Simple Strategies for Reducing Sample Loads in *In Vitro* Metabolic Stability High-Throughput Screening Experiments: A Comparison between Traditional, Two-Time-Point and Pooled Sample Analyses

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ABSTRACT: Higher-throughput ADME programs in early drug discovery are becoming common throughout the pharmaceutical industry as companies strive to reduce their compound attrition in later-stage development. Many of the ADME assays developed into higher-throughput formats rely on LC/MS analyses. Since the biological aspects of the assay are amenable to parallel processes using dense plate formats, the number of samples generated from these assays produce a large analysis load for serial LC/MS. Presented in this report are two novel strategies, including a sample pooling method and a two time-point method, that could be used in drug discovery to reduce the number of samples generated during multiple time-point *in-vitro* ADME assays. One hundred and sixty-three compounds were subjected to human microsomal incubations with full time-point method samples taken at $t = 0$, 5, 15, 30, and 45 min. The ER data correlation (R^2) between the full time-point method and the pooling method and two time-point methods were 0.98 and 0.97, respectively. Both methods have the potential to: 1. produce data of similar quality to traditional high throughput ADME assays, 2. be easily implemented, 3. shorten analytical run times, and 4. be reproducible and robust. J Pharm Sci 94:38–45, 2005

Keywords: higher throughput technologies; mass spectrometry; microsome; metabolism; mathematical model; regression analysis; pooling method; extraction ratio; HTS

INTRODUCTION

The advent of novel high-throughput synthetic chemistry approaches during drug discovery efforts has led to an increase in the number of compounds submitted for absorption, distribution, metabolism, and excretion (ADME) screening. As a result, the laboratories responsible for running these *in vitro* ADME assays have had to optimize high-throughput screening protocols to keep pace with increased compound submissions. Because the biological aspects of these assays are amenable to parallel processing using dense plate formats, the preparation protocols and sample capacities can easily be adjusted to handle large numbers of compounds. However, for those assays requiring an analytical endpoint not amenable to parallel detection, an increase in assay capacity equates to an increase in sample burden.

Many of the *in vitro* ADME assays operating in a high-throughput format rely on liquid chromatography/mass spectrometry (LC/MS) endpoints.[1] Because LC/MS is predominantly a serial technique, sample cycle time is finite which means an increase in the number of samples requiring

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analysis will lead to an increase in the time required to complete their analysis. With the primary goal of ADME support for early drug discovery programs being the generation of high-quality ADME data for the ever-increasing numbers of compounds considered to be lead material as quickly as possible, new techniques are continuously sought to minimize data turnaround time without decreasing the number of compounds screened.

A logical approach for reducing data turnaround during drug discovery is to develop methods that reduce the number of samples per assay but do not reduce the quality of data such that decision-making efforts are compromised. To date, several reports have described the development of sample pooling methods (PMs) for this purpose.[2–8] These PMs can be collected into three categories: cassette dosing, cassette analysis, and pooled analysis. In cassette dosing, compounds are combined before biological assay, assayed as mixtures, and analyzed as mixtures.[3] In cassette analysis, compounds are individually assayed biologically and samples from different compounds combined before analysis.[2,3,5] For pooled analysis, compounds are assayed separately with samples from an individual compound combined for analysis.[4–8] However, there are serious potential drawbacks for each of these PMs. Cassette dosing creates the potential for drug–drug interaction influencing the assay results.[3] Cassette analysis eliminates the drug–drug interaction potential, but results in an increase in assay limits of detection.[3] Pooled analysis requires sample volumes that are proportional to the time interval between adjacent time points to provide area under the curve information for a given compound.[4,5,7,8] Given the small assay volumes and unevenly spaced time intervals most often used for microsomal lability assays, this leads to prescribed sample volumes too small to be reproducible with parallel pipetting systems.[5]

A novel method for pooling samples from a multiple-time-point assay has been developed and evaluated. This method avoids the drug–drug interaction potential and sensitivity issues observed with cassette dosing and cassette analysis methods. In addition, the method allows the selection of sample volumes to pool based on assay convenience rather than on arbitrary formulae used in the published pooled analysis methods. For comparison, the simple strategy of reducing the number of time points to that which would produce the same number of samples as the PM was also evaluated. These two strategies could be used in drug discovery to reduce the number of samples generated during multiple-time-point *in vitro* ADME assays. Both methods have the potential to: 1. produce data of similar quality to traditional high-throughput ADME assays, 2. be easily implemented, 3. shorten analytical run times, and 4. be reproducible and robust.

METHODS

Human Liver P450 Microsomal Lability of Pfizer Compounds: Standard Microsomal Lability Time-Course Assay, PM, and Two-Time-Point Method (TTPM)

Human liver microsomes were prepared as a mix of individual liver microsome preparations. Microsomes were characterized for P450 concentration using the CO spectral difference method and for protein concentration using the Bradford protein assay method (Bio-Rad). In addition, major P450 isozyme activities (cyp1A2, 2C9, 2C19, 2D6, and 3A4) were determined using established protocols and specific marker substrates.[9,10]

Standard Microsomal Lability Time Course [Full Time-Point Method (FTPM)]

Polypropylene 96-well plates (Analytical Sales and Services Inc.) were used during this study. Frozen microsomes were thawed on ice for 15 min and diluted in assay buffer (70 mM KPO_4, 5 mM $MgCl_2$, pH 7.4) to a P450 concentration of 0.36 μM. Sample compounds dissolved in dimethyl sulfoxide were diluted with 9 volumes of acetonitrile/water (3:1 v/v %) and diluted to 5 μM in assay buffer. One hundred microliters of diluted microsomes was mixed with 30 μL of compound and incubated at 37°C for 20 min. A 15-μL cofactor mixture (4.4 mM nicotinamide adenine dinucleotide phosphate, 50 mM isocitric acid, 5 U/mL isocitrate dehydrogenase, 100 mM $MgCl_2$) was then added to complete the reaction mixture and aliquots were removed after 0, 5, 15, 30, and 45 min of additional incubation at 37°C. The final concentration of dimethyl sulfoxide in the assay was $\leq$0.1% v/v and of acetonitrile was $<$1% v/v. These concentrations were reported by Hickman et al.[11] and Easterbrook et al.[12] as having minimal to no effect on microsomal P450 activities. Each aliquot was mixed with 3× volume acetonitrile and centrifuged at 3000*g* for 5 min. Supernatant was analyzed by LC/MS/MS to quantitate