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Application Note

A Rapid Assay for p450 Inhibition/Induction Utilizing UPLC and Tandem Quadrupole MS

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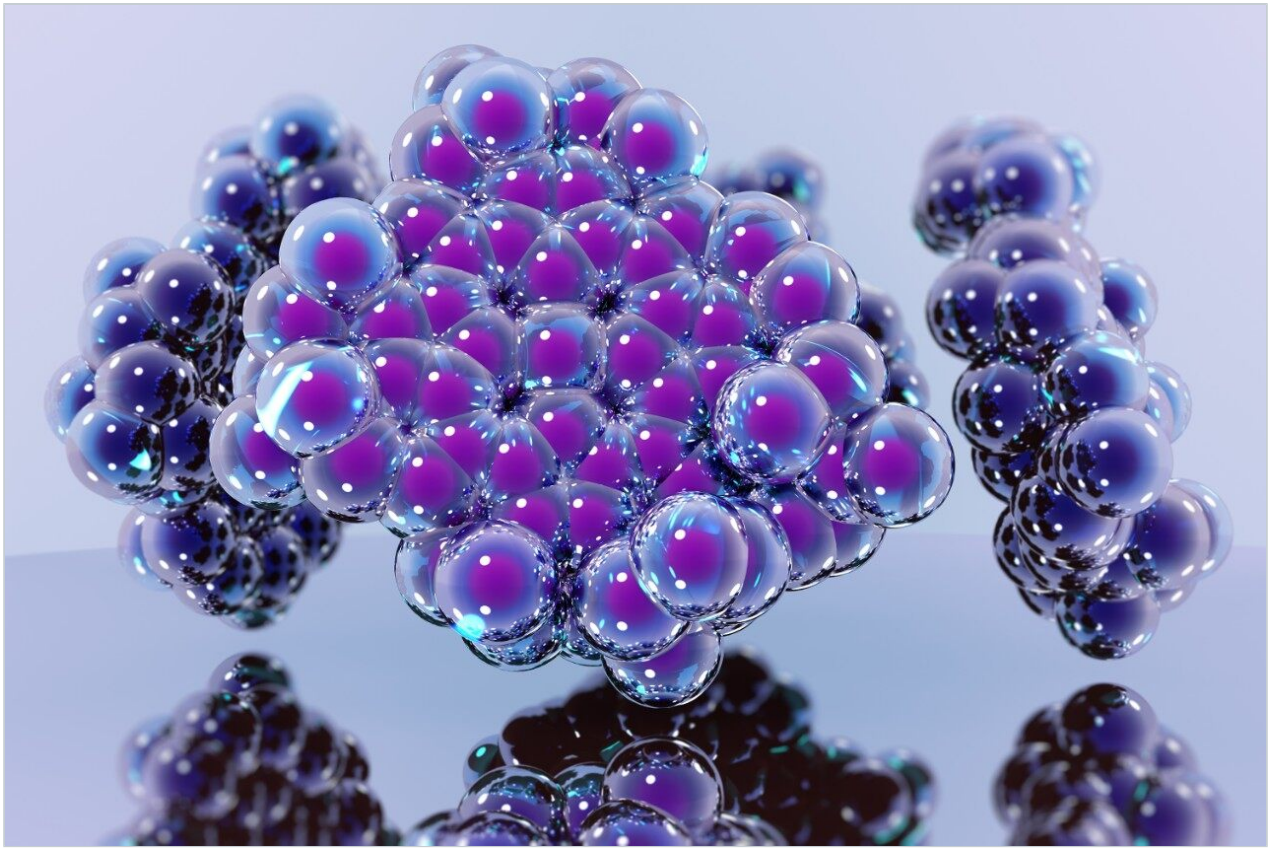

Abstract

In this application note, we investigate the use of the increased chromatographic efficiency of UltraPerformance LC (UPLC) and its sub-2-µm particles to deliver improved performance and faster analysis for toxicity studies.

Benefits

Increase throughput for the analysis of CYP 450 metabolites.

Introduction

A significant number of candidate medicines fall out of the development process due to toxicity. The later this occurs in the discovery/development process, the more costly it becomes. A number of high-profile medicines have been withdrawn from the market due to toxic events. These clinical events are often due to drug-drug interaction or unpredictable “idiosyncratic” events.

As part of the drug discovery and development process, it is critical to evaluate candidate drugs for possible toxicity/drug-drug interactions or inhibition/induction of metabolizing enzymes in the body. Failure to properly identify these potential toxic events can cause the compound to be withdrawn from the market at a significant loss in revenue.

The effect of a candidate medicine on the major drug metabolizing enzymes, Cytochrome p450 (CYP450), is studied in both discovery as part of the lead candidate evaluation process and in development to determine potential drug-drug interactions. These studies involve evaluating the effect of the candidate medicine on the drug metabolizing CYP450 enzymes by monitoring the changes in the concentrations on a set of probe substrate molecules. These assays are typically performed by HPLC/UV, or more recently by LC-MS/MS, with analysis times in the 5 to 10 minute range.[1-3]

In this application note, we investigate the use of the increased chromatographic

efficiency of UltraPerformance LC (UPLC) and its sub-2-µm particles to deliver improved performance and faster analysis for toxicity studies.

Figure 1. ACQUITY TQD with the TQ Mass Spectrometer.

Experimental

LC Conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm Column
Column temp.:	40 °C
Sample temp.:	4 °C
Injection volume:	1 µL

Flow rate:	800 µL/min
Mobile phase A:	0.1 % formic acid in H_2O
Mobile phase B:	0.1 % formic acid in MeOH
Gradient:	5 to 95% B over 0.25 min

MS Conditions

MS system:	Waters Quattro Premier or TQ Detector
Ionization mode:	ESI positive
Capillary voltage:	3.0 KV
Desolvation temp.:	450 °C
Desolvation gas:	900 L/Hr
Source temp.:	150 °C
Acquisition mode:	MRM Transition
Dwell time:	0.025
Inter channel delay:	0.005

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

Microsomal Incubation Conditions

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: phenacetin, coumarin, tolbutamide, s-mephytoin, bufuralol, midazolam, and test compounds were obtained by dissolving the compounds 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 substrates + 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 microsomeswere 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. Samples were then centrifuged at 2013 RCF and injected onto the UPLC-MS/MS system. Controls were included consisting of a quenched incubation at time 0 and an incubation containing no test compounds.

Results and Discussion

CYP450 is comprised of various drug metabolizing enzymes. The isoenzyme forms listed in Table 1 are responsible for the metabolism of more than 90 percent of the drugs on the market today.[4]

In order to determine the level of interaction between a potential drug candidate and the various CYP450 isoenzymes, various "cocktail" approaches have been developed whereby potential drug candidates are incubated with various probe substrates specific for the major CYP450 isoenzymes. The level of the major metabolite formed from the probe substrates is used to determine if any inhibition, induction, or drug-drug interaction has occurred. This approach provides a high-throughput analysis whereby levels of p450 inhibition/induction and drug-drug interaction can be determined.

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. Probe substrates and major metabolite for major CYP450 isoenzymes often utilized to determine drug interactions.

The goal for this assay was to develop a sensitive, gradient-based analysis whereby the major metabolites associated with the CYP450 probe substrates could be separated and detected with an analysis time of 30 seconds, while maintaining maximum resolutionbetween analytes. Reversed-phase chromatography utilizing common mobile phases and conditions were selected to be compatible with other commonly-used LC-MS ADME screening methods.

The best separation obtained utilizing this approach is shown in Figure 2. Average peaks widths of 1.4 seconds at peak base were observed containing greater than 20 scans per peak, ensuring that proper

quantification could be realized. The method obtained linear results for all the probe substrate major metabolites analyzed. An example is shown in Figure 3 for the calibration curve of acetaminophen. An R^2 value of 0.996 was obtained with similar results obtained for the other probe substrate major metabolites.

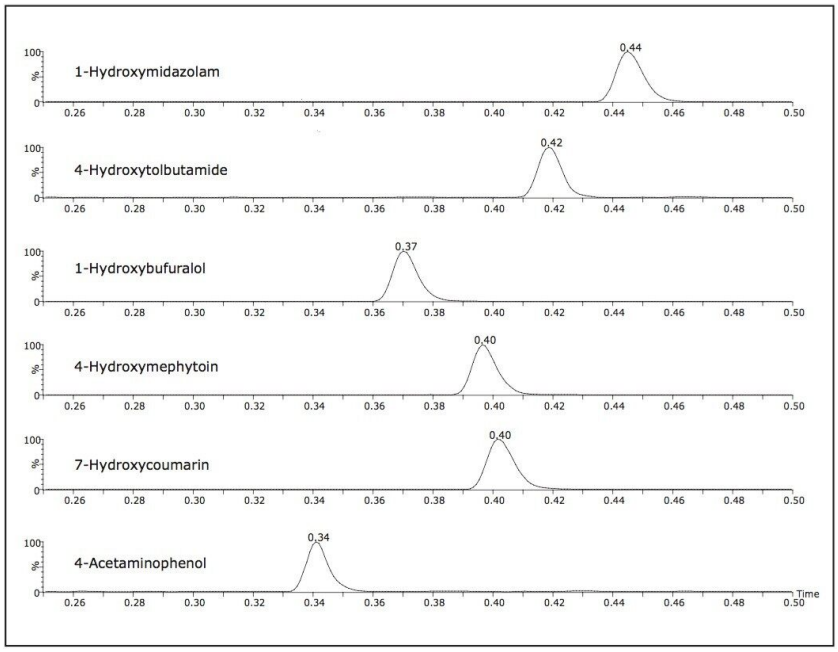

Figure 2. Separation of six CYP450 probe substrate major metabolites.

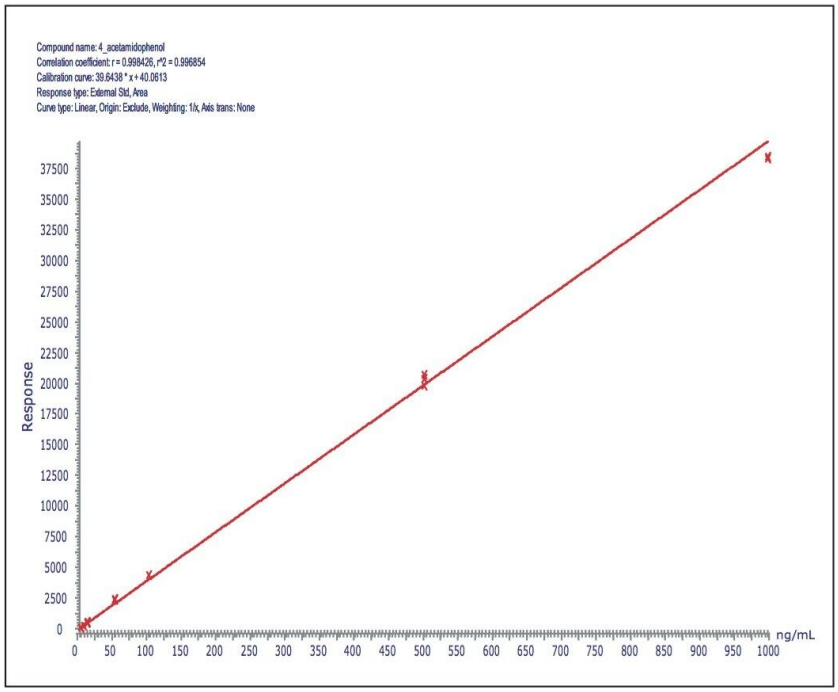

Figure 3. Calibration line for acetaminophen.

To illustrate the utility of this method, we tested nefazodone, a common pharmaceutical compound. Varying concentrations of nefazodone were incubated using the cocktail assay approach with the probe substrates listed in Table 1. The incubation was carried out over a 60-minute time period. The results of this experiment are shown in Figure 4.

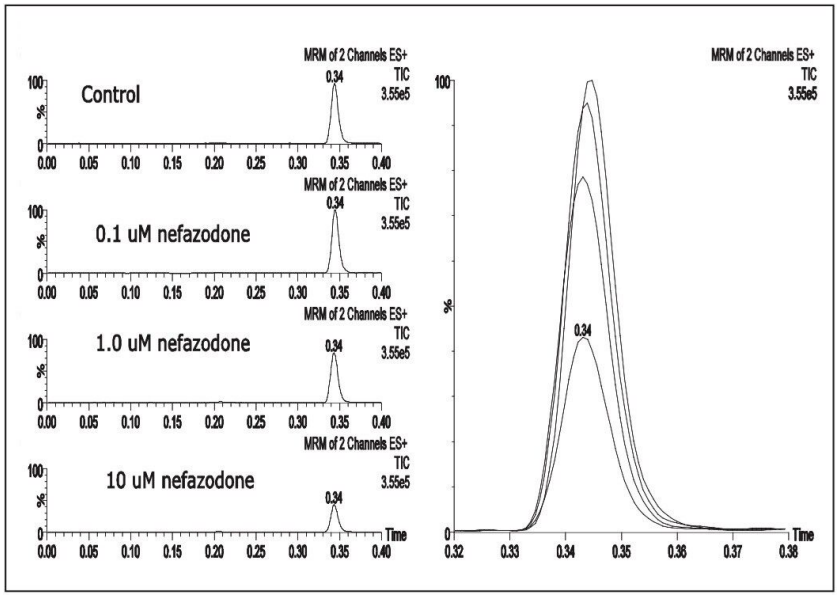

Figure 4. Effect of various concentrations of nefazodone on the level of acetaminophen measured, thereby evaluating the effect of nefazodone on phenacetin metabolism.

The data shows that as the concentration of nefazodone was increased, a reduction in the formation of acetaminophen was observed. This result indicates that nefazodone has a direct effect on the metabolism of phenacetin to acetaminophen. This inhibitory effect of nefazodone on the CYP450 isoenzyme 1A2, the isoenzymeresponsible for the conversion of phenacetin to acetaminophen, is in agreement with experimental data acquired by other investigators.[5]

Conclusion

The results of this study illustrate the utilization of chromatographic columns packed with sub-2-µm particles operating above 8000 psi, coupled with Tandem Quadrupole Mass Spectrometry, for the rapid measurement of potential p450 inhibition, induction, and drug-drug interactions.

Average peak widths of 1.4 seconds were observed, producing a rapid high-resolution separation under generic gradient conditions. Total ion chromatograms generated by Tandem Quadrupole Mass Spectrometer obtained quality spectra well above the required points-per-peak minimum to perform good quantification. The ability of the UPLC-MS/MS system operating with rapid generic gradients has been shown to increase

throughput for the analysis of CYP 450 metabolites.

This approach can be applied to other areas of bioanalysis where high throughput and sensitivity demands must be met. As well, since this UPLC-based approach can help labs pre-emptively determine candidate toxicity and drug-drug interactions, it enables organizations to be more confident in the viability of candidate medicines that do progress to late-stage clinical trials – and helping them avoid the costs and business impact of the failure of a drug candidate late in the development process.

References

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