RAPID MEASURE OF DRUG-DRUG INTERACTION UTILIZING GENERIC GRADIENT ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY

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INTRODUCTION

The ability to rapidly measure a drug candidate's effect on Cytochrome P450 (CYP450) is a key aspect in drug development. Information obtained from these analyses can predict potential drug-drug interaction and therefore determine clinical study direction. Typical assay protocol consists of multiple CYP450 probe substrates incubated with a selected drug candidate and CYP450, in the form of liver microsomes. Results of the assay, the amount of probe substrate metabolite formed with respect to control, are used to determine the level of interaction. Isocratic and generic gradient HPLC coupled with tandem mass spectrometry methods have been developed whereby the results of this assay are measured. These methods often have run times of up to five minutes or longer (1,2) and can exhibit peak width of up to 30 seconds at base as is the case during isocratic separations.(3) Isocratic separations further have drawbacks in that if multiple analytes are eluted at a single time point then multiple MRM channels must be monitored simultaneously in the MS method further reducing method sensitivity.

Columns packed with sub 2 µm particles have been shown to provide up to 8x faster analysis time and 3x increase in sensitivity over traditional HPLC. The work presented here shows the development of a high throughput, generic gradient, method for the rapid analysis of six CYP450 probe substrates metabolites in eight seconds.

p 450	Substrate	Major Metabolite
1A2	Phenacetin	4-Acetaminophenol
2A6	Coumarin	7-Hydroxycoumarin
2C8/9/10	Tolbutamide	4-Hydroxytolbutamide
2C19	S-mephenytoin	4-Hydroxymephenytoin
2D6	Bufuralol	1-Hydroxybufuralol
3A4	Midazolam	1-Hydroxymidazolam

Table 1: CYP450 probe substrates and associated major metabolite

METHODS

LC Conditions:

LC System:	Waters® ACQUITY UPLC® System
Column:	ACQUITY UPLC® BEH C_{18} 1.7 µm 2.1 x 50 mm
Column Temperature:	40.0 ˚C
Sample Temperature:	4.0 ˚C
Injection Volume:	1.0 µL
Flow rate:	800 µL/minute
Mobile phase A:	0.1 % Formic Acid
Mobile phase B:	0.1 % Formic Acid in Methanol
Gradient:	5-95 %B/0.25 minutes

MS Conditions:

MS System:	Waters Quattro Premier ®
Ionization Mode:	ESI Positive
Capillary Voltage:	3.2 KV
Desolvation Temperature:	450 ˚C
Desolvation Gas Flow:	900 L/Hr
Source Temperature:	130 ˚C
Acquisition Mode:	MRM
Dwell Time:	0.025 Seconds
Inter Channel Delay:	0.005 Seconds

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
4-acetaminophenol	152 > 110	24	14
7-hydroxycoumarin	163 > 107	35	23
4-hydroxytolbutamide	287 > 74	25	14
4-hydroxymephenytoin	235 > 150	25	18
1-hydroxybufuralol	278 > 186	25	20
1-hydroxymidazolam	342 > 324	30	20
paracetamol	181 > 110	25	21
coumarin	147 > 103	30	16
tolbutamide	271 > 91	25	31

Chemicals:

4-acetaminophenol, 7-hydroxycoumarin, 4-hydroxycoumarin, 4-hydroxytolbutamide, 4-hydroxymephenytoin, 1-hydroxybufuralol, 1-hydroxymidazolam, paracetamol, coumarin, tolbutamide, monobasic sodium phosphate, dibasicsodium phosphate, nefazodone and EDTA were all purchased from Sigma Chemical Co. (St. Louis, MO., US) Formic acid, methanol and acetonitrile were all purchased from Thermo Fisher Scientific (Pittsburgh, PA, US) rat liver microsomes and cofactors were purchased from BD Biosciences (Bedford, MA., US)

Microsomal Incubations:

Stored rat liver microsomes at –80.0˚C were thawed and resuspended in phosphate buffer at a ratio of 1:15 (v/v). 100 µM solutions of p450 substrates and test compounds were obtained by dissolving in phosphate buffer containing 0.5% EDTA (v/v). Cofactor solution was prepared by adding 1.6 mL of cofactor solution A and 0.32 mL of cofactor solution B to 10.9 mL of phosphate buffer. Incubations were carried out as follows: 100 µL p450 substates + 100 µL test compound + 100 µL cofacor solution + 100 µL phosphate buffer were combined and placed into a 2 mL 96 well plate and heated to 37.0 ˚C for 10 minutes. Rat liver microsomes were then added (100 µL) and the 96 well plate was then incubated at 37.0 ˚C while shaking for 60 minutes. The reaction was quenched by the addition of 500 µL of ice cold acetonitrile. Controls were included consisting of a quenched incubation at time 0 and an incubation containing no test compounds.

RESULTS

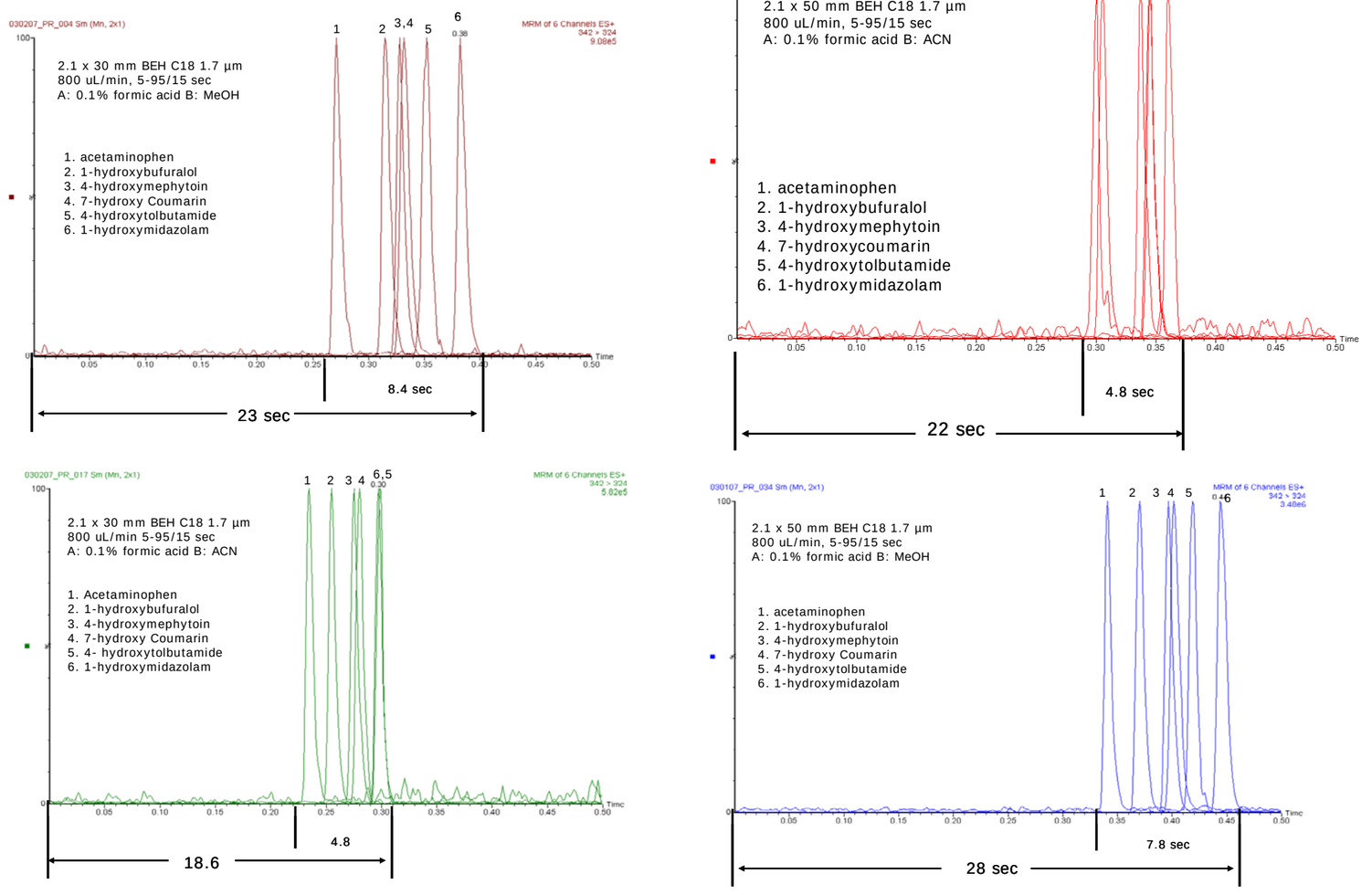

Figure 1. Generic gradient method development for rapid analysis of CYP450 probe substrate metabolites.

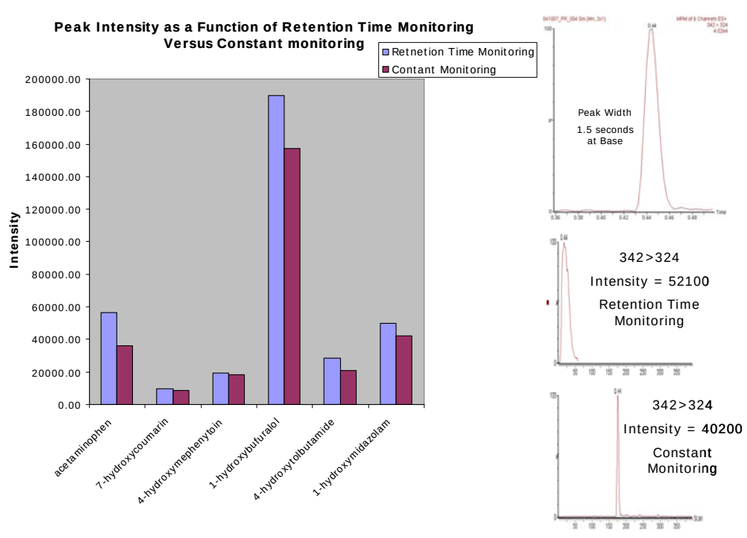

Figure 2. Method intensity increase using retention time monitoring versus constant monitoring.

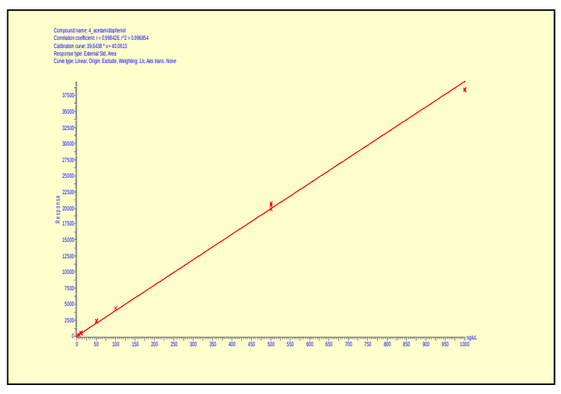

Figure 4. Calibration curve for acetaminophen.

Column Dimension	Column Packing	Seriel Number
2.1 x 50	BEH C 18 1.7 um	1423701135434

Analyte	RT Inj 1	RT Inj 2	RT Inj 3	RT Inj 4	RT Inj 5	RT Inj 6	Average	St Dev	% RSD
Acetaminophen	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.00	0.00
7-hydroxycoumarin	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.00	0.00
4-hydroxymepheytoin	0.41	0.40	0.40	0.40	0.40	0.40	0.40	0.00	1.02
1-hydroxybufuralol	0.38	0.39	0.38	0.38	0.38	0.38	0.38	0.00	1.07
4-hydroxytolbutamide	0.42	0.43	0.42	0.43	0.42	0.43	0.43	0.01	1.29
1-hydroxymidazolam	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.00	0.00

ACQUITY UPLC 2.1 x 50 BEH C18 1.7 um
Average Retention Times of Six Injections of CYP 450 probe metabolites

	serial # 1423701135434	serial # 1413627915209	serial # 1413627615223
Acetaminophen	0.35	0.34	0.34
7-hydroxycoumarin	0.41	0.40	0.40
4-hydroxymepheytoin	0.40	0.40	0.40
1-hydroxybufuralol	0.38	0.37	0.37
4-hydroxytolbutamide	0.43	0.42	0.42
1-hydroxymidazolam	0.46	0.44	0.44

Figure 3. Inter and intra column lot reproducibility.

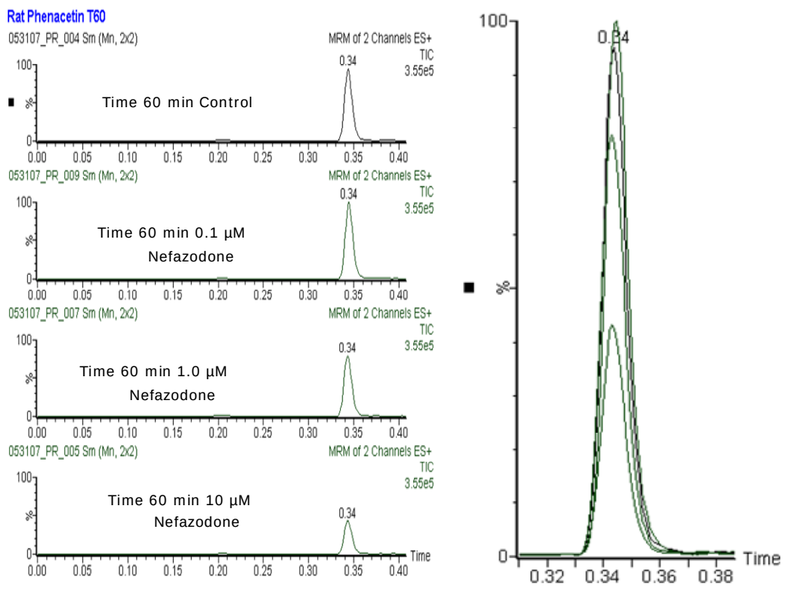

Figure 5. Measuring the effect of nefazodone of CYP450 1A2.

DISCUSSION

The goals for this assay was to develop a generic gradient analysis whereby the major metabolites associated with the CYP450 probe substrates could be separated and detected during an injection to injection cycle time of 1 minute, while maintaining maximum resolution between analytes. The generic method development approach used many common method parameters such as: 5-95 % organic per minute, C18 packing material, a column heater set at 40.0 ˚C and common organic modifiers such as methanol and acetonitrile. The best separation obtained utilizing this approach is shown in blue in Figure 1. Average peaks widths of 1.5 seconds were observed, Figure 2. Peaks of Isocratic separations often are wider than those separated during gradient elution. These types of separations further elute multiple analytes at once, requiring constant monitoring of multiple MRM channels during analysis further reducing method sensitivity. Examples of this is shown in Figure 2. Also shown is the ability of the mass spectrometer to acquire ample spectra across the narrow peak widths in order to perform good quantification. Due to the narrow peak widths and short analysis time obtained from this analysis, retention time shifts could lead to undetected or reduced measured signal causing erroneous results. Due to this fact, multiple columns containing the same lot of packing material as well a column containing a different lot of packing material were tested, Figure 3. The result of this test showed excellent retention time reproducibility between all column tested indicating that the system and column created a robust system for analysis. Last the effect of nefazodone was observed, Figure 5. Varying concentrations of nefazodone were incubated with phenacetin during a sixty minute time period to determine the effect of the conversion of phenacetin to acetaminophen by CYP450 isoenzyme 1A2.

CONCLUSION

- **A generic gradient UPLC/MS/MS method was developed for the high-throughput analysis of potential drug-drug interaction.**
- **Acquiring data using retention time monitoring versus constant monitoring showed increased method sensitivity.**
- **Excellent retention time reproducibility from inter and intra lot 2.1 x 50 mm columns packed with BEH C18 1.7µm packing material was observed.**
- **Nefazodone is an inhibitor of p450 isoenzyme 1A2.**

REFERENCES

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