxidative metabolites. Furthermore, some early metabolic information can be obtained from the stability studies to guide decision-making for structural modification. Therefore, predicted human PK and metabolic information gained from this approach can assist medicinal chemists to develop structure–activity relationships (SAR) to optimize

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ADME properties and increase the success rate of human drug discovery.

Liquid chromatography coupled with tandem mass spectrometry and NMR has greatly facilitated metabolite structure confirmation and elucidation [13–19]. Recently, Shou et al. described a novel approach in a screening mode to simultaneously perform metabolite detection and quantitative metabolic stability determination [20]. At the same time, developing efficient and reliable sample incubation, analytical preparation, and data reporting systems for in vitro microsome studies has been reported, which accelerates this process [24–28]. In this report we describe a new automated method based on a custom-designed 96-well format incubator integrated with a Beckman Biomek FX liquid handling system. This provides a different approach in comparison to those published methods. This modified Biomek FX liquid handling system has features such as a 96-channel pipetting pod, a Span-8 eight-channel pod, and a custom-designed heating block with 96 milled pockets sitting directly on the deck of the sample handler. These combined features allow this system to perform in vitro liver microsome stability studies in a 96-well format with 96-channel simultaneous pipetting to efficiently transfer reagents to the on-line incubator. The incubator design provides efficient thermal transfer between the heating block and the 96-well reaction plate, which is a key feature for accurately performing microsome stability studies. A custom-designed computer program is incorporated for data report generation after LC/MS/MS analysis of samples. Several applications of this in vitro liver microsome method for lead identification and modification in early-stage drug discovery are discussed.

2. Experimental

2.1. Materials

All compounds investigated were synthesized at Merck Research Laboratories, Rahway, NJ, and specific structures can not be released at this time. Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Fair Lawn, NJ). Ammonium formate, formic acid, dimethyl sulfoxide (DMSO), $MgCl_2$, potassium phosphate and 2,6-dichloroindophenol sodium hydrate were supplied by Aldrich (Milwaukee, WI) and testosterone, diazepam (DZP), EDTA and NADPH were purchased from Sigma (St. Louis, MO). Both the 96 square-well plates (2 mL/well) and the round-bottom 96 deep-well plates (1 mL/well) were obtained from Beckman Coulter (Fullerton, CA). Cryo-preserved liver microsomes were purchased from XenoTech (Lenexa, KS), stored at −80 °C, and thawed immediately before use.

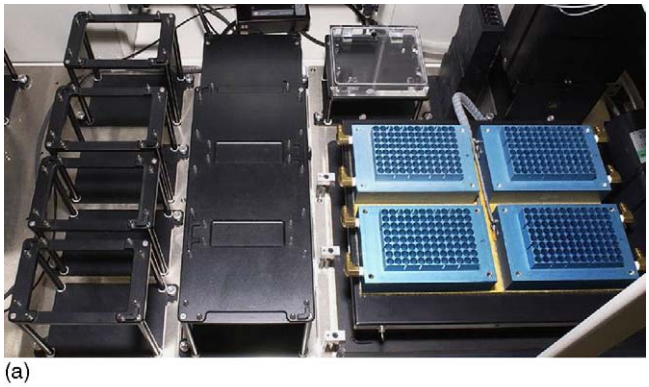
2.2. Biomek FX laboratory workstation

The Biomek FX features two pipetting capabilities on a single liquid-handling station. A multi-channel pod, which is based on air displacement technology, can rapidly, accurately and precisely aspirate and dispense very small volumes of solvent simultaneously to 96-positions. The Span-8 eight-channel pod, which is syringe air displacement based, provides 1–8 channel pipetting with independent volume control from 1 μL to 1 mL.

Reagents were transferred using Beckman AP250 pipet tips, which have a volume range of 1–200 μL. Automated Labware Positioners (ALPs) were installed on the deck of Biomek FX to secure and hold plates. A Biomek stacker carousel was integrated into the system to dispense and load labware onto the Biomek deck. Along with the gripper tool, the stacker carousel was used to expand the functionality of the system by storage of 96-well plates and disposable pipet tips.

2.3. J-KEM 96-well incubator

The J-KEM 96-well incubator was custom designed and was based on an orbital planar robotic shaker (RS2000) and heated reactors (KLS-120C), which were manufactured by J-KEM Scientific Inc. (St. Louis, MO). Four KEM-Lab heated reactors were mounted on the robotic shaker by J-KEM Scientific Inc. Each KEM-Lab heated reactor was an electrically heated aluminum block with pockets milled to provide a minimum air gap between it and the lowest 1.8 cm of each cylindrical, round-bottom well of the 96-well incubation plate (Beckman, 1 mL/well). Four digital temperature controllers were incorporated for precise, individual control of each reactor block temperature. A serial interface controller provided communications between the Biomek FX computer and the robotic shaker for controlling speed and home position detection. This custom designed incubator was mounted in-house on the deck of the Biomek FX (Fig. 1) and occupied six


(a)

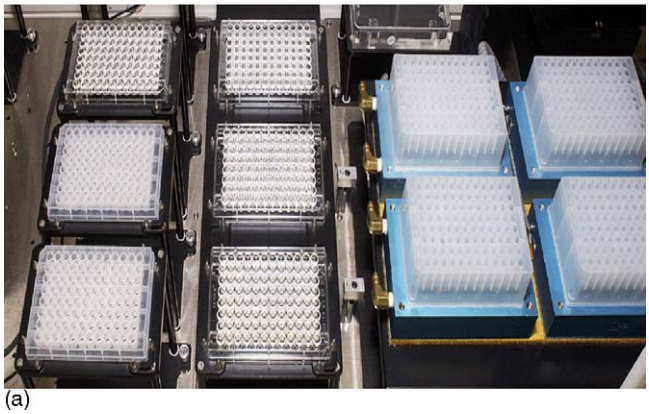

(a)

Fig. 1. The layout of the Biomek FX deck coupled with a 96-well format incubator (a) and the layout for liver microsome stability studies (b).

standard ALP positions. Thus, incorporating a Biomek stacker carousel in the system compensated for the elimination of the deck space that otherwise would have been needed to hold the very large supply of pipet tips required in our application. The AccuFrame, a tool for programming the ALP and labware positions on the deck of Biomek FX, was modified by adding four screws. Thereby it could be placed on the aluminum block of the JKEM incubator to program positions.

2.4. Microsome incubation procedure

Each test compound was incubated in an aqueous reaction mixture (200 μL total volume) consisting of animal or human liver microsomal protein and NADPH (1 mg/mL) in the presence of 100 mM potassium phosphate buffer (pH 7.4), 6 mM $MgCl_2$, and 1 mM EDTA. The final concentration of each substrate was 1 uM, and the microsomal protein concentration was 0.5 mg/mL (concentration of substrate and microsome are subject to change according to individual case). After incubation at 37 °C for a specific time period, the reaction was terminated by the addition of 500 μL of acetonitrile using the eight-channel pipetting tool, and 20 μL of an analytical internal standard in 50% aqueous acetonitrile. Mixing procedures were incorporated both upon starting the reaction by adding NADPH and upon adding ACN to stop the reaction. The delay between nominal and actual quenching times was less than one minute, which is acceptable in the drug discovery environment. The quenched reaction mixtures were centrifuged off-line at 1600 × *g* for 10 min, and 400 μL of the supernatant were transferred robotically to a 2-mL 96-well plate for reduction to dryness under a stream of nitrogen at 37 °C. After dryness was achieved, the residues were reconstituted in the chromatographic mobile phase for LC/MS measurement.

The layout of the reagent plates on the Biomek FX deck is shown in Fig. 2. Two types of studies are routinely conducted using this configuration. One involves three time points (including 0), eight compounds, and microsomes from one species per plate (Fig. 2a). The second involves eight time points (including 0), one compound, and liver microsomes from four species per plate (Fig. 2b). Three replicates are run for each time point.

The Biomek FX was programmed to use the 96-channel pod equipped with Beckman AP250 disposable tips for simultaneously transferring reagents in 96-position format to the incubation plate. The reagents and volumes were: 85 μL water, 50 μL buffer, 20 μL 5-mg/mL microsomal protein, 5 μL 20-μM substrate, and 40 μL 5-mg/mL NADPH. All solutions were aqueous except the substrate which was dissolved in 50% aqueous acetonitrile. The NADPH was added last to simultaneously initiate the reactions. Water was substituted for NADPH for the zero time-point samples. The Biomek FX stacker carousel and gripper tool were used to dispense, retrieve and load disposable tips onto the Biomek FX deck for reagent transferring, and the incubator was controlled to start planar orbital shaking at speed of about 200 rpm.

2.5. Liquid chromatography–mass spectrometry

All mass spectrometry was performed on a Sciex API 3000 triple quadruple instrument with a Turbo Ionspray interface (ABI Sciex, Toronto, Canada). Multiple reaction monitoring (MRM) transitions in the positive ion mode and Analyst Software (version 1.1) were used for quantification. The HPLC system consisted of two PE200 micro pumps and a PE200 autosampler (Perkin Elmer, Norwalk, CT). The stationary phase used was Atlantis dC18 (Waters Chromatography, Milford MA), 2.0 mm i.d. × 100 mm, with a 5-μm particle diameter. The mobile phase flow rate was 0.2 mL/min and the mobile phase consisted of a mixture of acetonitrile and water with 10 mM ammonium formate adjusted to pH 2.5 with formic acid. The typical run time for the application discussed in this paper was about 5 min, and retention time of the analytes was about 3.5–4 min under isocratic conditions.

2.6. Accuracy and precision

The accuracy and precision of Biomek FX multi-channel pod were measured by pipetting 2, 5 and 10 μL of 1 mg/mL 2,6-

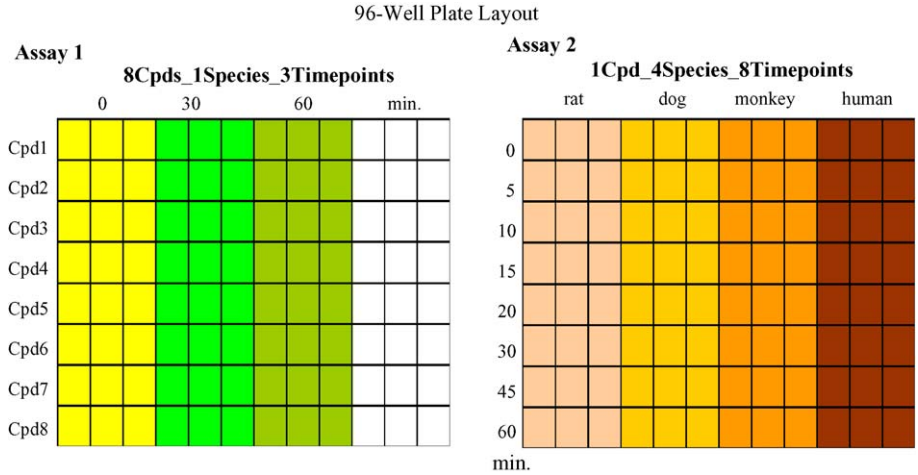


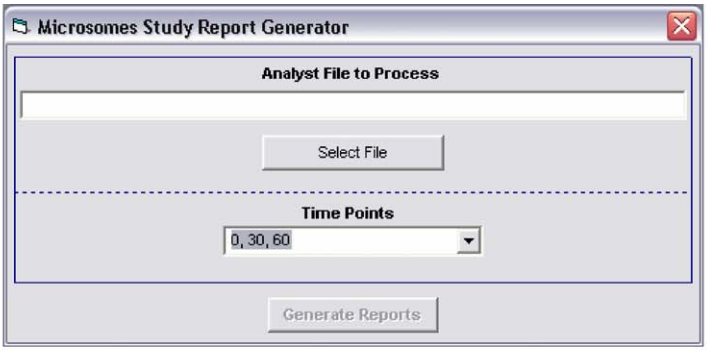
Fig. 2. The layouts of Assay 1 and Assay 2 on 96-well plates.

dichloroindophenol sodium hydrate into a 96-well flat-bottom, transparent microtiterplate containing 50 μL water. Three different solvents, water, 50% aqueous acetonitrile, and DMSO were separately used to prepare the 2,6-dichloroindophenol stock solutions at 1 mg/mL. Optical densities of the diluted solutions were determined using a 96-well UV reader, Spectramax Plus at 610 nM (Molecular Devices Corporation). Two certified and calibrated Rainin 20–200 μL multi-channel pipettors were used to perform similar manual pipetting to serve as a control reference. The average and standard deviation values of the UV readout from the 96 wells of each plate were calculated, and the resulting values for the Biomek FX multi-channel pod were evaluated relative to manual pipetting.

2.7. Microsome report generator

In liver microsome stability studies, the results are commonly presented as substrate disappearance over the incubation period. In the work presented here, graphs of the percentage drug remaining versus incubation time were used to generate stability reports. Large amounts of data are generated in the screening mode, and thus manual data processing and report generation can become a bottleneck in the overall process (average 30 min per compound). To address this, a user-friendly custom program was developed to read from a text file generated with Analyst software and then generate a final report containing both the data and stability plots. The programming language used was Microsoft Visual Basic 6.

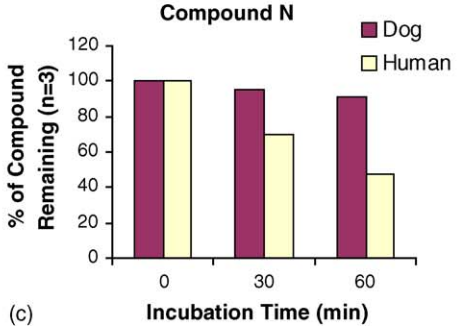
(a)



(b)

Analyte Peak Name	Analyte/IS	Average	Incubation time	Species	%	Analyte Retention Time	IS Retention Time
Compound N	26.78	26.05	0	Dog	100%	1.9	2.4
Compound N	26.14		0	Dog		1.9	2.4
Compound N	25.21		0	Dog		1.9	2.3
Compound N	25.59	24.85	30	Dog	95%	1.9	2.3
Compound N	25.18		30	Dog		1.9	2.3
Compound N	23.76		30	Dog		1.9	2.3
Compound N	23.36	23.83	60	Dog	91%	1.9	2.3
Compound N	25.49		60	Dog		1.9	2.3
Compound N	22.64		60	Dog		1.9	2.4
Compound N	23.90	22.76	0	Human	100%	1.9	2.4
Compound N	20.70		0	Human		1.9	2.4
Compound N	23.67		0	Human		1.9	2.4
Compound N	15.88	15.86	30	Human	70%	1.9	2.4
Compound N	15.48		30	Human		1.9	2.4
Compound N	16.22		30	Human		1.9	2.4
Compound N	11.80	10.84	60	Human	48%	1.9	2.4
Compound N	10.98		60	Human		1.9	2.4
Compound N	9.73		60	Human		1.9	2.4

(c)



(d)

Compound N	% of Compound Remaining	
Incubation Time (min)	1 uM Dog	1 uM Human
0	100	100
30	95	70
60	91	48

Fig. 3. The automated microsome stability study report generator (a), final metabolic stability plot (b) and report (c).

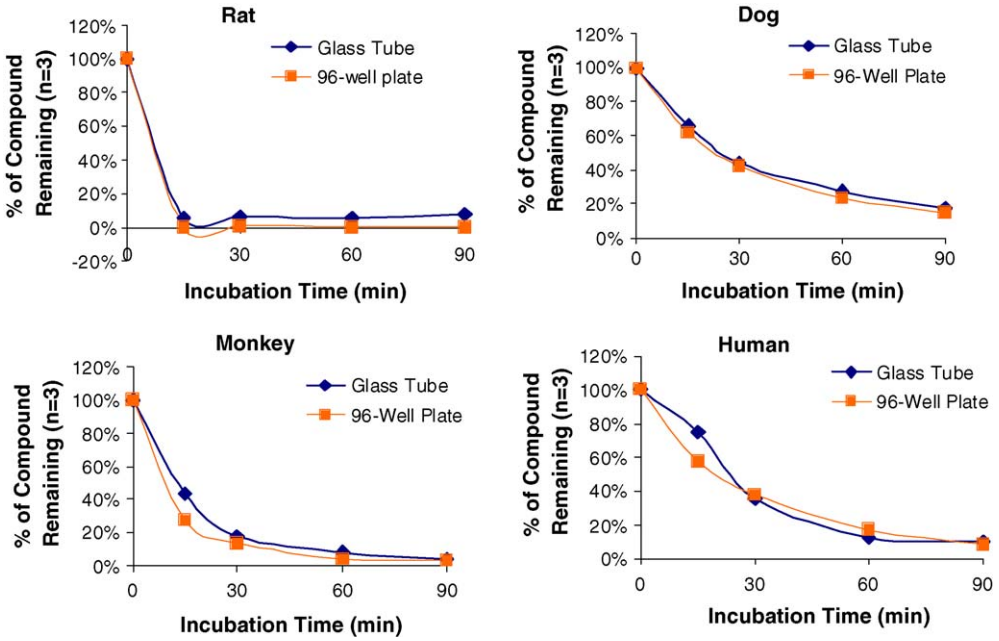


Fig. 4. A comparison study of individual glass tubes vs. 1-mL 96-well plates (with individual wells) for metabolic stability determinations of testosterone ($n = 3$ per time point). This metabolic stability study was conducted in either glass tubes or 1-mL 96-well plates (with individual wells) and incubated in a water bath shaker.

With this report generator, the user selects the text file through an open-file interface and enters the time points if not already displayed in the default drop down menu (Fig. 3). Additionally, a graph is created from the data table to aid interpretation of results. The structure of the compound (retrieved from a central database) is also displayed on the report. This program has the capability to generate a report from raw data within a minute for each compound and increases the throughput more than 10-fold. It also eliminates errors caused by manual data transfer.

3. Results and discussion

3.1. Assessment of on-line 96-well format incubator

Historically, liver microsome stability studies have generally been performed in individual glass vessels incubated in a water bath. This arrangement is not amenable to high-throughput automation. Using a 96-well format plate opens the possibility of incubating in a heated reactor block, which is easily adapted to the automation process. Our strategy here is to incorporate a 96-well format incubator on the deck of Biomek FX to eliminate the high degree of repetition in manual transferring of reagents and labeling individual tubes or vials.

Two important components of the 96-well incubator described here are the plates and heating reactors. The success of microsome incubation reaction depends heavily upon efficient thermal transfer between these two devices. Several different types of 96-well plates and heating reactors were evaluated. We performed metabolic stability determinations of testosterone in different types of 96-well plates with water bath heating as an incubator. The metabolic study of testosterone in glass tubes was used as a control. Our results showed that only 96-well plates with individual wells separated by air gaps can provide sufficient surface contact for efficient thermal transfer in a water bath (Fig. 4).

Robotic accessing of a reciprocating, liner water bath shaker is not easily accomplished. However, substituting a heating block for the water bath eliminates this problem. Many commercially available heating blocks have a flat surface that can provide only limited surface contact to the bottom of a 96-well plate. On the other hand, the 96-position KEM-Lab heated reactor has pockets milled to fit the individual wells of 1-mL round bottom 96-well deep plate. This distinct feature, similar to a water bath, can provide sufficient surface contact to improve heat distribution during incubation. The temperature of a reaction mixture in each individual well across the 96-well was measured, and 37 °C can be reached in less than 5 min.

Fig. 5 is the comparison of results obtained when diazepam was incubated in liver microsomes in the 96-position J-KEM incubator, a flat surface heating block (Eppendorf Thermomixer R), and a water bath shaker at 37 °C. The agreement of these results shows that the 96-position J-KEM incubator provides efficient heat transfer for good temperature control.

As described in Section 2, the incorporation of the KEM-Lab robotic shaker and 96-position reactor on the deck of the Biomek FX facilitates accessing most positions on the deck by both the multi-channel pod and the Span-8 pod. After the reactor is pre-equilibrated at 37 °C, a 96-well plate is added, and all reagents except NADPH are transferred by multi-channel pod to the reaction plates. This process takes slightly less than 5 min, but is sufficient for the mixture to reach 37 °C, thereby serving as a pre-incubation step before adding NADPH. This design provides more efficient transferring of chemical reagents and ensures that the reaction is initiated simultaneously across all 96 positions to improve accuracy and throughput.

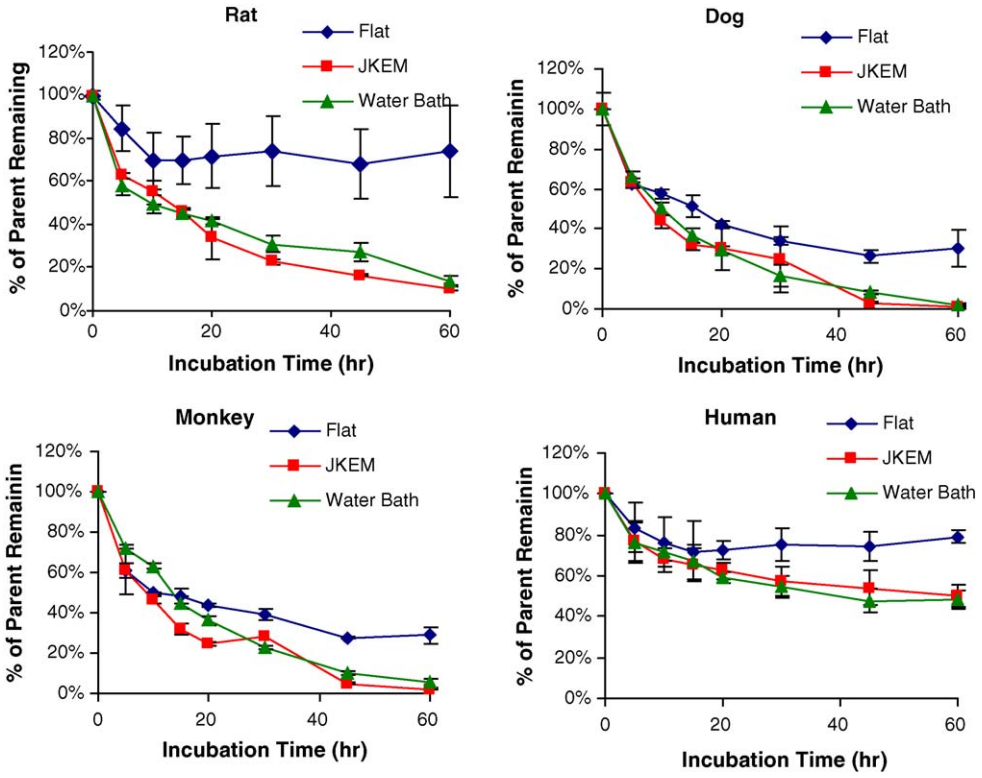


Fig. 5. A comparison study of metabolic stability of diazepam that was incubated in a water bath shaker, a J-KEM robotic incubator and a flat surface heating block ($n = 3$ per time point).

3.2. Accuracy and reproducibility of Biomek FX

Accurately transferring small volumes of substrate solution to the microsome reaction plate is essential, especially when the test compounds have very limited aqueous solubility and must be handled in organic solvents. Therefore, we performed an extensive evaluation of the pipetting performance of the multi-channel pod with AP250 tips. Liquid characteristics such as surface tension, viscosity and volatility will affect the accuracy of the pipetting process (aspirating, dispensing and mixing steps). Pipetting operations including tip touch, blowout, liquid level following, prewetting, trailing air gap, pod speed, aspirating and dispensing height, and mixing volume were adjusted according to the physical properties of each type of liquids. These liquid-specific values were then incorporated into the Biomek FX method to perform accurate pipetting. The accuracy and precision of the multi-channel pod with 100% aqueous solution, 50% aqueous acetonitrile solution, and 100% DMSO at 2, 5 and 10 μL were measured (see method discussion in Section 2). Table 1 is the summary of all accuracy and precision data of the Biomek FX multi-channel pod with AP250 tips. Even pipetting a volume as small as 2 μL with the multi-channel pod is accurate and precise across the 96 wells and meets the criteria for high throughput drug discovery in comparison to manual pipetting. More importantly, with this small volume of substrate solution, the percentage of organic solvent in the reaction is only about 1%, thereby ensuring proper microsome incubation conditions.

3.3. Application to drug discovery

Two assay types have been developed for microsome stability screening. Assay 1 has the capability to screen eight compounds with only two or three time points on one plate. In general, one or two control compounds with known microsome stability profiles are included on the same plate with the test compounds. The time points are 0, 30 and 60 min for the application discussed here,

Table 1
The relative accuracy and precision of Biomek FX multi-channel pod

Solvents	Transfer volume					
	2 μL		5 μL		10 μL	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
100% Water	–	–	4.9	8.2	3.3	6.9
ACN:Water (1:1)	–	–	7.4	5.5	5.1	3.7
100% DMSO	7.0	3.0	3.4	3.0	–	–

Biomek FX AP250 μL tips were used here.

Table 2
Percentage of compound remaining in dog and human liver microsomes

Incubation time (min)	Compound A		Compound B		Compound C		Compound D	
	Dog	Human	Dog	Human	Dog	Human	Dog	Human
0	100	100	100	100	100	100	100	100
30	83	101	105	79	61	15	91	27
60	79	93	93	76	40	4	71	14

Incubation time (min)	Compound E		Compound F		Compound G (control)		Compound H (control)	
	Dog	Human	Dog	Human	Dog	Human	Dog	Human
0	100	100	100	100	100	100	100	100
30	93	33	44	0	98	93	82	22
60	81	13	25	0	96	94	76	3

and triplicate samples are run for each time point. Assay 2 can provide detailed kinetic studies with up to eight time points. Usually, one compound and liver microsomes from four species per plate are evaluated with the Assay 2 format. The detailed plate layouts of each assay type are displayed in Fig. 2. The time for the Biomek FX to prepare two plates for either Assay 1 or Assay 2 is generally less than 5 min.

Assay 1 is more appropriate to high throughput requirements, and consequently we have applied it to lead compound screening in the early stage of drug-discovery. For example, in one drug discovery program we used this technique to identify first-pass hepatic metabolism as the dominant clearance pathway for many compounds over several structure classes in rat, dog and monkey. More than 100 compounds from these series were screened in both human and dog liver microsome stability studies**.** Compounds A–F are just few examples of these potent lead compounds, and their metabolic stability results are summarized in Table 2. Apparently, compounds A, B, D and E are quite stable in dogs, and compounds C–F are very unstable in humans. Since most drugs are intended for human diseases treatment, the ability to evaluate metabolic stability information in humans is important to gain preliminary clearance and half-life information in humans. The lack of stability in human liver microsomes for compounds C–F certainly indicates a potential liability when low clearance and reasonable half-life are key issues in a drug discovery program. In such a case, this information will be evaluated along with other in vivo and in vitro properties to allow significantly better decision-making at an early stage.

In addition, some preliminary metabolite information can be simultaneously acquired through this high throughput microsome stability screen approach. In the early stage of drug discovery authentic samples of potential metabolites are seldom available. However, the specificity and selectivity features provided by tandem MS and LC retention allow us to detect possible metabolites. This application has been advantageously used in one program where metabolic oxidation at a benzylic position (Fig. 6) was identified as major clearance route across rat, dog, monkey and human for one lead series of compounds. In order to support medicinal chemistry strategies to block this metabolically labile site and avoid long-lived active metabolites in preclinical species and in humans, we used a microsome screen approach to evaluate many potent analogs of this lead structure in rat and human liver microsomes along with a rat PK screen. As illustrated in Fig. 6, we included the predicated MS/MS transitions of metabolites ($[M+16]^+/[A+16]^+$ and $[M+16]^+/[B]^+$) along with MS/MS transition of analyte ($[M]^+/[A]^+$ and $[M]^+/[B]^+$) in our acquisition method to monitor interesting potent compounds and their metabolites in both microsome samples and rat PK samples. We observed the formation of an oxidative metabolite at the benzylic position in the in vitro rat and human liver microsome studies (Fig. 7). The oxidation metabolite data of these compounds from rat liver microsomes correlated well with the metabolic profiles obtained in the in vivo rat PK studies (data not shown here). The incorporation of a fluoride (compound CB) or methyl group (compound CC) at this benzylic position significantly enhanced the metabolic stability relative to compound CI. Consequently, no significant amount of benzylic oxidation metabolites was formed in both rat and human liver microsomes, as well as in rat PK studies. Thus**,** this high throughput screen in liver microsomes combined with a rat PK screen can assist medicinal chemists to rank compounds and perform structural modifications to enhance metabolic stability not only in preclinical species, but potentially in humans as well.

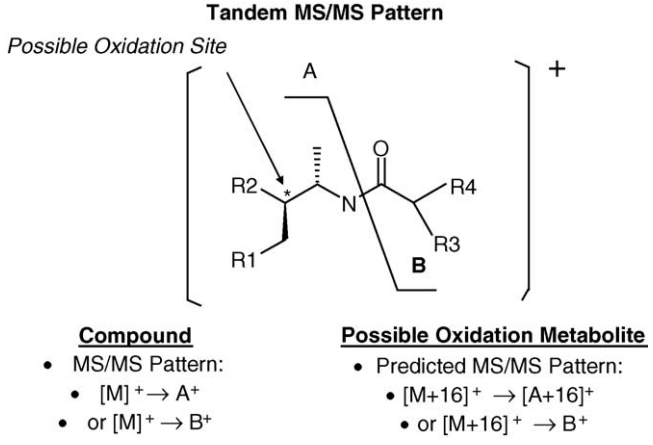


Fig. 6. The tandem mass patterns of compounds and their oxidation metabolites.

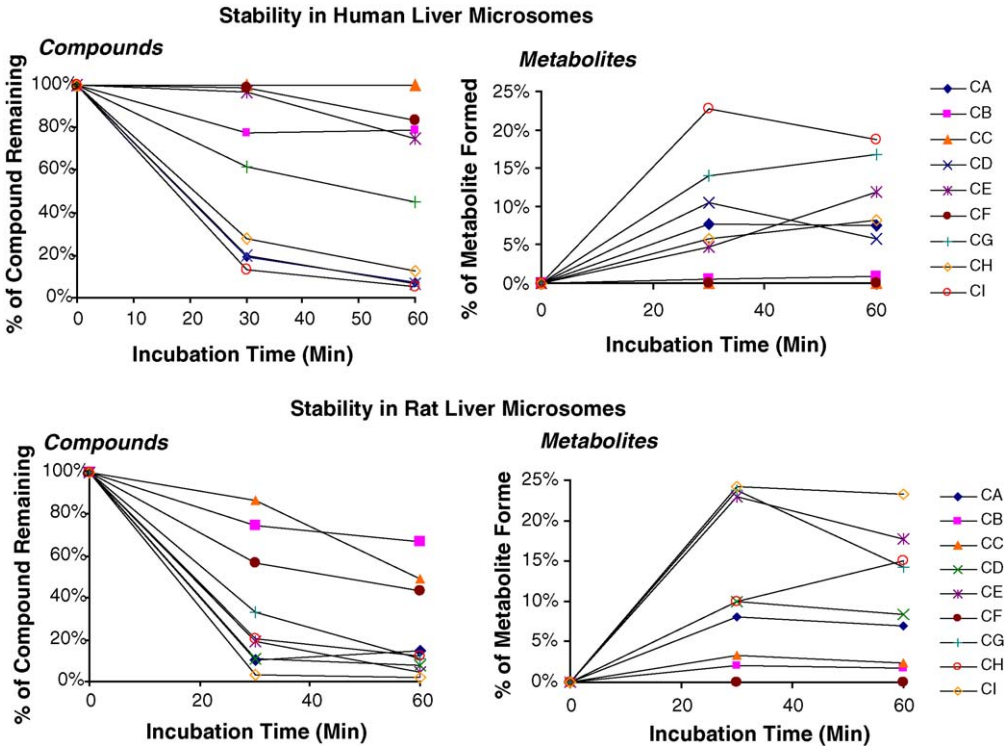


Fig. 7. The rat and human in vitro liver microsome stabilities of nine compounds and their oxidation metabolites.

4. Conclusion

In this report we have demonstrated a new design to combine a liquid handling system, Biomek FX, and a custom-designed J-KEM robotic shaker for performing high throughput microsome stability studies in drug discovery. The incorporation of this J-KEM incubator on the deck of Biomek FX enables the multi-channel pod to transfer chemical reagents to the incubator in a 96-position format. In this way, the reaction can be started simultaneously in all wells. The 96-position KEM lab reactor with pockets milled to the shape of the reaction plate bottom increases the thermal conductivity for microsome incubation. The entire process for preparing the microsome incubation reaction with this system is fast, automated, and accurate with a throughput of about 50 compounds per day tested in four different species. More importantly, it eliminates sample handling errors and the tedious labor involved in manually doing this work. Thus, we are able to significantly accelerate the throughput for determining the metabolic stability of compounds in liver microsomes as well as metabolite identity information in various species for programs where hepatic metabolic clearance has been confirmed as the major clearance pathway in the early stage of drug discovery. The information acquired from in vitro liver microsome studies along with in vivo data facilitates better prediction of pharmacokinetic properties, dosing regimens, and disposition characteristics in humans, and thereby accelerates strategic decision making for lead optimization in drug discovery.

5. Acknowledgements

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