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APPLICATIONS

Simultaneous Determination of Antidepressants and Metabolites in Urine Using Ultra-High Performance Liquid Chromatography – Tandem Mass Spectrometry (UHPLC-MS/MS) using a Kinetex® Core-Shell C18 HPLC/UHPLC Column

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Introduction

Antidepressants are drugs used to treat clinical depression, as well as anxiety disorders. According to the pharmacological mechanism of action (MOA), antidepressants are divided into seven classes. These include tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs), among others. Antidepressant overdose could lead to severe side effects or even death. This is especially true with TCAs, a class of antidepressants exhibiting inhibitory effects on a wide range of neurologic pathways in the human brain. As such, it is imperative to evaluate patient adherence as well monitor abuse.

Although clinical laboratories can use immunoassay to evaluate antidepressant dosage and misuse, LC/MS/MS methodology is preferred because of its selectivity and robustness. In this study, a simple sample preparation procedure and a rapid, sensitive, specific Ultra-High Performance Liquid Chromatography – Tandem Mass Spectrometry (UHPLC-MS/MS) method has been developed for quantifying several antidepressants, including amitriptyline, bupropion, citalopram, clomipramine, doxepin, duloxetine, fluoxetine, imipramine, paroxetine, and venlafaxine as well as five major metabolites, nortriptypline, norfluoxetine, desipramine, hydroxybupropion, and o-desmethylvenlafaxine, in human urine samples.

Reagents and Chemicals

Primary reference standards and deuterated internal standards were purchased (Cerilliant Corporation, Round Rock, TX). A mixture of standard solution was prepared in acetonitrile and then added to drug-free urine to make multi-concentration levels of calibrators. The internal standards mixture includes amitriptyline-d3, clomipramine-d3, desipramine-d3, doxepin-d3, imipramine-d3, nortriptyline-d3, bupropion-d9, fluoxetine-d6, paroxetine-d6, and venlafaxine-d6, and it was prepared in acetonitrile and added in urine samples, standards, and controls.

Sample Preparation

A simple "dilute and shoot" urine sample extraction was carried out by a Tomtec™ Quadra 4™ liquid hander (Hamden, CT) in 96-well collection plates to increase throughput. Samples were treated with beta-glucuronidase to hydrolyze glucuronide conjugates, followed by dilution and centrifugation.

LC/MS/MS Conditions

LC/MS/MS was performed using a Kinetex® 2.6 µm core-shell C18 50 x 3.0 mm HPLC/UHPLC column and a Shimadzu® Nexera™ UHPLC system (Kyoto, Japan) with an upper pressure limit of 1300 bar. A triple quadrupole API 3200™ system (AB SCIEX, Framingham, MA), equipped with an electrospray source, was used for mass spectrometric detection. The MS spectra were recorded in multiple-reactions monitoring and scheduled MRM™ algorithm. MRM Transitions and Ionization Source Parameters are listed in **Table 1**.

Analytical Method

Column:	Kinetex® 2.6 µm C18	
Dimensions:	50 x 3.0 mm	
Part No.:	00B-4462-Y0	
Mobile Phase:	A: 2 mM Ammonium acetate with 0.075 % (v/v) acetic acid (pH 4.5)	
	B: 2 mM Ammonium acetate with 0.075 % (v/v) acetic acid and acetonitrile	
Flow Rate:	0.8 mL/min	
Temperature:	Ambient	
Instrument:	Shimadzu Nexera UHPLC	
Detector:	AB SCIEX API 3200™ MS/MS, ESI+	
Gradient:	**Time (min)**	**% B**
	0.0	18
	0.40	18
	1.10	45
	1.70	60
	2.10	95
	2.50	95
	2.54	18
	3.50	18

Ionization Source Parameters:

Gas 1 & Gas 2	50
CAD	6
Cur	35
IS	3000
Temp	550

Table 1.
MRM Transitions & Retention Times

Analyte Peak Name	Q1	Q3	Analyte Retention Time (min)
Amitriptyline	278.0	91.0	2.29
Bupropion	240.1	184.1	1.88
Citalopram	325.1	109.1	2.03
Clomipramine	315.1	86.2	2.42
Desipramine	267.1	208.2	2.17
o-Desmethylvenlafaxine	264.1	107.2	1.27
Doxepin	280.0	107.1	2.08
Duloxetine	298.0	154.2	2.21
Fluoxetine	310.1	148.0	2.30
Hydroxybupropion	256.1	238.1	1.57
Imipramine	281.0	86.2	2.23
Norfluoxetine	296.0	134.1	2.24
Nortriptyline	264.1	233.2	2.22
Paroxetine	330.0	192.2	2.12
Venlafaxine	278.1	260.2	1.81

Figure 1.
Representative chromatogram for ten antidepressants and five of their major metabolites
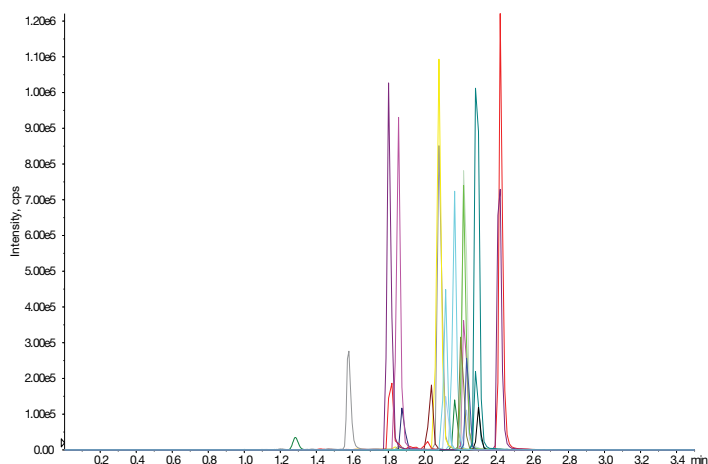
Results

Linearity was evaluated by analyzing samples at 10 concentration levels over the reportable range of assay for three days. Linear Regression using the Analyst™ software ("1 / x*x" weighting) was used to determine slope and correlation coefficient. Results are shown in **Table 2**. Intra-day and inter-day precision and accuracy were determined by analyzing quintuplicate samples at three levels of concentrations. Results are shown in **Table 3** and **Table 4**.

The carry-over was also estimated by injecting blank samples immediately following the highest concentration, and was less than 15 % of lower limit of quantitation (LLOQ), which can be acceptable. Bench-top stability was assessed by reinjection of same samples within 24-48 hours, and was within compliance range. In addition, cross reactivity was investigated against all our current test panels of drugs.

Table 2.
Summary of Reportable Range and Linearity Correlation

Drug	Cut-off (ng/mL)	Reportable Range (ng/mL)	Slope	Linearity Correlation Data (r)	y-intercept
Amtriptyline	50	12.5-7500	0.97	0.997	27
Bupropin	10	2.5-1500	1.02	0.998	2.45
Citalopram	10	2.5-1500	1.04	0.993	3.89
Clomipramine	50	12.5-7500	1.11	0.995	2.21
Desipramine	50	12.5-7500	1.11	0.998	41.28
o-Desmethylvenlafaxine	10	2.5-1500	0.9	0.992	9.04
Doxepin	50	12.5-7500	0.89	0.998	69.57
Duloxetine	5	12.5-7500	0.94	0.994	32.93
Fluoxetine	20	12.5-7500	1.05	0.995	0.95
Hydroxybupropion	10	2.5-1500	0.99	0.993	1.05
Imipramine	50	12.5-7500	0.94	0.997	31.65
Norfluoxetine	10	12.5-7500	1.16	0.998	86.59
Nortriptyline	50	12.5-7500	0.88	0.993	85.48
Paroxetine	10	12.5-7500	1.03	0.997	14.56
Venlafaxine	20	2.5-1500	1.03	0.999	4.54

Table 3.
Intra-day Precision and Accuracy

| Drug | Concentration (ng/mL) | Intra-day | | | | | |
| | | Day 1 | | Day 2 | | Day 3 | |
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Amitriptyline	100	5.9	109.0	2.7	102.4	5.4	97.0
	250	3.3	109.7	3.4	111.0	4.3	108.9
	750	4.0	102.3	3.0	103.1	7.2	97.8
Bupropion	20	8.7	105.2	7.3	108.9	11.7	94.5
	50	5.5	108.5	5.4	111.4	5.1	109.4
	150	5.8	96.6	6.5	102.2	2.3	101.5
Citalopram	20	6.0	111.4	3.7	104.3	6.8	100.4
	50	5.1	109.7	5.7	110.2	3.9	97.9
	150	4.7	103.6	5.0	105.7	3.9	91.8
Clomipramine	100	2.1	102.2	2.3	103.2	7.4	94.3
	250	3.5	101.5	2.9	105.5	7.4	100.8
	750	4.1	99.7	2.6	106.8	5.0	98.5
Desipramine	100	7.5	101.6	5.0	104.2	5.9	97.4
	250	3.5	107.1	4.5	112.3	6.1	106.1
	750	8.7	98.2	3.4	104.4	4.6	95.9
o-Desmethylvenlafaxine	20	7.3	104.2	1.9	111.4	2.8	100.3
	50	4.0	115.2	1.5	119.1	4.1	113.1
	150	1.0	100.1	2.0	109.0	9.2	103.2
Doxepin	100	0.7	110.8	3.5	108.2	5.6	103.3
	250	3.1	115.4	1.4	118.0	5.0	114.6
	750	0.7	100.4	2.3	106.8	1.9	98.4

Table 3. (continued)
Intra-day Precision and Accuracy

Drug	Concentration (ng/mL)	Intra-day					
		Day 1		Day 2		Day 3	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Duloxetine	100	6.8	110.7	7.5	109.6	7.0	105.8
	250	2.2	111.8	6.3	109.9	11.7	111.1
	750	6.1	103.9	5.9	95.3	4.2	105.8
Fluoxetine	100	9.2	100.1	4.3	102.8	7.1	98.1
	250	5.2	95.9	5.5	103.6	5.5	108.1
	750	10.7	98.5	2.5	105.8	3.7	103.5
Hydroxybupropion	20	4.4	102.9	4.0	107.7	4.6	100.6
	50	2.6	108.8	1.9	112.1	5.3	116.0
	150	2.7	95.2	1.9	104.5	4.6	108.3
Imipramine	100	3.1	109.4	4.1	115.6	2.3	96.9
	250	1.1	110.7	2.1	122.5	3.9	112.4
	750	4.1	100.3	4.0	111.4	4.4	95.1
Norfluoxetine	100	5.9	88.9	2.9	99.8	7.0	99.6
	250	6.6	92.4	2.0	97.4	6.3	110.7
	750	6.6	92.0	4.1	100.9	5.2	106.0
Nortriptyline	100	4.2	101.9	2.7	117.0	3.9	102.7
	250	5.3	105.1	3.4	119.9	5.8	111.4
	750	4.2	97.4	1.8	112.0	4.2	102.0
Paroxetine	100	7.9	106.8	6.1	107.7	2.6	101.7
	250	6.2	104.2	5.1	105.9	6.7	107.8
	750	4.5	104.4	4.7	100.2	6.2	97.2
Venlafaxine	20	9.7	101.5	7.9	100.4	11.0	98.8
	50	2.3	105.5	3.2	116.3	5.8	110.3
	150	3.5	96.0	3.1	105.2	3.8	99.0

Table 4.
Inter-day Precision and Accuracy

| Drug | Concentration (ng/mL) | Inter-day | |
		CV (%)	Accuracy (%)
Amitriptyline	100	6.7	102.8
	250	3.5	109.9
	750	5.3	101.0
Bupropion	20	10.5	102.9
	50	5.1	109.8
	150	5.3	100.4
Citalopram	20	6.9	105.4
	50	7.3	106.0
	150	7.8	100.1
Clomipramine	100	5.8	99.9
	250	5.1	102.6
	750	5.3	101.8
Desipramine	100	6.4	101.1
	250	5.1	108.5
	750	6.5	99.6
o-Desmethylvenlafaxine	20	6.2	105.3
	50	3.8	115.8
	150	6.3	104.4
Doxepin	100	4.6	107.4
	250	3.5	116.0
	750	4.1	102.0
Duloxetine	100	6.7	108.5
	250	7.2	110.9
	750	6.9	101.5

Table 4. (*continued*)
Inter-day Precision and Accuracy

Drug	Concentration (ng/mL)	Inter-day	
		CV (%)	Accuracy (%)
Fluoxetine	100	6.9	100.3
	250	7.1	102.5
	750	6.3	102.9
Hydroxybupropion	20	5.0	103.7
	50	4.3	112.3
	150	6.2	103.2
Imipramine	100	8.1	107.3
	250	5.3	115.2
	750	8.1	102.4
Norfluoxetine	100	7.5	96.1
	250	9.4	100.1
	750	7.5	100.2
Nortriptyline	100	7.5	107.2
	250	7.2	112.1
	750	6.8	104.3
Paroxetine	100	6.2	105.4
	250	5.8	106.0
	750	5.6	100.3
Venlafaxine	20	8.9	100.2
	50	5.6	110.7
	150	5.1	100.3

Discussion and Conclusion

Accuracy and precision for all antidepressants and metabolites in reportable range were obtained. The intra-day and inter-day variability was less than 20 %. Negative cut-off values and reportable ranges for urinary analysis can be acceptable.

A new method for detecting 10 antidepressants and 5 of their major metabolites in urine using ultra-high performance liquid chromatography – tandem mass spectrometer (UHPLC-MS), and "dilute-shoot" sample extraction procedures has been established and validated. This assay is suitable for the confirmatory test of the patient antidepressant drug compliance.

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5 μm Minibore Columns (mm)			SecurityGuard™ ULTRA Cartridges‡
Phases	50 x 2.1	100 x 2.1	3/pk
C18	00B-4601-AN	00D-4601-AN	AJ0-8782
			for 2.1 mm ID

5 μm MidBore™ Columns (mm)			SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	3/pk
C18	00B-4601-Y0	00D-4601-Y0	AJ0-8775
			for 3.0 mm ID

5 μm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
					for 4.6 mm ID

2.6 μm Minibore Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	75 x 2.1	100 x 2.1	150 x 2.1	3/pk
C18	00A-4462-AN	00B-4462-AN	00C-4462-AN	00D-4462-AN	00F-4462-AN	AJ0-8782
						for 2.1 mm ID

2.6 μm MidBore™ Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	3/pk
C18	00A-4462-Y0	00B-4462-Y0	00C-4462-Y0	00D-4462-Y0	00F-4462-Y0	AJ0-8775
						for 3.0 mm ID

2.6 μm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phases	30 x 4.6	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	3/pk
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	AJ0-8768
						for 4.6 mm ID

1.7 μm Minibore Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
C18	00A-4475-AN	00B-4475-AN	00D-4475-AN	00F-4475-AN	AJ0-8782
					for 2.1 mm ID

1.7 μm MidBore Columns (mm)			SecurityGuard ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	3/pk
C18	00B-4475-Y0	00D-4475-Y0	AJ0-8775
			for 3.0 mm ID

‡ SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

> Kinetex Core-Shell Technology HPLC/UHPLC columns also come in a varity of other particle sizes and stationary phases. For more info, please visit www.phenomenex.com/Kinetex

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