Application Note

Forensic Toxicology

Ultrafast Analysis of a Tricyclic Antidepressant Drug Panel in Human Serum

By the Agilent RapidFire high-throughput triple quadrupole mass spectrometry system

Authors

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Abstract

Quantitative analysis of tricyclic antidepressant drugs (TCAs) in forensic laboratories traditionally relies on HPLC and immunoassay, however, interfering substances, false positives, and cross-reactivity to other compounds may compromise results. An efficient, fast, accurate, and sensitive SPE/MS/MS method with a wide calibration range was developed for the simultaneous quantitation of eight antidepressant drugs in human serum (Amitriptyline, Nortriptyline, Imipramine, Desipramine, Doxepin, Nordoxepin, Clomipramine, and Norclomipramine). This method uses protein precipitation followed by dilute and shoot on the SPE/MS/MS system, enabling analysis of all eight TCAs at 13 seconds per sample producing >10x savings in analysis time and solvent consumption compared to typical HPLC methods.

Introduction

Analysis of tricyclic antidepressants could be necessary in forensic cases such as driving under the influence of drugs, cases of violent crime, sexual assault cases, and unknown cause of death cases. Traditional quantitative measurement methods for antidepressant drugs analysis use HPLC, recently LC/MS/MS and other technologies.[1] The need for greater throughput and faster turn-around times has increased demands on these traditional technologies. The RapidFire High-throughput Mass Spectrometry system is an ultrafast SPE/MS/MS system capable of analyzing samples with cycle times under 13 seconds per sample. In this study, an ultrafast SPE/MS/MS method was developed for the simultaneous analysis in human serum of eight TCAs (Figure 1) (Amitriptyline, Nortriptyline, Imipramine, Desipramine, Doxepin, Nordoxepin, Clomipramine, and Norclomipramine) with much faster sample cycle times and similar analytical results compared to HPLC and LC/MS/MS assays.

A simple protein precipitation methodology followed by dilute and shoot analysis by RapidFire SPE/MS/MS allows for the accurate and precise measurement of these analytes in human serum over a linear range of 10 to 500 ng/mL. Samples were analyzed on the RapidFire SPE/MS/MS system at 13 seconds per sample providing a much higher-throughput method of analysis. This new ultrafast method has the speed and accuracy necessary for an efficient quantitative workflow.

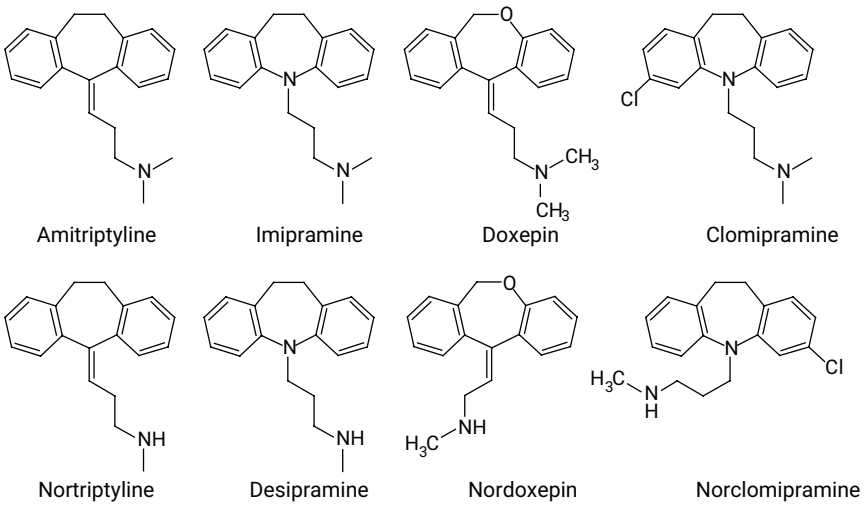

Figure 1. Chemical structures of the eight TCAs.

Experimental

RapidFire/MS/MS conditions

RapidFire Conditions	
Buffer A (Pump 1)	0.1% formic acid in LC/MS grade water, 1.5 mL/min flow rate
Buffer B and C (Pumps 2 and 3)	0.1% formic acid in LC/MS grade methanol, 1.25 and 0.8 mL/min flow rate respectively
Aqueous Wash	HPLC grade water
Organic Wash	HPLC grade acetonitrile
Injection Volume	10 µL
SPE Cartridge	RapidFire cartridge C (reversed-phase C18, G9205A)
RF State 1	600 ms
RF State 2	2,500 ms
RF State 3	0 ms
RF State 4	7,000 ms
RF State 5	800 ms
Triple Quadrupole Conditions	
Gas Temperature	300 °C
Gas Flow	10 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Nebulizer	45 psi
Nozzle Voltage	500 V
Capillary Voltage	3,500 V
Peak Width	0.07

RapidFire/triple quadrupole conditions

The Agilent RapidFire/MS/MS system consisted of the following modules:

- Agilent RapidFire 360
- Agilent 6460 Triple Quadrupole Mass Spectrometer using MassHunter Triple Quadrupole Acquisition Software with Qualitative Analysis, Quantitative Analysis, and RapidFire Acquisition software

Samples were analyzed at a rate of 13 seconds per sample. Quantitative and qualitative ions for all eight TCAs and internal standards were monitored simultaneously in all experiments (Table 1). Agilent MassHunter Quantitative Software automatically calculated qualifier ion ratios.

Chemicals and reagents

All of the analytes (TCAs) and all of the stable-labeled isotopic internal standards were purchased from Cerilliant, Round Rock, TX. The three levels of quality controls were obtained from Utak Laboratories, Valencia, CA. All other solvents and reagents were purchased from Fisher Scientific.

Sample preparation

The samples, calibrators (10, 100, 250, and 500 ng/mL), and QC levels (50, 200, and 400 ng/mL) were prepared using the following procedure. First, 150 µL of sample was added to a 1.5 mL micro centrifuge tube. Next, 150 µL of 0.2 M zinc sulfate was added and the sample was gently mixed.

Methanol containing the deuterated internal standard (200 ng/mL), 300 µL, was added next, followed by vigorous vortexing for 30 seconds. The samples were then centrifuged at 13,000 rpm for 10 minutes. A 100 µL portion of the supernatant from each tube was added into a corresponding well of a deep well plate containing 900 µL of LC/MS grade water. The plate was then sealed with an Agilent PlateLoc Thermal Microplate Sealer and mixed prior to RapidFire/MS/MS analysis.

Data analysis

System control and data acquisition were performed by MassHunter Triple Quadrupole Data Acquisition software.

Calibration curves were constructed using linear least squares regression with 1/X weighting for the multiple reactions monitoring (MRM). The quantitation using MassHunter Quantitative software was performed by chromatographic peak area ratio to a known concentration of the internal standards.

Table 1. MRM Transitions.

Compound Name	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy	CAV
Clomipramine-d3	318.2	89.1	5	110	13	4
Clomipramine Quant	315.2	86.1	10	80	13	4
Clomipramine Qual	315.2	58.1	10	80	49	4
Norclomipramine Quant	301.2	72.1	10	100	13	4
Norclomipramine Qual	301.2	44.1	10	100	49	4
Imipramine-d3	284.2	89.1	5	100	13	4
Doxepin-d3	283.2	107.1	5	115	21	4
Amitriptyline-d3	281.2	91.1	5	110	25	4
Imipramine Quant	281.2	86.1	10	75	13	4
Imipramine Qual	281.2	58.1	10	75	45	4
Doxepin Qual	280.2	115.0	10	115	50	4
Doxepin Quant	280.2	107.1	10	115	21	4
Amitriptyline Qual	278.2	117.1	10	115	21	4
Amitriptyline Quant	278.2	91.0	10	115	25	4
Desipramine Quant	267.2	72.1	10	90	13	4
Desipramine Qual	267.2	44.1	10	90	50	4
Nordoxepin Quant	266.2	235.1	10	100	13	4
Nordoxepin Qual	266.2	107.0	10	100	21	4
Nortriptyline Quant	264.2	233.2	10	100	13	4
Nortriptyline Qual	264.2	91.1	10	100	25	4

Results and discussion

Samples were prepared by spiking TCAs into drug-free human serum followed by a protein crash with zinc sulfate/methanol containing the internal standards and then diluting samples 10-fold with water. Samples were then analyzed through SPE/MS/MS using the RapidFire/MS/MS system and a reversed-phase C18 cartridge at 13 seconds per sample (Figure 2).

This RapidFire/MS/MS methodology is capable of throughputs greater than 260 samples per hour providing a high-throughput and very efficient mode of analysis. Carryover was assessed by analyzing the AUC of a matrix blank injection immediately following the highest calibrator and calculated as a % of the mean peak area of the lowest calibrator. No significant carryover (<20% of the 10 ng/mL calibrator or <1% of the 500 ng/mL calibrator) was determined for all of the AEDs (Figure 2). When measuring higher concentrations of TCAs (>500 ng/mL), using one blank injection between wells by injecting a strong organic solution (e.g. 50/25/25 methanol/IPA/ACN) is recommended.

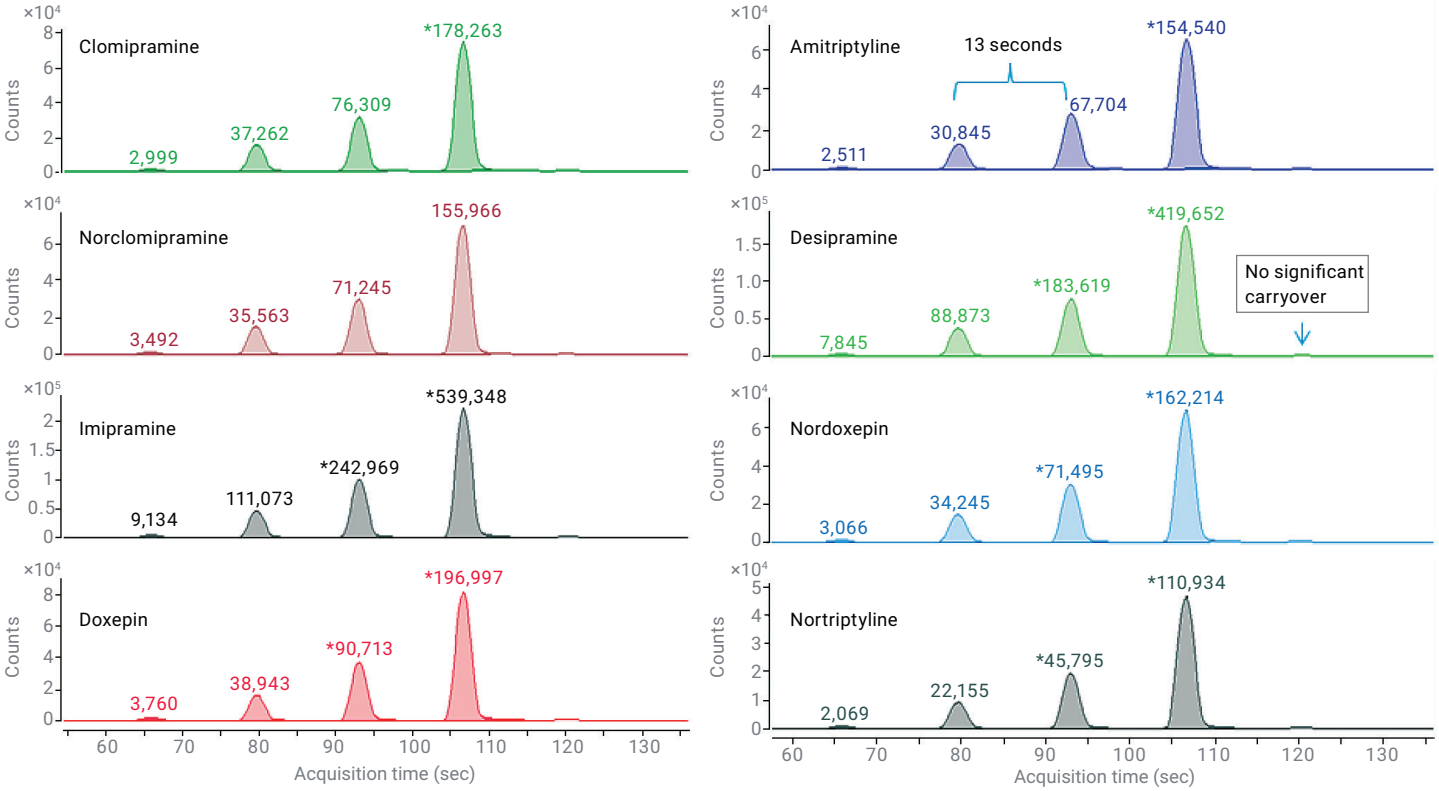

Figure 2. Representative calibration curve data for each of the eight TCA analytes showing the injection to injection interval of 13 seconds. Carryover assessment using a matrix blank immediately after the highest calibrator for all analytes shows no significant carryover was observed for any of the analytes.

Standard curves, consisting of each TCA spiked into serum, had excellent linearity within the measured range (10 to 500 ng/mL) with an R^2 value greater than 0.995 (Figure 3).

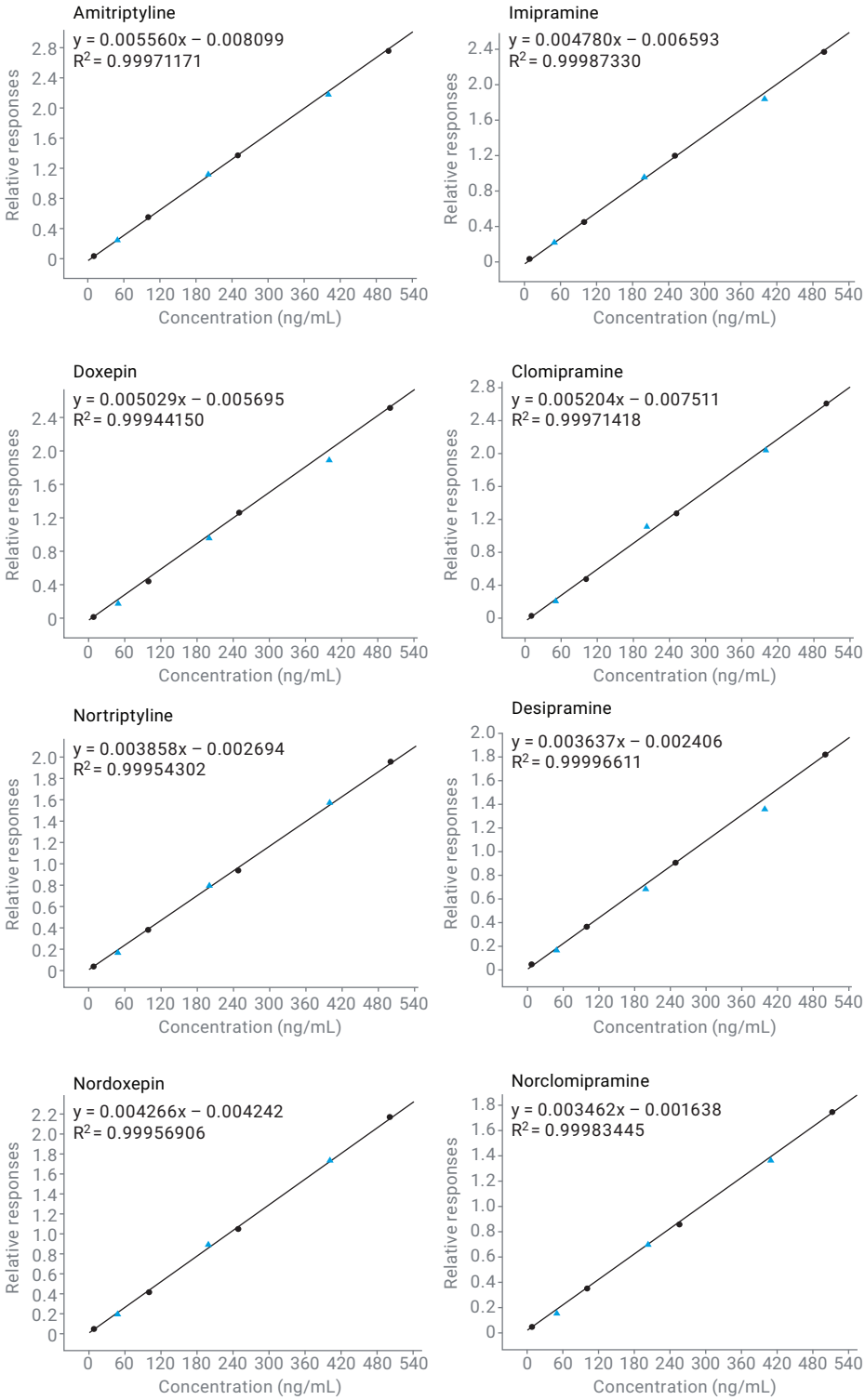

Figure 3. Representative calibration curves showing linear range 10 to 500 ng/mL for each of the eight TCA analytes. Dark circles are calibrators and blue triangles are QC standards.

QC standards for each TCA were run over a series of days to establish both intra- and interday precision and accuracy values. The accuracies determined were within 10% and coefficient of variation values were all less than 6% for concentrations within the measured range (Table 2).

Table 2. Interday and intraday accuracy and precision data for the QC standards.

Amitriptyline (ng/mL)	Interday % Accuracy (n = 6)	Interday % Precision (n = 6)	Intraday % Accuracy (n = 6)	Intraday % Precision (n = 6)
50	99.1	3.3	98.7	5.4
200	107.6	1.5	104.8	2.2
400	96.9	2.9	102.1	1.9
Nortriptyline (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	97.3	2.3	97.3	2.7
200	105.9	3.9	101.9	0.4
400	96.2	2.9	98.7	2.8
Imipramine (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	103.7	2.9	102.4	2.6
200	103.9	0.8	98.9	2.2
400	94.6	2.3	98.5	3.9
Desipramine (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	98.1	1.6	97.3	2.3
200	97.6	1.4	96.6	0.8
400	91.8	1.5	92.9	1.5
Doxepin (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	92.2	2.0	99.8	3.3
200	102.6	2.2	100.4	1.5
400	97.2	2.3	96.4	1.5
Nortdoxepin (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	97.4	4.8	99.4	4.9
200	102.9	2.9	103.1	1.7
400	97.6	1.3	101.7	1.4
Clomipramine (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	97.6	2.6	97.0	3.6
200	107.2	2.5	102.7	4.8
400	104.2	1.4	100.9	5.0
Norclomipramine (ng/mL)	**Interday % Accuracy (n = 6)**	**Interday % Precision (n = 6)**	**Intraday % Accuracy (n = 6)**	**Intraday % Precision (n = 6)**
50	91.5	1.5	97.8	4.0
200	98.1	1.6	99.9	2.4
400	97.5	1.7	97.3	3.5

The reproducibility of the method was evaluated by measuring >2,000 sequential injections of all eight TCAs spiked into serum. The instrument response was stable for each of the TCA analytes with coefficient of variation ranging from 2.2 to 4.2% showing the robustness of the RapidFire system, SPE cartridge lifetime and consistency of quantitation for the analytes in the panel. As an example, the data for Norclomipramine can be found in (Figure 4) where the precision over 2,000 injections was 3.1%.

This procedure, consisting of a protein crash followed by dilute and shoot sample preparation and quick analysis on RapidFire/MS/MS, provides a very efficient method of screening and quantitating tricyclic antidepressant drugs in human serum compared to traditional HPLC or LC/MS/MS methods.

Conclusion

A panel of eight tricyclic antidepressant drugs including Amitriptyline, Nortriptyline, Imipramine, Desipramine, Doxepin, Nordoxepin, Clomipramine, and Norclomipramine was quickly, accurately, and precisely measured in serum using a simple protein precipitation protocol and the Agilent RapidFire/MS system. Samples were analyzed in 13 seconds per sample, providing a high-throughput method capable of analyzing more than 260 samples per hour. This methodology provides comparable results to HPLC and LC/MS/MS, but at >10x the speed and efficiency of typical LC/MS/MS methods. Therefore, this method provides a very efficient mode for the forensic screening and quantitation of these eight TCAs in serum when compared to traditional analytical methods.

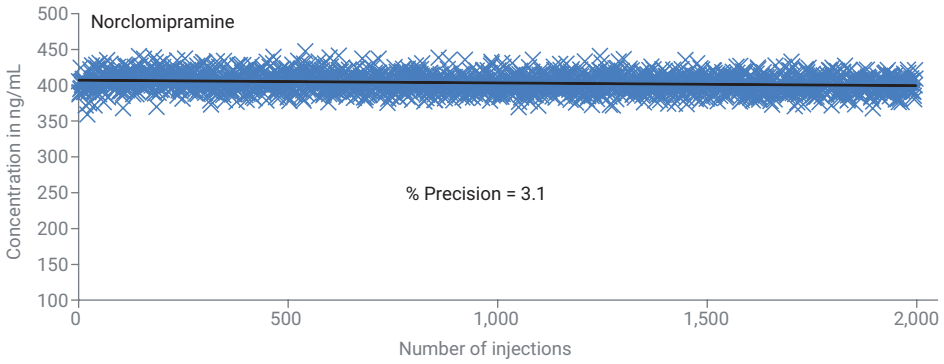

Figure 4. Reproducibility evaluation using sequential injections of the high quality control.

References

1. Titier, K. *et al.* Quantification of Tricyclic Antidepressants and Monoamine Oxidase Inhibitors by High-Performance Liquid Chromatography-Tandem Mass Spectrometry in Whole Blood. *Journal of Analytical Toxicology* **2007**, *31*, 200–207.

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