

A Rapid Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Method for Quantifying the Metabolites of Seven Major Probe Substrates in Human Liver Microsomes



Poster#: P30

Xiaotong Li, Chunli Zhu, Xinxin Wen, Ling Sun, Lei Tian, Zhiyu Li, Lifang Jiang, Lili Xing, Yi Tao, Liang Shen
DMPK Service Department, WuXi AppTec, 31 Yiwei Road, Waigaoqiao Free Trade Zone, Shanghai, P.R. China, 200131

Abstract

Cytochrome P450 (CYP) family of enzymes are commonly involved in important drug-drug interactions (DDIs). The FDA recommends an *in vitro* CYP inhibition assay for the 7 major human hepatic CYP isoforms: CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A. Compared to the way individual metabolites are investigated separately with a single probe, cassette incubation and analysis, known as the “cocktail” approach, can simultaneously detect multiple metabolites from probe substrates to improve efficiency and throughput, which involves simultaneous determination of acetaminophen (CYP1A2), hydroxyl-bupropion (CYP2B6), n-desethyl-amodiaquine (CYP2C8), 4'-hydroxy-diclofenac (CYP2C9), 4'-hydroxymephenytoin (CYP2C19), dextrophan (CYP2D6) and 1'-hydroxy-midazolam (CYP3A4/5) [1-2]. This cassette analysis method faces many challenges such as interference separation issues, the solvent effect of acetaminophen, the sensitivity of 4'-hydroxymephenytoin, etc. To overcome these challenges, we have developed a rapid and robust method in this study.

Table 1. List of substrates, metabolites and internal standards

CYP450	Substrate	Metabolite (Analytes)	Internal Standard
1A2	Phenacetin	Acetaminophen	Acetaminophen-D ₄
2B6	Bupropion	Hydroxyl-bupropion	Hydroxyl-bupropion-D ₆
2C8	Amodiaquine	N-Desethyl-amodiaquine	N-Desethyl-amodiaquine-D ₅
2C9	Diclofenac	4'-Hydroxy-diclofenac	4'-Hydroxy-diclofenac-D ₄
2C19	S-Mephenytoin	4'-Hydroxy-mephenytoin	4'-Hydroxy-mephenytoin-D ₃
2D6	Dextromethorphan	Dextrophan	Dextrophan-D ₃
3A4	Midazolam	1'-Hydroxy-midazolam	1'-Hydroxy-midazolam- ¹³ C ₃

Objective

The objective of this study is to establish a rapid, highly sensitive and selective method that can simultaneously detect these metabolites, thereby assessing the potential compound inhibition of the seven major CYPs during the drug discovery stage.

Method

Sample Preparation

Human liver microsomes were incubated with 7 substrate cocktails. 200 μL samples were added with 400 μL acetonitrile (containing 7 stable isotope labeled internal standards), then mixed well and centrifuged for 20 min at 4000 rpm. 100 μL supernatant was mixed with 200 μL water and then injected into LC-MS/MS.

LC-MS/MS Condition

Table 2. Retention times and ion transitions for metabolites

Metabolites	Retention Time(min)	Monoisotopic Mass(Da)	Ion Transitions (m/z)
Acetaminophen	1.08	151.1	152.1 > 110.1
Hydroxyl-bupropion	1.63	255.1	256.4 > 238.0
N-Desethyl-amodiaquine	1.30	327.1	328.2 > 283.2
4'-Hydroxy-diclofenac	2.25	311.0	312.0 > 231.0
4'-Hydroxy-mephenytoin	1.61	234.1	235.1 > 150.2
Dextrophan	1.59	257.2	258.2 > 157.2
1'-Hydroxy-midazolam	1.84	341.1	342.1 > 203.1

Conclusion

- The selectivity, linearity, intra-day accuracy and precision were evaluated, and the results showed the high reliability and reproducibility of the method.
- A novel and rapid LC-MS/MS method was established for simultaneous quantification of 7 metabolites in CYP inhibition assay within 2.5 minutes, which was 3 times faster than the method reported in the literature.

References

[1] D. Spaggiari, L. Geiser, Y. Daali, S. Rudaz. A cocktail approach for assessing the in vitro activity of human cytochrome P450s: an overview of current methodologies. J Pharm Biomed Anal. 101 (2014) 221–237 .
[2] K.A. Youdim, K.C. Saunders, A review of LC-MS techniques and high-throughput approaches used to investigate drug metabolism by cytochrome P450s, J. Chromatogr. B 878 (2010) 1326–1336.

Table 3. Analytical condition

LC System	Shimadzu Nexera UHPLC-40
MS System	SCIEX Triple Quad™ 6500+
Column	Waters ACQUITY HSS T3, 1.8 μm, 2.1 mm X 100 mm
Mobile Phase A	2% formic acid in water
Mobile Phase B	2% formic acid in acetonitrile
Column Temperature	40 °C
Flow Rate	700 μL/ min
Run Time	2.5 min
Injection Volume	15 μL
Ionization Mode	ESI positive ion mode

Results

Chromatograms

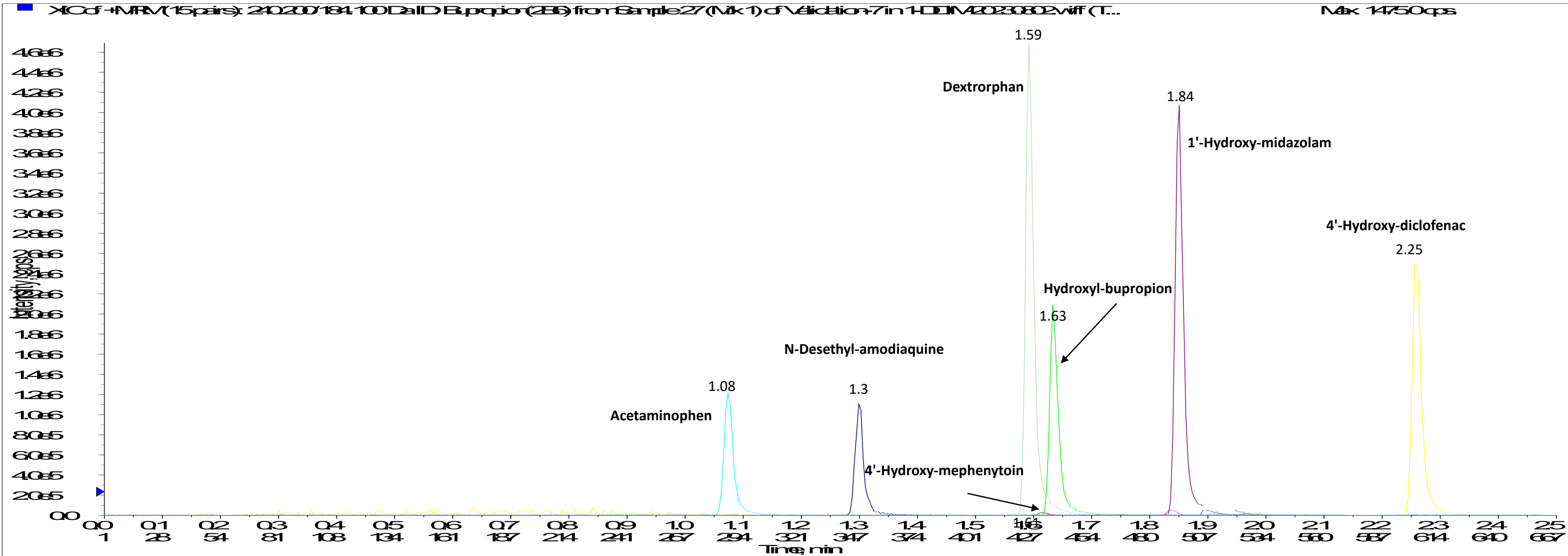


Figure 1. The chromatograms of 7 metabolites.

Table 4. The calibration curve and linearity range of 7 metabolites

Metabolite	Slope (a)	Intercept (b)	Correlation coefficient (r)	Linearity range (nM)
Acetaminophen	0.01591	-0.03097	0.99748	15.0 ~ 4000
Hydroxyl-bupropion	0.07546	-0.01447	0.99757	1.5 ~ 400
N-Desethyl-amodiaquine	0.01781	-0.01352	0.99820	6.0 ~ 1600
4'-Hydroxy-diclofenac	0.00214	-0.00622	0.99775	30.0 ~ 8000
4'-Hydroxy-mephenytoin	0.00943	0.00324	0.99607	1.2 ~ 320
Dextrophan	0.05263	-0.03427	0.99609	3.0 ~ 800
1'-Hydroxy-midazolam	0.08433	-0.00133	0.99696	3.0 ~ 800

Table 5. Intra-day accuracy and precision results for 7 metabolites (n=6).

Metabolite	QC Samples	Theoretical concentration (nM)	Mean (nM)	Accuracy* (%)	CV** (%)	Metabolite	QC Samples	Theoretical concentration (nM)	Mean (nM)	Accuracy* (%)	CV** (%)
Acetaminophen	LLOQ	15.0	17.3	115.3	8.8	1'-Hydroxy-midazolam	LLOQ	3.0	3.1	101.7	6.8
	LQC	45.0	47.6	105.8	5.3		LQC	9.0	9.2	102.2	7.0
	MQC	400.0	411.0	102.8	3.1		MQC	80.0	83.2	104.0	2.8
	HQC	3200.0	3008.0	94.0	5.8		HQC	640.0	618.0	96.6	3.0
Hydroxy Bupropion	LLOQ	1.5	1.8	117.3	7.3	N-Desethyl Amodiaquine	LLOQ	6.0	6.7	111.3	11.8
	LQC	4.5	4.6	103.1	7.8		LQC	18.0	18.1	100.6	10.8
	MQC	40.0	41.8	104.5	5.5		MQC	160.0	167.0	104.4	10.5
	HQC	320.0	302.0	94.4	3.0		HQC	1280.0	1185.0	92.6	6.3
Dextrophan	LLOQ	3.0	3.6	121.3	9.7	4'-Hydroxy-diclofenac	LLOQ	30.0	34.9	116.3	4.9
	LQC	9.0	9.4	104.4	9.0		LQC	90.0	96.8	107.6	2.4
	MQC	80.0	74.8	93.5	5.0		MQC	800.0	845.0	105.6	6.2
	HQC	640.0	565.0	88.3	4.7		HQC	6400.0	5838.0	91.2	5.7
4'-Hydroxy-mephenytoin	LLOQ	1.2	1.3	104.2	19.6	* Accuracy(%) must be within (100±25)%. **CV(%) must be within 20%. LLOQ: Lower Limit of Quantification, LQC: Low Quality Control, MQC: Medium Quality Control, HQC: High Quality Control , CV: Coefficient of Variation.					
	LQC	3.6	2.9	79.2	15.5						
	MQC	32.0	32.6	101.9	6.7						
	HQC	256.0	236.0	92.2	7.3						

