

# Ultrafast Analysis of Six Antiepileptic Drugs in Serum by the Agilent RapidFire High-Throughput Mass Spectrometry System

## Application Note

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### Abstract

Clinical research laboratories traditionally rely on HPLC and immunoassays, and more recently, LC/MS/MS for quantitative analysis of antiepileptic drugs (AEDs). An efficient, fast, accurate, and sensitive SPE/MS/MS method, with a wide calibration range, was developed for the simultaneous quantitation of six antiepileptic drugs in human serum [lamotrigine, zonisamide, gabapentin, pregabalin, MHD (oxcarbazepine metabolite), and levetiracetam]. This method employs protein precipitation followed by dilute and shoot on the SPE/MS/MS system, enabling analysis of all six AEDs in 14 seconds per sample. As a result, >10x savings in analysis time and solvent consumption was achieved compared to typical LC/MS/MS methods.

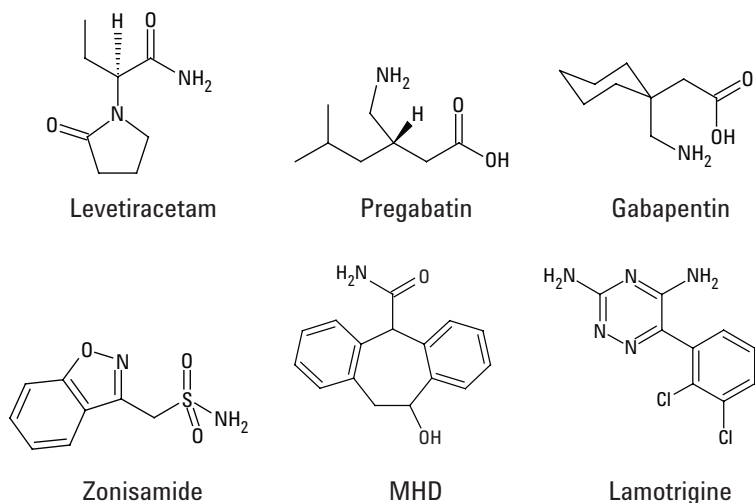


Figure 1. Chemical structures of the six AED analytes.



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## Introduction

Traditional measurement methods for clinical research in quantitative antiepileptic drug analysis use HPLC, LC/MS/MS, and other technologies.<sup>1</sup> 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 15 seconds per sample. In this application note, we developed an ultrafast SPE/MS/MS method for simultaneous analysis in human serum of six AEDs (Figure 1): MHD (oxcarbazepine metabolite), gabapentin, lamotrigine, levetiracetam, pregabalin, and zonisamide with much faster sample cycle times and similar analytical results compared to LC/MS/MS and HPLC 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 (1–100 µg/mL). Samples were analyzed on the RapidFire SPE/MS/MS system at 14 seconds per sample providing a much higher throughput method of analysis. This ultrafast method has the speed and accuracy necessary for an efficient quantitative workflow.

## Experimental

### 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 Agilent MassHunter Triple Quadrupole Acquisition Software (B.04.01) with Qualitative Analysis (B.04.00), Quantitative Analysis (B.04.00), and RapidFire Acquisition Software.

Samples were analyzed at a rate of 14 seconds per sample. Quantitative and qualitative ions for all six AEDs and internal standards were monitored

simultaneously in all experiments (Table 1). Agilent MassHunter Quantitative Software automatically calculated qualifier ion ratios.

Table 1. 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 (pump 2)	50 % LC/MS grade methanol:50 % LC/MS grade isopropanol; 1.25 mL/min flow rate					
Buffer C (pump 3)	50 % LC/MS grade methanol:50 % LC/MS grade isopropanol; 1.25 mL/min flow rate					
Aqueous wash	HPLC grade water					
Organic wash	HPLC acetonitrile					
Injection volume	10 µL					
SPE cartridge	RapidFire cartridge C (reversed-phase C18)					
RF state 1	600 ms					
RF state 2	3,000 ms					
RF state 3	0 ms					
RF state 4	6,000 ms					
RF state 5	1,500 ms					
Triple quadrupole conditions						
Gas temperature	350 °C					
Gas flow	8 L/min					
Sheath gas flow	9 L/min					
Capillary voltage	3,000 V					
Peak width	0.03					
Sheath gas temperature	400 °C					
Nebulizer	45 psi					
Nozzle voltage	500 V					
Compound	Q1	Q3	Dwell	Fragmentor	CE	CAV
Lamotrigine quant	256	211	15	145	26	2
Lamotrigine qual	256	108.9	15	145	57	2
MHD quant	255.1	194	15	85	17	2
MHD qual	255.1	165	15	85	57	2
Zonisamide quant	213	132	15	85	13	2
Zonisamide qual	213	51.1	15	85	61	2
Gabapentin-d10	182.2	164.1	15	110	9	2
Gabapentin quant	172.1	154.1	15	110	9	2
Gabapentin qual	172.1	137	15	110	13	2
Levetiracetam-d6	177.1	132	15	90	9	2
Levetiracetam quant	177.1	126	15	80	13	2
Levetiracetam qual	171.1	69	15	80	29	2
Pregabalin-d6	166.2	148.1	15	105	5	2
Pregabalin quant	160.1	55.1	15	105	13	2
Pregabalin qual	160.1	83	15	105	21	2

## Chemicals and reagents

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

## Sample preparation

The samples, calibrators (1, 6.25, 12.5, 25, 50, and 100 mcg/mL) and QC levels were prepared using the following procedure. First, 200  $\mu$ L of sample was added to a 1.5 mL micro centrifuge tube. Next, 50  $\mu$ L of methanol was added and the sample was gently mixed. Acetonitrile, 400  $\mu$ L, was added next, followed by vigorous vortexing for 10 seconds. The samples were

then centrifuged at 13,500 rpm for 5 minutes. A portion of the supernatant from each tube (20  $\mu$ L) was added into a corresponding well of a deep well plate containing 980  $\mu$ L of LC/MS grade water and deuterated internal standards (200 ng/mL). 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 spectral peak area ratio to a known concentration of the internal standards.

## Results and Discussion

Samples were prepared by spiking AEDs into drug-free human serum followed by a protein crash with methanol and acetonitrile and then diluting samples 50-fold with water containing internal standards. Samples were then analyzed through SPE/MS/MS using the RapidFire/MS/MS system and a reversed-phase C18 cartridge at 14 seconds per sample (Figure 2). This RapidFire/MS/MS methodology is capable of throughputs greater than 250 samples per hour providing a high-throughput and very efficient mode of analysis. Carryover was assessed by analyzing the AUC of the blank calculated as a % of the mean peak area of the 1 mcg/mL samples. No significant carryover (0 %) was determined for all of the AEDs (Figure 2). When measuring higher concentrations of AEDs (>100 mcg/mL), we recommend using one blank injection between wells by injecting a strong organic solution (50:50) (Methanol:IPA).

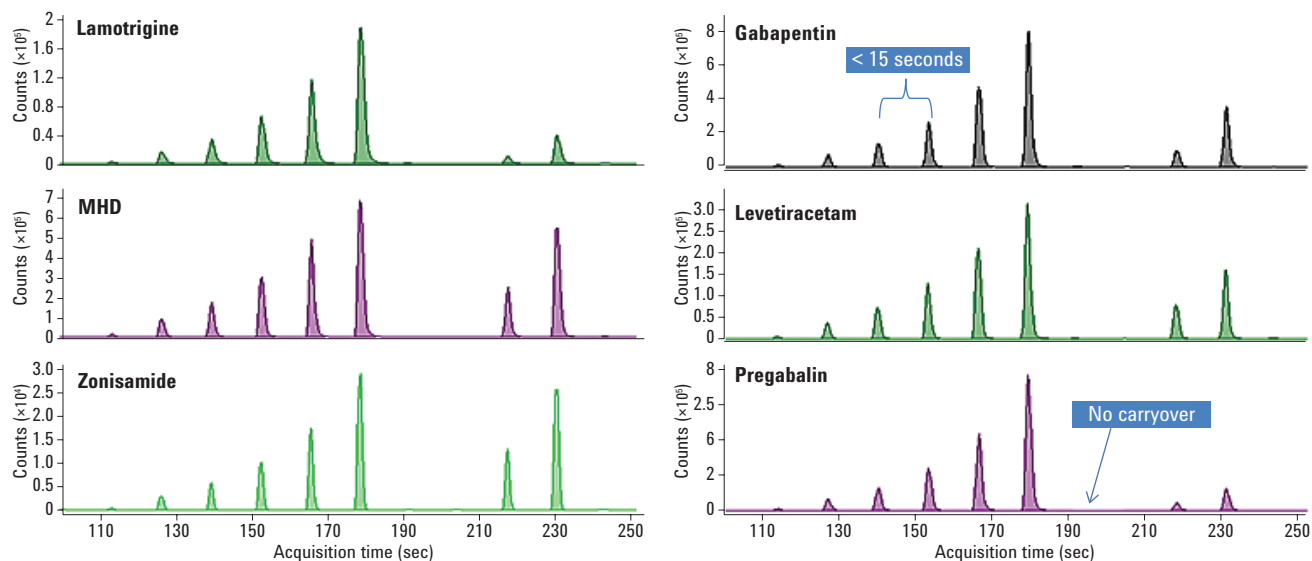


Figure 2. Representative calibration curve data for each of the six AED analytes showing the injection to injection interval of 14 seconds. Carryover assessment using a matrix blank immediately after the highest calibrator for all analytes shows no significant carryover was observed for any of the analytes.

Standard curves consisting of each AED spiked into serum had excellent linearity within the measured range (1–100 mcg/mL) with an R<sup>2</sup> value greater than 0.995 (Figure 3).

QC standards for each AED were run over a series of days to establish both intra and interday precision and accuracy values. The accuracies determined were within 7 % and coefficient of variation values were all less than 7 % for concentrations within the measured range (Table 2).

Table 2. Intraday and interday accuracy and precision data for the QC standards.

	Interday % Accuracy (n=6)	Interday % Precision (n=6)	Intraday % Accuracy (n=6)	Intraday % Precision (n=6)
<b>Lamotrigine (µg/mL)</b>				
4.5	95.1	2.7	99.3	3.1
20	102.6	1.9	101.9	3.5
<b>Gabapentin (µg/mL)</b>				
10	95.6	0.6	95.2	2.9
40	100.0	0.5	96.6	2.9
<b>Pregabalin (µg/mL)</b>				
5	92.9	1.2	93.6	4.6
15	94.0	1.1	93.8	2.3
<b>MHD (µg/mL)</b>				
21	105.7	2.1	100.7	2.3
65	105.1	1.8	102.6	2.1
<b>Levetiracetam (µg/mL)</b>				
16	105.9	1.3	105.9	4.3
44	105.1	1.0	103.6	1.3
<b>Zonisamide (µg/mL)</b>				
32	105.5	2.9	100.9	3.8
70	103.8	6.0	100.3	3.1

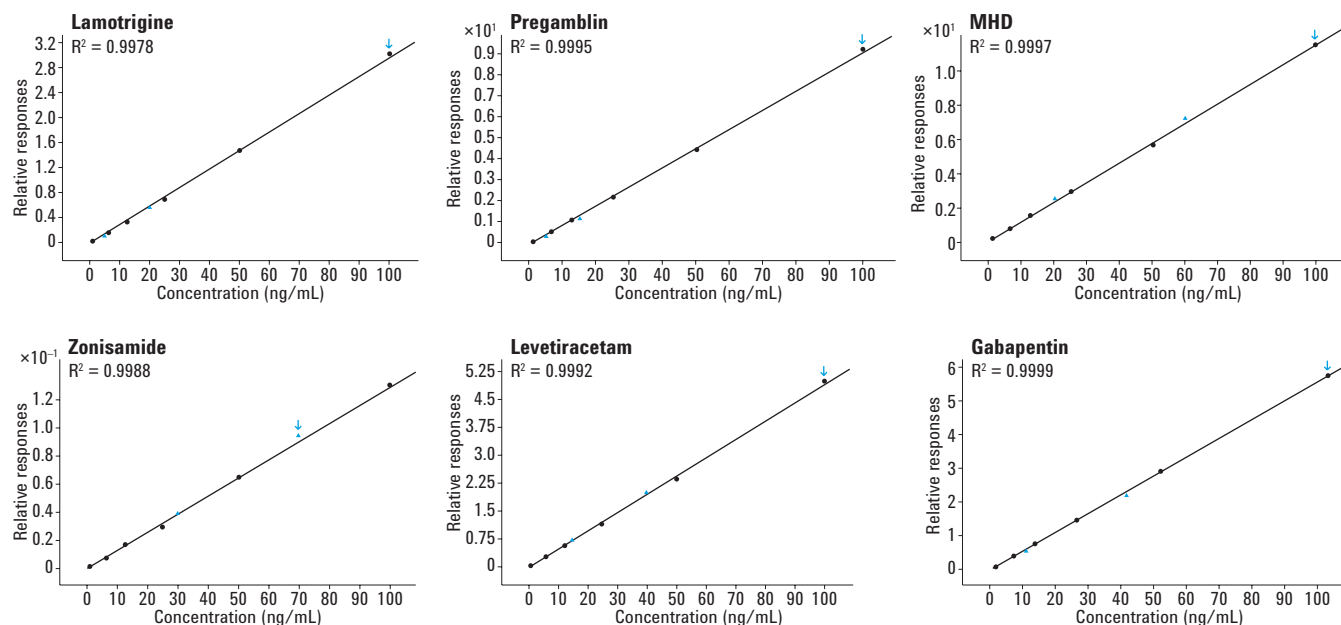


Figure 3. Representative calibration curves showing linear range from 1–100 mcg/mL for each of the six AED analytes. Dark circles are calibrators and blue triangles are QC standards.

The reproducibility of the method was evaluated by measuring >2,000 sequential injections of all six AEDs spiked into serum. The instrument response was stable for each of the six analytes with coefficient of variation ranging from 1.6–7 % 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 Levetiracetam can be found in Figure 4 where the precision over >2,000 injections was 1.63 %.

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 antiepileptic drugs in human serum compared to traditional HPLC or LC/MS/MS methods.

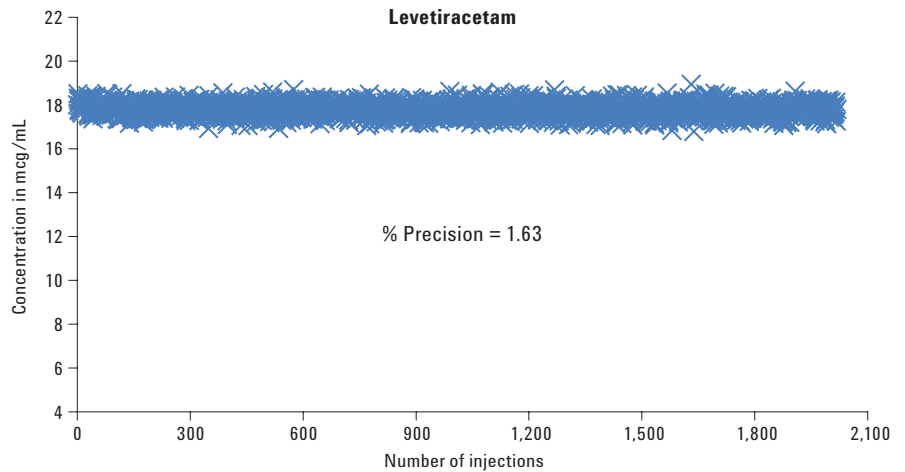


Figure 4. Repeatability evaluation using sequential injections of Levetiracetam.

## Conclusions

A panel of six antiepileptic drugs including Lamotrigine, Zonisamide, Gabapentin, Pregabalin, MHD (Oxcarbazepine metabolite), and Levetiracetam was quickly, accurately, and precisely measured in serum using a simple protein precipitation protocol and the Agilent RapidFire/MS system. Samples were analyzed at 14 seconds per sample, providing a high-throughput method capable of analyzing more than 250 samples per hour. This methodology provides comparable results to 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 screening and quantitating these six AEDs in serum compared to traditional analytical methods.

## Reference

1. Subramanian, M. Birnbaum, A. K., and Rimmel, R. P. High-Speed Simultaneous Determination of Nine Antiepileptic Drugs Using Liquid Chromatography-Mass Spectrometry. *Ther. Drug Monit.*, **2008**, 30(3):347-356.

[www.agilent.com/lifesciences/rapidfire](http://www.agilent.com/lifesciences/rapidfire)

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