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Automating Metabolic Stability Assays and Analyses using a Robotic Autosampler and LC/MS/MS Platform

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Abstract

The in vitro metabolic stability of potential drug candidates is routinely examined at an early stage of drug discovery. Metabolic stability is a simple, well-established screening technique used to predict the in vivo hepatic clearance of a drug based on due to metabolism. With the ever-increasing chemical libraries, today's drug metabolism and pharmacokinetic (DMPK) laboratories are constantly being asked to increase the number of molecules that can be assayed to decrease the time needed to identify lead drug candidates.

Automating the entire metabolic stability assay and subsequent LC-MS/MS analysis provides the high throughput necessary for DMPK laboratories. The GERSTEL MPS robotic autosampler performs syringe transfer of all liquids involved in the metabolic stability procedure including temperature-controlled incubation of the samples for defined time periods. Additional sample preparation steps are performed as needed. The resulting extracts from the automated method were introduced into an Agilent Ultivo LC/MS/MS instrument.

Introduction

The liver is the principal organ involved in mammalian metabolism of xenobiotics including drug compounds. Microsomes are isolated through differential centrifugation of liver tissue homogenate and are principally derived from the membranes of the endoplasmic reticulum. These microsomes provide an enriched source of membrane bound drug metabolizing enzymes including the cytochrome P450 (CYP) superfamily and uridine glucuronosyl transferase (UGT) enzymes.

To study and assess the metabolism of chemical drug compounds, DMPK laboratories can perform in vitro studies using liver microsomes. Microsomal (metabolic) stability assays are defined as the percentage of parent drug compound lost over time in the presence of liver microsomes. The general assay for cytochrome P450 and other NADPH dependent enzymes involves incubation of the drug candidate with liver microsomes along with the necessary buffers and cofactors. Sample aliquots are removed from the incubation at specific time intervals and the reactions stopped using cold acetonitrile. The samples are then centrifuged, and the supernatant is analyzed to evaluate the metabolic stability of the drug. Precise control over the incubation temperature, solution storage temperature, and reproducible sampling are critical for these experiments.

As a result of this study, we were able to show that an in vitro metabolic stability assay and subsequent sample preparation method can be successfully automated using the GERSTEL MPS robotic sampler for a variety of model drug compounds in microsomes. Using this method, analytes can be rapidly and reproducibly isolated from microsome samples using an automated procedure coupled to LC-MS/MS analysis using the Agilent Ultivo Triple Quadrapole Mass Spectrometer. Linear calibration curves resulting in R^2 values of 0.99 or greater were acheived upon the complete automated procedure. Time-course studies for model drug compounds in microsomes were examined. Coupling the sample preparation method to the LC-MS/MS provides the high throughput required for this type of metabolic stability study.

EXPERIMENTAL

Materials. All stock solutions for the compounds listed in Table 1 were purchased from Cerilliant. An intermediate analyte stock solution was prepared by combining the analyte stock solutions with acetonitrile, at appropriate concentrations, to evaluate the different drug compounds. Individual subtrate samples for each compound were prepared at a concentration of 5 mM each, respectively, in DMSO.

Table 1. Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		Fragmentor Voltage [V]		Collision Energy [V]		Ret. Time [min]	High Std Conc. [ng/mL]
Dextromethorphan	272.2	171	147.1	152	152	40	25	3.05	5000
Diazepam[2]	285.1	193	154	189	189	45	47	4.07	5000
d_5-Diazepam	290.1	198.1	154	149	149	36	28	4.07	-
d_3-Imipramine	284.3	89.1	61.1	100	100	10	45	3.34	-
Imipramine[1]	281.3	86	58	100	100	10	45	3.34	5000
Phenacetin	180	138.1	110	100	100	10	10	3.02	5000
Midazolam[2]	326.1	291.1	249.1	170	170	29	41	3.09	5000
Verapamil	455.5	165	150	158	158	20	30	3.39	5000

1- d_3-Imipramine used as internal standard
2- d_5-Diazepam used as internal standard

Deuterated analogues, d_3-imipramine and, d_5-diazepam, were purchased from Cerilliant. An internal standard stock solution containing the deuterated internal standards was prepared in acetonitrile at a concentration of 1000 ng/mL. Table 1 shows which deuterated internal standard was used with each respective analyte during quantitation.

High concentration calibration standards and intermediate QC samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solution using (1:1) water: acetonitrile to give the concentrations listed in Table 1. Calibration standards were then prepared using a dilution ratio strategy from the high concentration sample of 1:2:2.5:2:2:2.5:2. The high and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:1.67:10.

Male, CD-1, mouse liver microsomes (20 mg/mL, #452701), and male, Sprague Dawley, rat liver microsomes (20 mg/mL, #452501) were purchased from Corning Discovery Labware, Inc. NADPH Regenerating System Solutions A (#451220) and B (#451200) were also purchased from Corning Discovery Labware, Inc. The NADPH Regenerating Solution A contains 26 mM NADP+, 66 mM glucose-6-phosphate, and 66 mM magnesium chloride in water. The NADPH Regenerating Solution B contains 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. When combined, solutions A and B can be used for NADPH requiring oxidase assays. All other reagents and solvents used were reagent grade.

Instrumentation. All automated PrepSequences were performed using a MPS robotic^{PRO} sampler with the GERSTEL CF-200 Centrifuge Option and Heated Agitator as shown in Figure 1. All analyses were performed using an Agilent 1260 HPLC with an Agilent Poroshell 120 EC-C18 column, (3.0 x 50 mm, 2.7 µm) and an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source (all from Agilent Technologies). Samples, stop solution, substrates, microsomes, and NADPH regeneration

solutions were stored within a Peltier Cooled tray at 4°C throughout the automated process. Sample injections were made using the GERSTEL LCMS Tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 2 µL stainless steel sample loop.

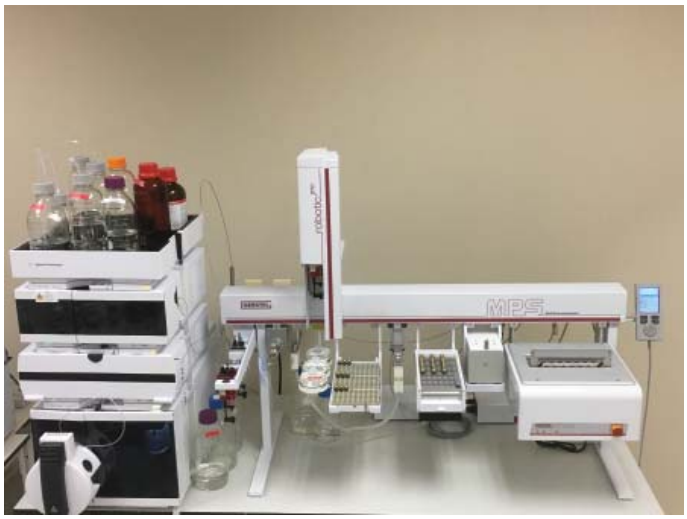


Figure 1. MPS robotic^{PRO} Multi-Purpose Sampler with the GERSTEL CF-200 centrifuge option.

Automated Prep Sequence. The automated microsomal stabilty experiment followed industry standard experimental conditions [1], which included the steps detailed below. Negative controls were performed using the same steps, minus the cofactors, in order to exclude substrate disappearance due to causes other than those induced by the presence of cofactors.

1. The MPS adds 100 µL of working internal standard in acetonitrile to time-course collection vials with inserts held within the Peltier Cooled Tray Holder at 4°C.
2. The MPS adds the following to the microsomal stability incubation vial:
   a. 713 µL water.
   b. 200 µL 0.5 M potassium phosphate, pH 7.4 (100 mM final conc.)
   c. 50 µL NADPH Regenerating System Solution A.
   d. 10 µL NADPH Regenerating System Solution B
   e. 2 µL of 5 mM substrate in DMSO (10 µM final conc.)
3. The MPS moves the incubation vial to the Heated Agitator (37°C and 250 rpm) for 10 minutes to pre-warm vials.
4. The MPS adds 25 µL liver microsomes to the incubation vial to begin the time-course study.
5. The MPS immediately withdraws 100 µL for the 0 min time-course sample and transfers the aliquot into the collection vial being held in the Peltier Cooled Trayholder, containing the 100 µL of working internal standard in acetonitrile.
6. The MPS continues the incubation (37°C and 250 rpm) throughout the remaining time-course experiments.
7. The MPS withdraws 100 µL aliquots from the incubation vial at each of the following time points and transfers these to individual time-course vials held in the Peltier Cooled Tray holder at 4°C, each containing the 100 µL of working internal standard in acetonitrile:
   a. 5, 10, 20, 30, 40, 50, and 60 minutes.
8. Once the time-course experiment is complete, the MPS centrifuges the time-course vials at 2000 g for 10 minutes.
9. The MPS transfers 100 µL aliquots of the supernatants to individual clean empty vials with insert.
10. The MPS injects the samples individually into the LC-MS/MS for analysis.

Analysis conditions LC

Pump:	gradient (800 bar), flowrate = 0.5 mL/min	
Mobile Phase:	A - 0.1 % formic acid in water	
	B - 0.1 % formic acid in acetonitrile	
Gradient:	Initial	5 % B
	0.5 min	5 % B
	1.5 min	30 % B
	3.5 min	70 % B
	4.5 min	95 % B
	6.49 min	95 % B
	6.5 min	5 % B
Run time:	8 minutes	
Injection volume:	2.0 µL (loop over-fill technique)	
Column temperature:	55°C	

Analysis conditions MS

Operation:	electrospray positive mode
Gas temperature:	350°C
Gas flow (N_2):	5 L/min
Nebulizer pressure:	35 psi
Sheath gas heater:	400°C
Sheath gas flow (N_2):	11 L/min
Capillary voltage:	4000 V
Nozzle voltage:	500 V
Delta EMV:	0 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

Results and Discussion

Figure 2 shows representative mass chromatograms for all substrates being analyzed, along with their respective qualifier ion transitions, from a low QC sample.

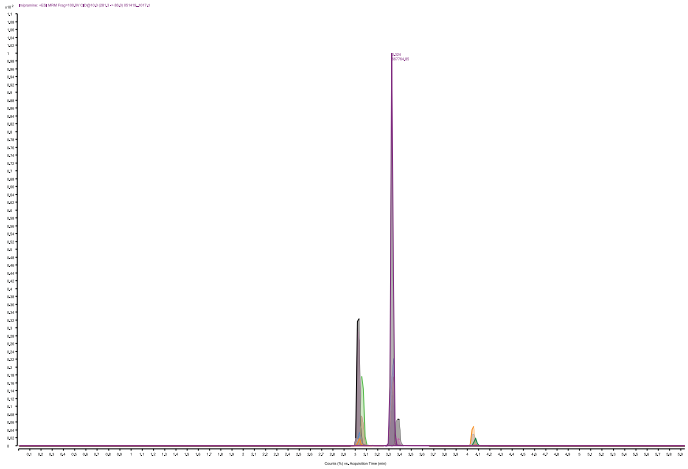
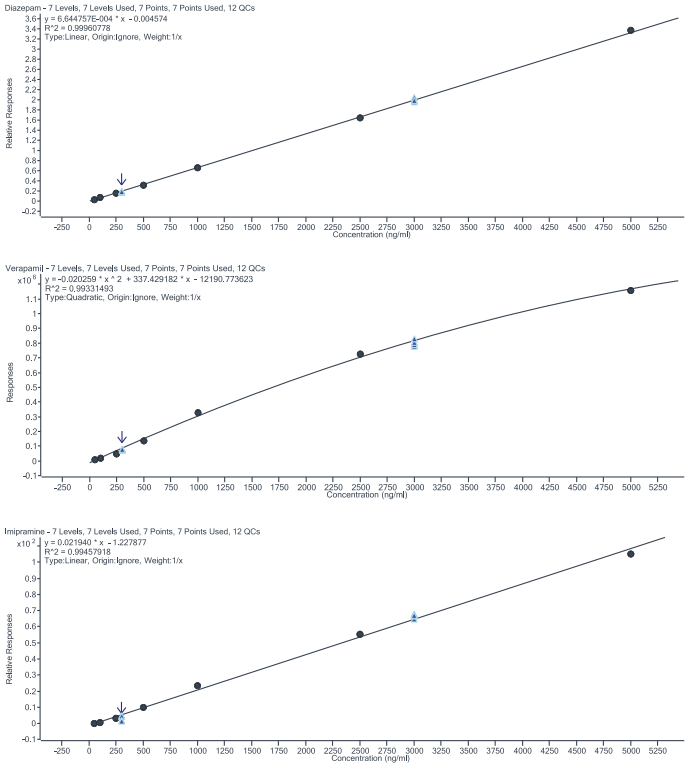


Figure 2. Mass chromatograms overlay for low QC sample.

Representative calibration curves are shown in Figures 3 A-C. Regression analysis for all substrate compounds analyzed within this method resulted in R^2 values of 0.99 or greater.



Figures 3a-c. Representative calibration curves: diazepam, imipramine, and verapamil.

The accuracy and precision of the method was measured for all substrate compounds analyzed using QC samples at two concentrations. Table 2 shows the resulting accuracy and precision data for all compounds. Accuracy data averaged 95.6 % (range: 73.8 % - 113 %) and precision data averaged 2.78 % RSD (range: 1.48 % -5.29 %) for all compounds analyzed.

Table 2. QC sample accuracy and precision table.

Compound	QC Level	Exp. Conc. [ng/mL]	Ave. Conc. [ng/mL]	Ave. Prec. [%]	Ave. Acc. [%]
Dextromethorphan	low	300	267	5.29	89.0
	high	3000	3174	2.32	106
Diazepam	low	300	279	2.34	93.1
	high	3000	2995	1.48	99.8
Imipramine	low	300	262	1.52	87.3
	high	3000	3099	1.57	103
Midazolam	low	300	222	4.46	73.8
	high	3000	3158	2.25	105
Phenacetin	low	300	339	3.17	113
	high	3000	2775	3.70	92.5
Verapmil	low	300	264	2.37	87.9
	high	3000	2911	2.84	97.0

Figures 4 and 5 show representative time-course results for various substrates in either mouse or rat liver microsomes from the automated microsomal stability assays performed. These data provide evidence that the automated microsomal stability assays and associated LC-MS/MS analyses can be readily automated using the GERSTEL MPS robotic^{PRO} sampler.

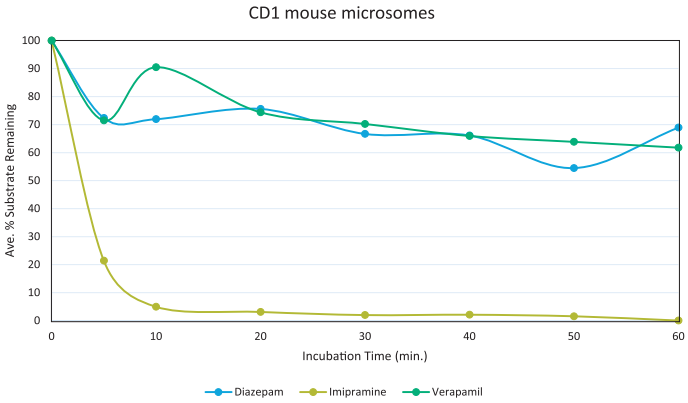


Figure 4. Representative time-course results for substrates in CD1 mouse microsomes.

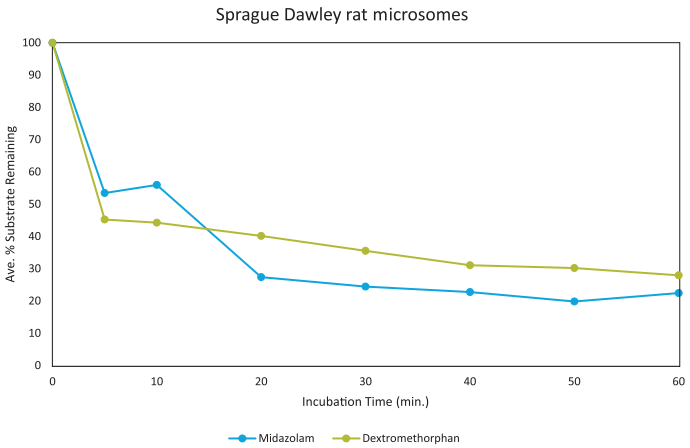


Figure 5. Representative time-course results for substrates in Sprague Dawley rat microsomes.

Conclusions

As a result of this study, we were able to show:

- Automated microsomal stability assays were shown to be readily automated using the GERSTEL MPS robotic^{PRO} sampler with subsequent LC-MS/MS analysis using an Agilent Ultivo Triple Quadrupole Mass Spectrometer.
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved for the determined compounds .
- The LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 95.6 % (range: 73.8 % - 113 %) and precision data averaged 2.78 % RSD (range: 1.48 % - 5.29 %) for all compounds.

References

[1] Corning Discovery Labware Inc., Mammalian Liver Microsomes, Guidelines for Use, TF000017 Rev. 2.0, Retrieved April 2019 from https://certs-ecatalog.corning.com/life-sciences/product-descriptions/452701.pdf.

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