

Metabolic Stability Screen for Drug Discovery Using Cassette Analysis and Column Switching

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Abstract: *In vitro* metabolic stability assays are used to screen compounds for stability in the presence of various drug metabolizing enzymes, usually cytochrome P450 in liver preparations (e.g., liver microsomes). High-throughput metabolic stability assays using pooling methods have been developed to keep pace with screening requirements at the lead ADME optimization stage. In our laboratory, we have improved the metabolic stability assay using the cassette analysis method, column switching, and incorporated time saving techniques in method development to yield a robust method which reduces data turnaround time, increases compound throughput, and maximizes mass spectrometer usage. This method can determine metabolic stability using microsomes or hepatocytes from any species. We describe our findings following incubation of 40 different compounds with human liver microsomes and analysis by the cassette and discrete analysis methods. Similar metabolic stability results were obtained using the cassette analysis and discrete analysis method. An overall 70% time savings was achieved by pooling four new compounds into one sample for method development/MS optimization, cassetting four samples into one sample to minimize the number of injections on LC/MS/MS analysis, and using a column switching system to analyze the samples, which results in a two-fold decrease in the LC/MS/MS analysis time.

Key Words: Metabolic stability, *in vitro*, liver microsomes, ADME, column switching, and cassette analysis.

INTRODUCTION

In the lead optimization stage of drug discovery, there is a growing need to assess absorption, distribution, metabolism, and excretion (ADME) properties of molecules while optimizing compounds for selectivity and potency [1]. A large number of compounds of varying chemical structure require testing for ADME properties, such as metabolic stability. Since in most cases the liver is the major site of metabolism, screening compounds for metabolic stability toward liver preparations is important and is usually one of the primary screens. High-throughput metabolic stability assays have been developed to keep pace with screening requirements. Liquid chromatography/mass spectrometry (LC/MS/MS) is used to analyze the samples due to high sensitivity and selectivity of the technique. LC/MS/MS analysis, in general, is a time- and resource-consuming technique. The need to rapidly identify lead compounds requires as short an interval between sample submission and data reporting as possible. To meet these needs, various high-throughput *in vitro* techniques and rapid and robust analytical methods are used to reduce the total number of samples to be analyzed, analysis time, and data turnaround time while maintaining data quality [2-9]. Pooling methodology, such as pooled analysis (compounds incubated separately then time-points of the same compound combined into one sample for analysis) and cassette analysis (compounds incubated separately followed by combining each time point for multiple compounds), do decrease the number of samples to analyze and

reduce the time for analysis compared to traditional discrete dosing methods, but they have limitations. For example, pooled analysis may result in a low limit of quantitation and/or limitation of data/parameters obtained. Cassette analysis may have a problem with limit of quantitation because of the dilution of the samples. However, mass spectrometers are becoming more highly sensitive; therefore, the limit of quantitation is less of a concern in the pooling methods. Analytical methods, such as fast LC/MS/MS analysis, which utilizes higher mobile phase flow rates and/or shorter columns or cartridges, and parallel LC/MS/MS, which uses multiple HPLC columns simultaneously connected to a single mass spectrometer (e.g., column switching), have been developed to minimize the HPLC runtimes and maximize mass spectrometer usage. In our laboratory, we have optimized the metabolic stability assay using the cassette analysis method, column switching, and time saving techniques to yield a method which 1) reduces sample analysis time, 2) increases throughput, 3) is easily implemented, 4) is reproducible, and 5) maintains data quality.

MATERIALS AND METHODS

Magnesium chloride, β -nicotinamide adenine dinucleotide phosphate (NADP⁺), glucose-6-phosphate (G6P), D-glucose-6-phosphate dehydrogenase (G6PDH), dimethylsulfoxide (DMSO) and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium phosphate buffer (KPi; pH 7.4 at 1.0 M) was provided by Media Preparation Facility at Genentech, Inc. The KPi buffer was diluted in water to 100 mM. HPLC grade water and acetonitrile (ACN), trifluoroacetic acid (TFA) and formic acid (FA) were purchased from J.T. Baker (Phillipsburg, NJ). Pooled liver microsomes (HLM; lot MGU; 20 mg/mL) prepared by mixing

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microsomes from individual male and female human donors were purchased from *In Vitro* Technologies (Baltimore, MD) and stored at -70°C .

Compounds for metabolic stability screening and internal standards (IS) were provided by the Department of Medicinal Chemistry at Genentech, Inc. Four IS were chosen based on structural similarity to the compounds selected for stability screening. Compounds were diluted to 1 mM in DMSO in amber glass vials from the 100 mM stock solution in DMSO. The Chemical Plate was prepared by further dilution to 10 μM in KPi buffer (Fig. (2)). Solutions were mixed thoroughly prior to addition to incubations.

Ten different cassette analysis groups (4 compounds in each group) were created from 40 different compounds to be analyzed by LC/MS/MS. The design of each cassette of compounds was chosen to avoid interferences considering molecular weights and potential metabolites. This was accomplished using a Microsoft Excel spreadsheet that calculates molecular weights of metabolites arising from common

metabolic pathways. Compounds were not included in the same cassette group if they or their possible Phase I metabolites (i.e., +16, +32, +/-14) had molecular weights within 2 amu of each other. Other potential metabolites that required further understanding of the molecular structure, such as dealkylation or hydrolytic reactions that are compound-specific, were also considered. Once the cassette groups were established, each compound was assigned one of four IS. The four compounds in a cassette group each were assigned a different IS to minimize variability resulted from cassetting.

The NADPH-regenerating system (NADPH-RS) was prepared by dissolving 7.5 mg of NADP^+ and 15.2 mg of G6P in 993 μL of 30 mM magnesium chloride and kept on ice. G6PDH (6.8 μL ; 0.8U) was added just before use.

Incubation Conditions

Fig. (1) depicts the overall incubation scheme. Incubations were performed in 0.65 mL polypropylene Micro-

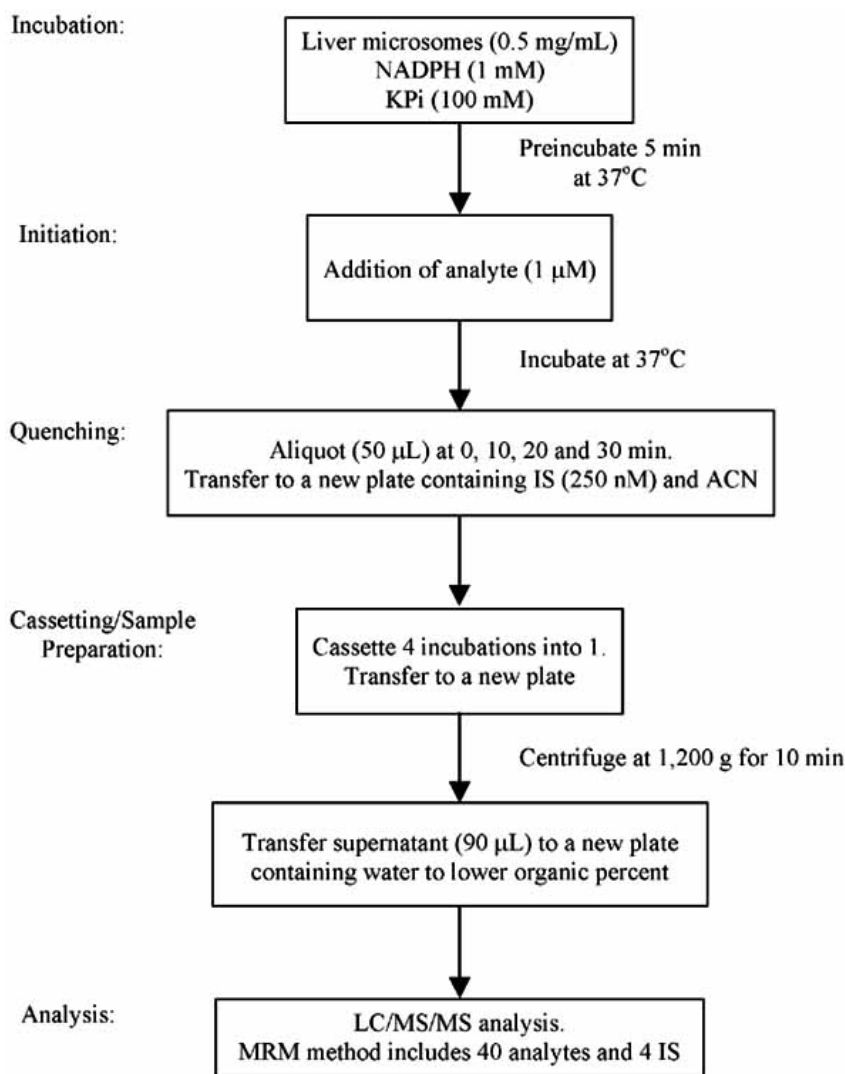
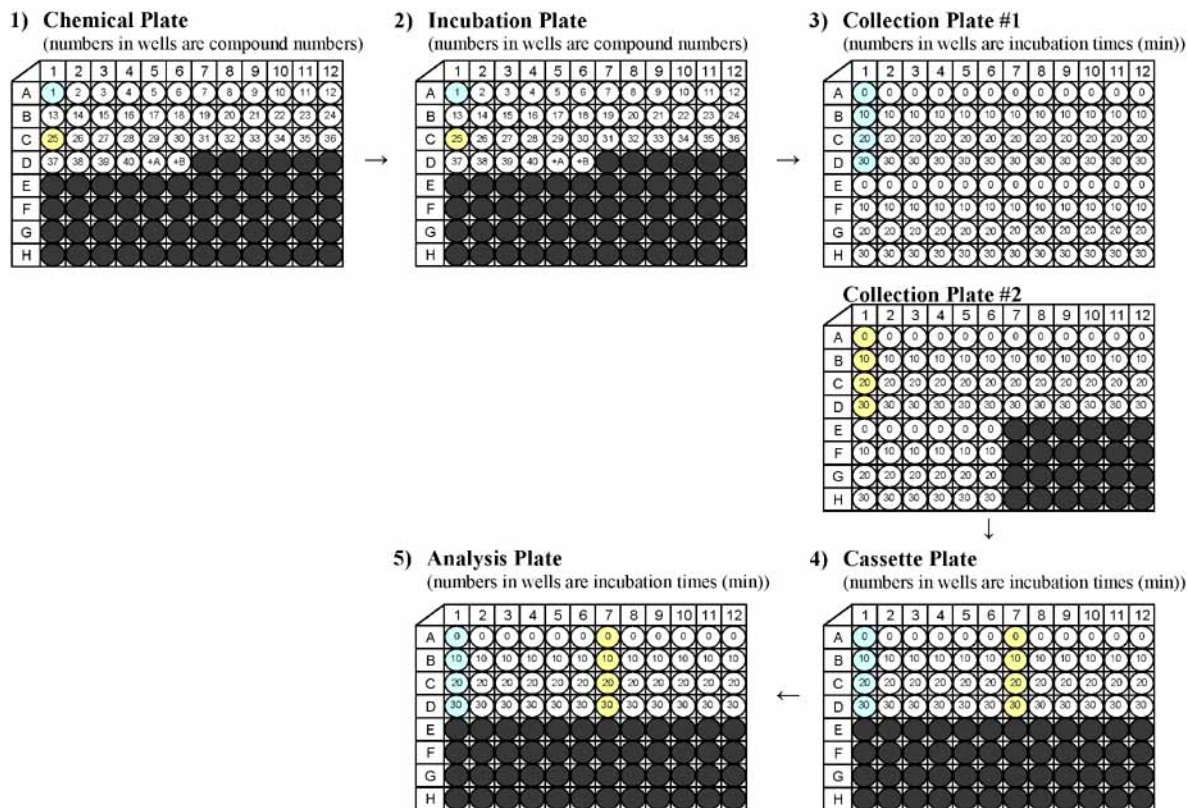


Fig. (1). Overall incubation and sample analysis workflow for sample cassetting. The information in parentheses is final incubation concentrations. The total incubation volume is 250 μL .

Tubes™ from National Scientific Supply Co., Inc. (Claremont, CA) arranged in a 96-well plate format. Human liver microsomes were thawed, diluted 1:4 (v/v) in KPi (pH 7.4, 100 mM), and placed on ice. An aliquot of the liver microsomal suspension was added to the cofactor mixture and preincubated for 5 min in a 37°C Precision water bath incubator (Jouan, Inc., Westchester, VA). The Chemical Plate containing 10 μM test compounds in KPi was generated as shown in Fig. (2). The reaction was initiated with addition of

25 μL of 10 μM test compound in KPi. Incubations (250 μL total volume) contained test compound (1 μM) containing 0.1% DMSO, liver microsomes (0.5 mg/mL), and NADPH-RS. This was performed in the Incubation Plate shown in Fig. (2). Incubations were carried out for 30 min, and aliquots were taken at 0, 10, 20 and 30 min. At each time-point, an aliquot of 50 μL was transferred to Collection Plates containing 100 μL of IS (250 nM) and 0.5% TFA dissolved in ACN to quench the reaction. For discrete analysis, each



1) Chemical Plate – Each test compound (10 μM in KPi) and the two positive controls (50 μM (testosterone) and 10 μM (proprietary compound)) are put into individual wells as illustrated below. The compounds and two positive controls are labeled in each well 1-40 and +A and +B, respectively. For example, compound 1 is highlighted blue and compound 25 is highlighted yellow. Wells highlighted in black indicate wells not used.

2) Incubation Plate – To initiate the reaction, 25 μL of 10 μM test compounds from the Chemical Plate is added to the Incubation Plate containing HLM (0.5 mg/mL) and NADPH (1 mM) in 100 mM KPi, which was preincubated for 5 min at 37°C. Each well contains compound (1 μM final concentration), HLM, and NADPH in KPi. For example, the incubation containing compound 1 is highlighted blue and the incubation containing compound 25 is highlighted yellow. Wells highlighted in black indicate wells not used.

3) Collection Plate – After initiation of reaction, 50 μL samples from the Incubation Plate was collected at 0, 10, 20, and 30 min and transferred to the Collection Plates, which contain IS (250 nM) in ACN. For example, wells highlighted blue in Collection Plate #1, column 1, rows A-D are the 0, 10, 20, and 30-min samples (respectively) for compound 1 and wells highlighted yellow in Collection Plate #2, column 1, rows A-D are the incubation samples from compound 25. Wells highlighted in black indicate wells not used.

4) Cassette Plate – Four samples are then cassetted into one sample. For example, the wells highlighted blue in column 1, rows A-D are the 0, 10, 20, and 30-min incubation samples (respectively) for compounds 1-4 and wells highlighted yellow in column 7, rows A-D are the incubation samples for compounds 25-28. This plate is centrifuged and transferred to the Analysis Plate. Wells highlighted in black indicate wells not used.

5) Analysis Plate – The supernatants are transferred to the Analysis Plate that contains water to reduce the percentage of ACN. For example, wells highlighted blue in column 1, rows A-D are the 0, 10, 20, and 30-min incubation samples (respectively) for compounds 1-4 and wells highlighted yellow in column 7, rows A-D are the incubation samples for compounds 25-28. This plate is sealed and placed in the autosampler for LC/MS/MS analysis. Wells highlighted in black indicate wells not used.

Fig. (2). Overall incubation plate formats.

sample was individually analyzed. For cassette analysis, samples (30 μL) were removed from the Collection Plate and placed into the Cassette Plate according to the cassette groups established above. Each well in the Cassette Plate contained 4 compounds and 4 IS at each specific incubation time. The precipitated microsomal protein was removed by centrifugation at 2,000 \times g for 10 min. The supernatants (90 μL) were transferred to the Analysis Plate in which the wells contained 180 μL of water to bring the organic percent to the initial HPLC conditions. The positive controls were not cassetted prior to analysis.

Positive Controls

Two positive controls were used for this assay. One was testosterone at 50 μM , which was considered labile in our assay having a human hepatic intrinsic clearance (CL_{int}) greater than 48 mL/min/kg. The other positive control was a compound (at 1 μM) from a similar scaffold as the other 40 compounds that was moderately stable in liver microsomes (CL_{int} value between 8.9 mL/min/kg and 48 mL/min/kg).

Sample Analysis

The samples (50 μL injection volume) were analyzed using a Cohesive LX-2 Series system from Cohesive Technologies (Franklin, MA) coupled to a QTRAP 2000 mass spectrometer from Applied Biosystems Instruments (ABI; Foster City, CA) equipped with turboion spray. The Cohesive Technologies Multiplexing system consisted of two Agilent 1100 Series binary pumps from Agilent Technologies (Palo Alto, CA), LX-2 switching valve system, and a

CTC PAL autosampler from LEAP Technologies (Carrboro, NC). The HPLC column was Gemini C18 5 μm 50 X 2 mm from Phenomenex Inc. (Torrance, CA) and the LC flow rates were 0.35 mL/min. The HPLC mobile phases consisted of 0.1% FA in water (mobile phase A) and 0.1% FA in ACN (mobile phase B). The HPLC gradient started at 5% mobile phase B and was held for 0.1 min. Mobile phase B was increased linearly to 98% over 1.35 min and was held at 98% for 0.15 min. The mobile phase then was returned to initial conditions to equilibrate for 0.6 min prior to the next injection. The total LC/MS/MS run time was 2 min. The samples were analyzed in positive mode using the multiple reaction monitoring mode (MRM).

Method Development

Compounds in each cassette group (4 compounds per cassette group; 10 cassette groups total) were pooled at 10 μM each in 80:20 water:ACN (v/v) for LC method development and MS optimization. Each cassette group containing 4 pooled compounds were infused into the MS and MS conditions for each compound were optimized in positive MS/MS mode. After the 40 compounds were optimized for MS parameters using automated quantitative optimization in Analyst software, a script was developed to merge the ten MRM methods and generate one method for subsequent data acquisition and sample analysis. The MS method we used contained 44 different MRM transitions for 40 analytes and the four IS with their respective MS parameters. The mass spectrometer interface temperature was 450°C with dwell time of 25 msec for each analyte.

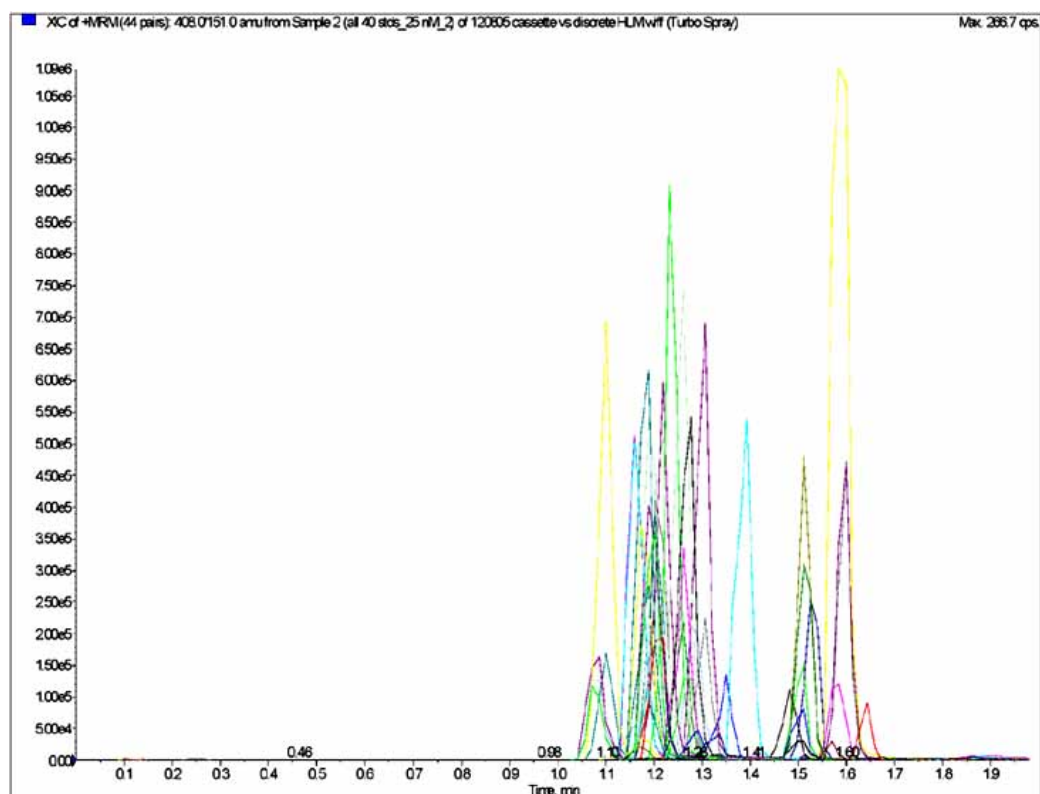


Fig. (3). Representative LC/MS/MS chromatogram in the MRM scan mode of 40 compounds standards at 25 nM. Detection of each analyte was ensured before analysis of the incubation samples.

Once the MS method was generated, the pooled standards (25 nM each compound) were injected and analyzed simultaneously using a short, generic HPLC method described above for LC/MS/MS method development. Fig. (3) shows a representative MRM chromatogram of the pooled standards in all cassette groups. Detection of each analyte was ensured before analysis of the incubation samples. This was done by making sure a peak for each MRM transition in the MS method was present in the MRM chromatogram. Also, the range of analyte retention times was noted in order to program the Cohesive valve/column switching system and MS scan window. This approach reduced the time for method development compared to the conventional approach that analyzes one compound per analysis. Complete separation of compounds in each cassette group was not essential because of the selectivity of the MS.

Data Analysis

Peak areas for all analytes were integrated using Quant Wizard of the version 1.4.1 Analyst Software from ABI and were exported to a Microsoft Excel spreadsheet designed to calculate *in vitro* metabolic stability parameters. The integrated peak areas of the compounds at 0, 10, 20, and 30 min were divided by the respective peak areas of the IS. The percent of parent remaining was calculated by normalizing the peak area ratio of parent to IS at 0 min. Observed rate constant (k_{obs}) for parent degradation was calculated by determining the slope of the line of the graph of the natural log of percentage parent remaining versus time of incubation. CL_{int} was calculated using the following equation [10]:

$$CL_{int} = \frac{K_{obs} (\text{min}^{-1}) \times \text{incubation volume (mL)}}{\text{microsomal protein (mg)} \times \text{liver wt. (g)} \times (\text{liver wt./body wt. (g/kg)})}$$

Physiological parameters used were from established methods [11-14].

RESULTS AND DISCUSSION

The metabolic stability data obtained from utilizing discrete analysis and cassette analysis methods were compared. The analysis of 40 compounds using the two methods resulted in comparable CL_{int} values. The graph of CL_{int} generated from each of the methods had R^2 of 0.9317 (Fig. (4)). The compounds selected in this study had a wide range of physical and chemical properties. The similar CL_{int} observed demonstrate the utility and robustness of the cassette analysis method.

The high throughput metabolic stability method using the cassette analysis method reduced the number of samples to be analyzed by 75% compared to using the discrete analysis method. In the study described here, the number of samples generated following incubation of the 40 compounds using the discrete method was 160 samples to be analyzed by LC/MS/MS compared to 40 samples (cassette analysis). Reducing the number of samples to be analyzed resulted in an increased compound throughput in the metabolic stability assay and a decreased data turnaround time, amount of consumables used, amount of money spent, and mass spectrometer time needed.

The analysis of samples was performed using a Cohesive Technologies LX-2 system, which reduced the analysis time by half and maximized mass spectrometer usage. The LX-2 system consisted of two columns that were running on two separate HPLC pumps. Once the analytes of interest were eluted off the column to the MS, that column was switched to waste for column wash time and re-equilibration to the

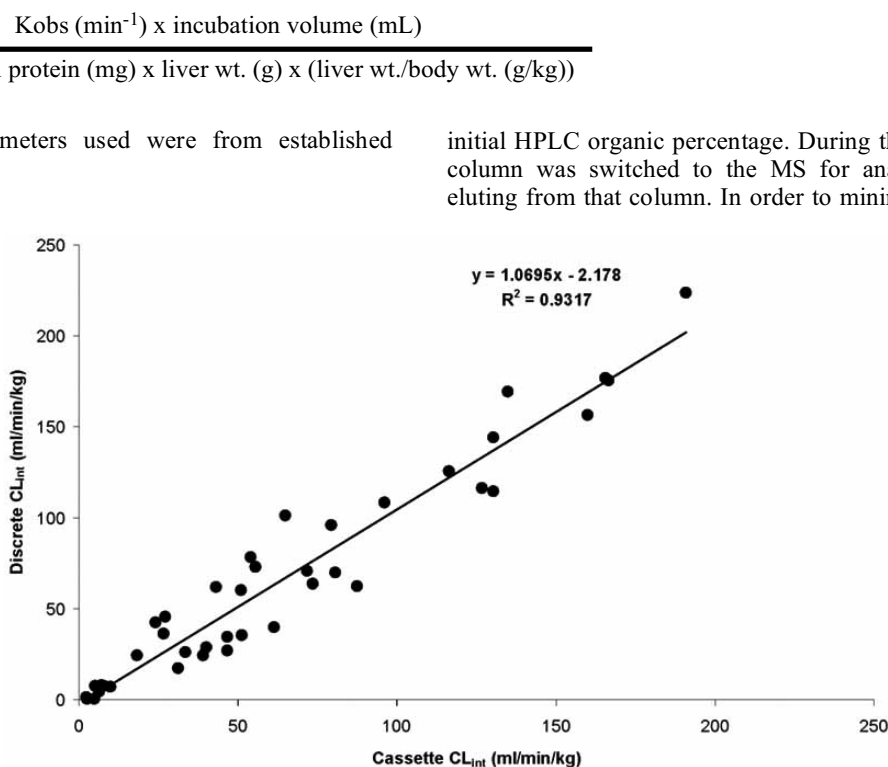


Fig. (4). Comparison of the *in vitro* metabolic stability data from cassette analysis with discrete analysis.

the retention time, peak shape, and peak area that can occur using a multiplexing system, the injection of samples were staggered so that the same compounds at each incubation time-point were run on the same column.

The overall time saving using the method development, MS optimization, and cassette analysis method was approximately 3-fold (70% time savings) while maintaining data quality. We obtained this time saving by pooling four new compounds into one sample for infusion for method development and MS optimization, cassetting four samples into one sample to minimize the number of injections for LC/MS/MS analysis, and using the Cohesive LX-2 system to analyze the samples. This latter step decreased the LC/MS/MS analysis time two-fold. Time was also saved by cassetting the incubated samples prior to centrifugation. In this way, there was only one step that required careful transfer of solution without disrupting the protein pellet and since the cassetting was already done, there were fewer wells that needed to be transferred. The use of individual IS for each compound helped maintain data quality which is potentially degraded by transferring steps and potential variability during sample analysis. Also, two positive controls were incorporated in the metabolic stability assay to assess the enzymatic activity of the microsomal incubation.

CONCLUSIONS

The high-throughput metabolic stability assay using the cassette analysis method and column switching described here has become a valuable addition to our routine metabolic stability assays using microsomes and hepatocytes (derived from humans and animals) in support of drug discovery programs. Following extensive use, this method has proved to be reproducible, reliable, and universal, and it requires

minimal time for method development. For each new compound, the method development required was only to determine an appropriate precursor/product ion transition for MRM detection. Because of the reduced amount of time to perform the assay and analyze the samples using the cassette analysis method and column switching, we are able to report the metabolic stability data as early as one day after the initiation of the study.

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