Simultaneous LC/MS/MS Quantitation of 20 Antiepileptic Drugs in Human Serum

Application Note

Clinical Research

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Abstract

Monitoring antiepileptic drugs can be challenging due to the large size of this drug class as well as the disparate concentrations at which those drugs may be present in human serum. Therefore, a quality assay must be able to monitor many compounds simultaneously, over several orders of magnitude. Liquid chromatography-mass spectrometry (LC/MS/MS) is particularly well suited to this type of analysis. A highly sensitive and specific method was developed for the quantitation of 20 antiepileptic drugs in human serum. Samples were prepared through a simple protein precipitation/dilution protocol. Analytes could be quantified over a wide dynamic range, and accuracy and reproducibility metrics as well as R^2 values were excellent.

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Introduction

A major strength of liquid chromatography-mass spectrometry (LC/MS/MS) as a detection method is that it allows the concurrent monitoring of multiple analytes in a single injection. This study used an LC/MS/MS analytical method to quantify a panel of 20 antiepileptic drugs in human serum. Conversely, historic assays have traditionally monitored a smaller number of compounds due to large concentration differences between similar analytes. Compounds included in the panel were: acetylretigabine, carbamazepine-10,11-epoxide, carbamazepine, 10,11-dihydro-10-hydroxy-carbamazepine, felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, phenobarbital, phenytoin, pregabalin, retigabine, rufinamide, tiagabine, topiramate, valproic acid, vigabatrin, and zonisamide. The analytical method used the Agilent 6460 Triple Quadrupole Mass Spectrometer to detect compounds over a wide range of concentrations simultaneously. The calibration concentrations ranged from 12 ng/mL to 200,000 ng/mL for the various analytes. Top concentrations ranged from 1.5 to 200 µg/mL.

Samples were created by spiking drug standards into clean human serum. Samples and controls were prepared for analysis through a simple protein precipitation protocol followed by dilution in water. Injection, separation of analytes, column cleaning, and column re-equilibration were accomplished in less than 10 minutes. Two transitions were monitored for each of the 20 compounds of interest, including valproic acid. Fifteen isotopically labeled internal standards were included to account for differential suppression, and ensure accurate and reproducible quantitation across the chromatogram. A transition for phospholipids was also monitored to verify minimal interference from this class of endogenous molecules.

Calibration curve accuracies were within 20 % of the expected concentration at the lowest calibration level, and well within 15 % at all higher levels. Reproducibility was good, with all CVs <15 %, and most well under 10 %. All R^2 values were >0.997, with some compounds displaying a linear response across their concentration range, and others requiring a quadratic fit.

Experimental

Chemicals and reagents

Human serum, used for matrix-matched calibrators, was from UTAK Laboratories (Valencia, CA). Standards and internal standards were bought from Sigma-Aldrich (St. Louis, MO) and Cerilliant Corporation (Round Rock, TX). Sample preparation and LC solvents were from Sigma-Aldrich (St. Louis, MO) and Honeywell Riedel-de Haën (Seelze, Germany).

Sample preparation

To achieve the top concentration sample, clean human serum was spiked with standards of the 20 drugs. Seven lower concentration samples were created by serial 1:2 dilution into clean serum. Forty microliters of sample or control were mixed with 40 µL of ISTD solution in methanol, and 120 µL of pure methanol. After being vortexed for 30 seconds, samples were spun for 10 minutes at 10,000 rpm. Then, 50 µL of supernatant were added to 450 µL of water, and 4 µL were injected onto the LC/MS system.

Data analysis

System control and data acquisition were performed by Agilent MassHunter Acquisition Software (B.08.00). MS/MS transitions were obtained using MassHunter Acquisition Optimizer software to determine optimal parent and fragment ions, fragmentor voltages, and collision energies upon injection of a neat solution of each individual compound or internal standard. Data were analyzed using Agilent MassHunter Quantitative Analysis Software(B.08.00) and Qualitative Analysis Software (B.07.00).

LC Configuration and parameters

Configuration		
Agilent 1290 Infinity II high speed pump (G7120A)		
Agilent 1290 Infinity autosampler (G4226A)		
Agilent 1290 Infinity autosampler thermostat (G1330B)		
Agilent 1290 Infinity II multicolumn thermostat (G7116B)		
Needle wash	50:50 Isopropanol:methanol	
Autosampler temperature	4 °C	
Injection volume	4 µL	
Guard column	Agilent Poroshell 120 EC-C18, 2.1 × 5 mm, 2.7 µm, guard column (p/n 821725-911)	
Analytical column	Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm, LC column (p/n 695775-902)	
Column temperature	50 °C	
Mobile phase A	2 mM Ammonium acetate in water	
Mobile phase B	2 mM Ammonium acetate in methanol	
Flow rate	0.4 mL/min	
Gradient	Time (min)	%B
	0.0	10
	1.0	10
	5.0	50
	6.2	60
	6.3	95
Stop time	7.5 minutes	
Post time	1.5 minutes	

MS Configuration and parameters

Configuration	
Agilent 6460 Triple Quadrupole Mass Spectrometer with Agilent Jet Stream	
MS/MS mode	Dynamic MRM
Ion mode	Positive and Negative
Drying gas temperature	350 °C
Drying gas flow	12 L/min
Nebulizer pressure	50 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Nozzle voltage	0 V
Capillary voltage, positive	3,500 V
Capillary voltage, negative	2,500 V
Delta EMV, positive	100 V
Delta EMV, negative	800 V
Q1/Q2 resolution	0.7/0.7 Unit
Dwell time	Variable

MS/MS Compound information for analytes and internal standards

Compound	ISTD?	Precursor ion	Product ion 1	Product ion 2	RT (min)	Delta RT (min)	Fragmentor (V)	Collision energy (V)	CAV	Polarity
10,11-Dihydro-10-hydroxycarbamazepine		255.1	237.0	194.0	5.64	0.92	80	8/20	4	+
Carbamazepine		237.1	194.1	193.3	6.74	0.90	146	16/36	5	+
Carbamazepine D10	✓	247.2	204.1		6.68	0.92	152	20	4	+
Carbamazepine 10,11 epoxide		253.1	210.0	180.1	5.77	0.82	94	12/28	5	+
Carbamazepine 10,11 epoxide $^{13}C_6$	✓	259.1	186.1		5.77	0.78	97	32	4	+
Felbamate		178.1	117.1	91.1	4.72	0.90	71	15/25	5	+
Gabapentin		172.1	154.1	137.1	2.30	1.08	106	12/16	2	+
Gabapentin D10	✓	182.2	164.1		2.22	1.10	91	12	4	+
Lacosamide		251.1	108.0	91.1	4.53	1.02	80	4/20	4	+
Lacosamide ^{13}C D3	✓	255.3	108.0		4.51	0.92	88	4	4	+
Lamotrigine		256.0	210.9	43.0	5.12	1.06	154	28/40	3	+
Lamotrigine ^{13}C $^{15}N_4$	✓	261.0	46.0		5.12	1.02	157	48	4	+
Levetiracetam		171.1	154.0	126.0	2.90	0.94	71	4/12	3	+
Levetiracetam D6	✓	177.1	132.1		2.86	1.06	71	16	4	+
N-Acetylretigabine		274.1	256.1	109.0	6.80	1.20	120	12/36	4	+
Oxcarbazepine		253.1	208.0	180.0	6.03	0.82	120	16/32	3	+
Oxcarbazepine $^{13}C_6$	✓	259.1	214.0		6.03	0.70	120	16	4	+
Phenobarbital		231.1	188.1	42.0	5.17	0.86	91	8/24	2	–
Phenobarbital D5	✓	236.1	42.0		5.15	0.74	91	16	4	–
Phenytoin		251.1	208.0	102.0	6.37	0.92	105	12/20	4	–
Phenytoin D10	✓	261.2	105.9		6.32	0.68	105	20	4	–
Phospholipids		184.0	184.0		4.00	8.00	250	0	3	+
Pregabalin		160.1	142.1	55.1	2.09	1.14	89	8/24	2	+
Pregabalin D6	✓	166.2	148.1		2.04	1.02	88	8	4	+
Retigabine		304.2	230.0	109.0	6.84	0.68	123	16/36	4	+
Retigabine D4		308.2	113.0		6.80	0.86	126	36	4	+
Rufinamide		239.1	127.0	101.0	4.70	1.02	100	20/50	3	+
Tiagabine		376.1	247.0	111.0	7.17	0.72	143	16/32	3	+
Tiagabine D6	✓	382.2	253.1		7.16	0.72	149	16	4	+
Topiramate		338.1	96.0	78.0	5.51	1.02	150	20/20	4	–
Topiramate D12	✓	350.1	78.0		5.46	0.90	150	20	4	–
Valproic acid		225.1/143	143.0		5.30	0.92	95	11/15	3	–
Valproic acid D6	✓	231.0	149.0		5.30	0.41	95	11	4	–
Vigabatrin		130.1	113.0	71.1	0.64	0.84	71	8/16	2	+
Zonisamide		211.0	147.0	118.9	3.80	0.80	86	8/12	4	–
Zonisamide $^{13}C_6$	✓	217.1	125.0		3.79	0.90	83	12	4	–

Results and Discussion

Linearity

The method used the abilities of LC/MS/MS to simultaneously detect multiple compounds spanning a wide range of concentrations (Figure 1). The calibration concentrations ranged from 12 ng/mL to 200,000 ng/mL for the various analytes. Top concentrations ranged from 1.5 to 200 µg/mL, and are given, along with curve fit parameters, in Table 1. All R^2 values were >0.997, with some compounds displaying a linear response across their concentration range, and others requiring a quadratic fit (Figure 2).

Table 1. Top calibration concentrations and curve fit parameters.

Compound	Top concentration (µg/mL)	Curve fit	Average R^2 (n = 3)
10,11-dihydro-10-hydroxycarbamazepine	40	Quadratic	0.9998
Carbamazepine	50	Quadratic	0.9980
Carbamazepine 10,11 epoxide	25	Quadratic	0.9998
Felbamate	80	Linear	0.9987
Gabapentin	30	Quadratic	0.9989
Lacosamide	20	Quadratic	0.9995
Lamotrigine	20	Quadratic	0.9992
Levetiracetam	100	Quadratic	0.9987
N-Acetylretigabine	3.5	Quadratic	0.9994
Oxcarbazepine	5	Quadratic	0.9987
Phenobarbital	40	Quadratic	0.9988
Phenytoin	40	Quadratic	0.9990
Pregabalin	20	Quadratic	0.9996
Retigabine	5	Quadratic	0.9998
Rufinamide	40	Quadratic	0.9995
Tiagabine	1.5	Quadratic	0.9973
Topiramate	30	Quadratic	0.9994
Valproic acid	200	Linear	0.9974
Vigabatrin	180	Quadratic	0.9996
Zonisamide	40	Linear	0.9996

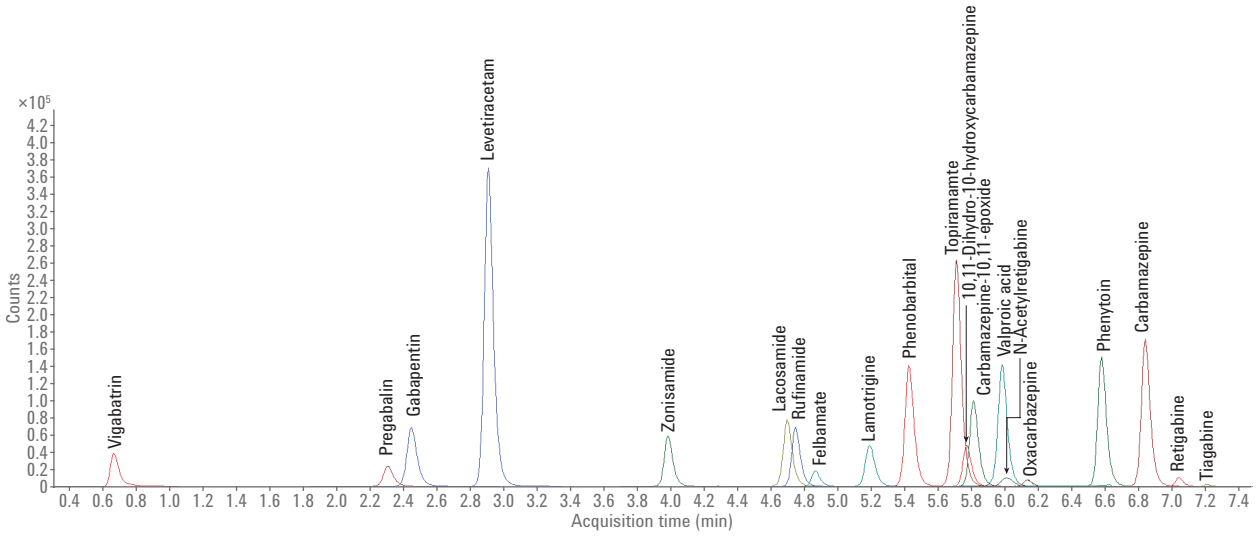

Figure 1. Example dMRM chromatogram showing elution of the 20 compounds.

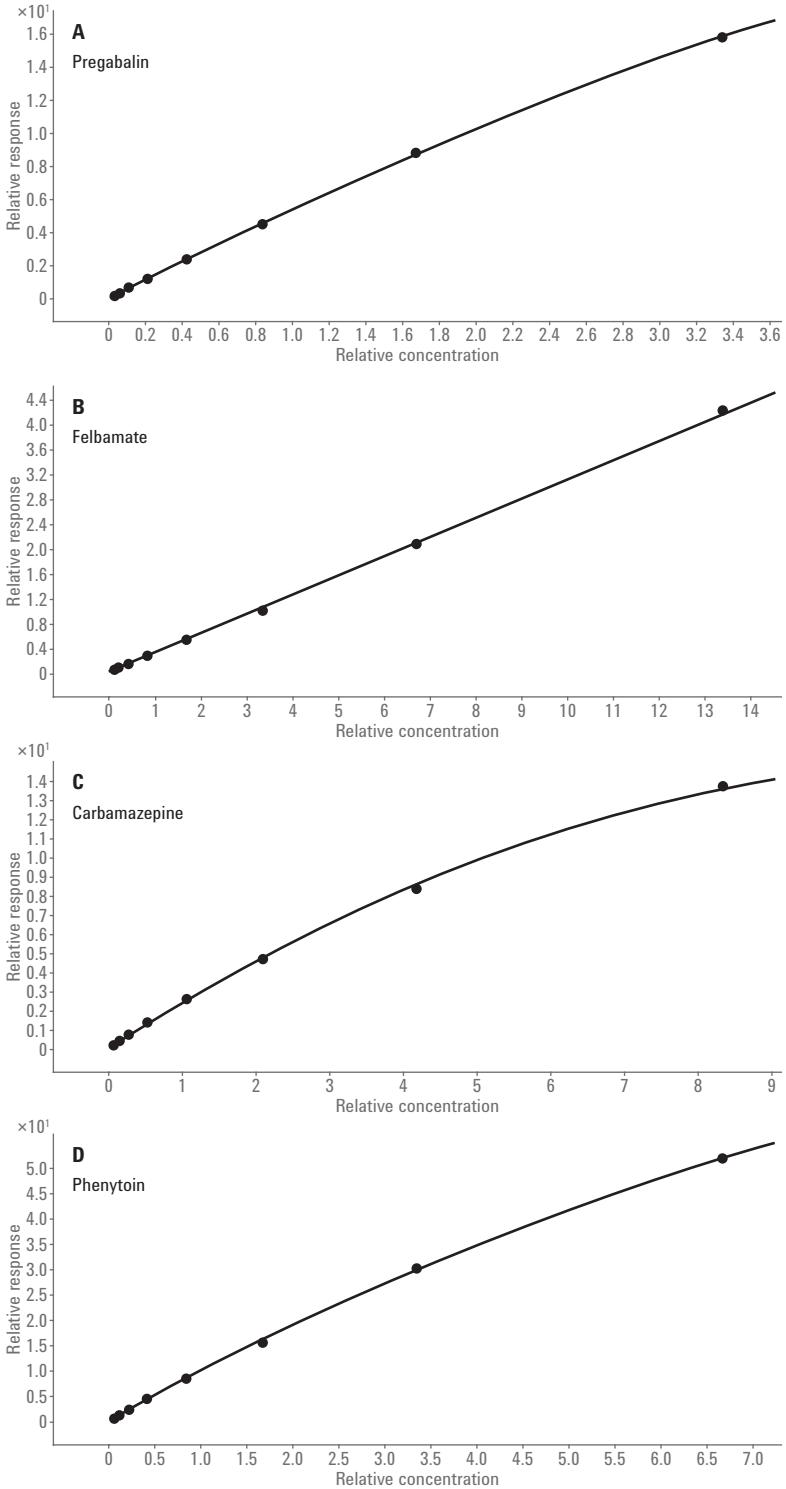

Figure 2. Example calibration curves for compounds dispersed throughout the chromatogram and of both polarities. All calibration curves employed a 1/x weighting factor.

Accuracy and reproducibility

Calibration curve accuracies were within 20 % of the expected concentration at the lowest level, and well within 15 % at all higher levels. Reproducibility was good, with all CVs <15 %, and most well under 10 %. Table 2 gives the values for three replicate curves run on the same day, and Table 3 presents accuracy average and CV values for three replicate curves run on three consecutive days.

Table 2. Accuracy and reproducibility for curves analyzed on the same day (n = 3).

	10,11-Dihydro-10-hydroxycarbamazepine		Carbamazepine		Carbamazepine 10,11 epoxide		Felbamate		Gabapentin		Lacosamide		Lamotrigine	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	95.4	3.8	83.5	2.0	96.1	1.9	92.7	7.2	88.1	4.4	92.3	3.0	97.1	4.8
2	99.2	0.1	100.8	2.7	99.3	2.9	100.0	4.6	98.5	2.8	98.0	2.1	97.0	3.1
3	102.6	1.5	108.4	1.3	101.8	1.0	101.1	1.2	106.9	1.4	105.1	0.8	101.9	0.1
4	102.0	3.0	107.8	2.0	102.1	0.8	101.6	3.6	105.4	1.1	103.9	1.1	101.8	1.3
5	102.5	2.0	104.6	1.6	102.1	1.4	103.4	5.0	104.7	2.1	103.0	2.1	105.0	7.1
6	99.2	0.3	97.2	2.2	99.6	0.7	102.0	4.0	99.0	1.1	99.6	0.9	97.9	0.7
7	98.8	1.1	92.7	3.1	98.7	1.2	100.2	3.9	96.0	0.8	97.4	0.2	99.0	2.7
8	100.3	0.3	103.0	2.0	100.3	0.2	98.9	3.2	101.6	0.4	100.8	0.1	100.3	0.7

	Levetiracetam		N-Acetyl-retigabine		Oxcarbazepine		Phenobarbital		Phenytoin		Pregabalin		Retigabine	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	87.1	2.2	97.2	6.0	100.7	6.4	101.1	2.2	100.3	2.5	94.2	3.7	95.7	2.1
2	98.5	1.9	103.5	7.3	92.6	2.0	93.8	1.3	96.7	2.6	97.9	1.4	100.4	4.3
3	106.3	0.7	98.3	3.0	102.2	4.9	101.7	5.4	101.1	0.7	103.3	2.8	102.1	2.2
4	107.1	0.8	99.6	6.8	101.3	4.5	101.9	4.9	100.1	2.1	103.5	1.6	101.8	3.1
5	104.9	0.7	102.8	0.1	105.2	4.9	102.9	3.3	104.8	3.3	103.5	1.2	100.5	1.4
6	99.3	1.3	98.6	4.4	99.5	4.4	99.8	4.3	96.3	4.1	99.1	1.5	100.4	1.0
7	95.4	1.6	100.1	2.2	98.2	5.1	98.3	5.7	100.9	4.2	97.8	0.6	98.8	1.3
8	101.8	0.3	100.0	0.3	100.3	1.0	100.5	1.5	99.8	1.5	100.7	0.2	100.3	0.4

	Rufinamide		Tiagabine		Topiramate		Valproic acid		Vigabatrin		Zonisamide	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	91.9	2.7	91.3	11.7	98.4	3.5	89.0	10.2	89.6	3.6	105.1	6.7
2	99.5	1.7	98.6	13.8	96.6	2.3	97.2	3.4	101.6	1.6	96.0	2.2
3	104.5	2.1	104.2	10.0	101.1	2.3	100.5	3.4	104.4	2.2	96.2	3.4
4	103.1	0.6	109.1	9.3	102.3	3.4	101.8	7.6	104.1	2.5	99.7	1.2
5	103.0	2.8	100.1	2.4	101.9	4.6	107.2	3.5	102.3	1.2	101.5	2.8
6	99.8	1.0	96.2	7.2	101.9	0.7	104.7	2.7	99.6	0.8	101.4	2.1
7	97.4	0.5	100.6	6.7	97.1	1.4	103.2	3.0	97.8	1.1	100.8	1.7
8	100.8	0.2	100.0	1.4	100.7	0.2	96.3	2.6	100.6	0.3	99.2	1.1

Table 3 Accuracy and reproducibility for curves analyzed on consecutive days (n = 3).

	10,11-Dihydro-10-hydroxycarbamazepine		Carbamazepine		Carbamazepine 10,11 epoxide		Felbamate		Gabapentin		Lacosamide		Lamotrigine	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	94.4	5.6	81.8	1.9	92.7	6.2	92.9	6.1	87.5	5.1	91.9	4.0	92.8	8.2
2	100.2	2.1	100.0	3.0	99.1	2.6	101.3	4.3	100.5	5.3	99.0	3.2	98.7	5.4
3	104.6	2.8	109.7	0.7	104.8	1.7	100.8	1.8	106.4	1.0	105.3	1.1	102.9	0.7
4	100.4	2.3	108.0	2.4	102.7	3.1	103.2	3.2	103.8	2.8	103.1	1.5	102.4	2.4
5	101.7	2.2	105.8	2.3	102.8	1.4	104.8	4.0	105.7	2.4	103.8	1.8	107.4	7.5
6	99.4	0.4	98.2	0.5	99.0	1.3	98.0	2.7	98.4	1.2	98.0	1.4	97.4	0.9
7	99.2	1.7	94.6	2.0	98.5	2.1	98.6	0.9	96.4	2.6	98.4	1.8	97.9	3.8
8	100.2	0.4	103.4	1.4	100.4	0.5	100.4	1.1	101.6	0.9	100.6	0.5	100.7	1.0

	Levetiracetam		N-Acetyl-retigabine		Oxcarbazepine		Phenobarbital		Phenytoin		Pregabalin		Retigabine	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	84.1	4.4	98.2	6.9	97.5	8.7	97.1	6.0	95.7	5.9	93.0	5.5	94.4	2.7
2	101.4	3.3	101.3	3.6	97.6	3.2	99.6	5.0	96.3	2.7	100.0	2.8	100.6	4.1
3	106.6	0.7	98.0	3.0	105.0	10.0	103.2	7.8	101.3	1.8	103.9	3.1	100.9	3.2
4	107.0	2.7	105.0	2.8	97.6	5.5	98.6	5.3	106.6	5.8	101.4	2.7	103.6	4.3
5	104.6	1.9	99.3	3.1	104.1	10.4	103.4	2.2	104.4	3.3	103.8	2.5	102.8	1.5
6	99.6	0.8	96.4	2.6	98.3	5.3	98.2	2.5	96.2	1.0	99.6	1.2	98.5	1.6
7	95.2	2.4	102.1	1.8	99.7	5.9	99.7	3.6	99.2	1.9	97.7	2.6	99.0	1.7
8	101.7	0.8	99.7	0.4	100.1	1.1	100.3	1.0	100.7	0.8	100.7	0.7	100.3	0.4

	Rufinamide		Tiagabine		Topiramate		Valproic acid		Vigabatrin		Zonisamide	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	88.7	5.5	93.0	13.6	100.4	5.9	91.6	14.6	89.0	2.9	101.3	8.1
2	100.7	5.4	107.1	5.5	98.6	0.9	94.6	5.1	101.7	1.2	101.0	3.7
3	104.5	2.2	97.3	5.8	101.0	2.1	99.9	2.4	104.4	2.3	98.8	1.3
4	104.3	2.0	105.9	5.4	99.1	5.9	104.4	4.7	104.3	2.7	96.9	2.4
5	105.9	2.2	98.9	5.4	100.8	5.0	106.1	6.9	102.9	0.6	98.8	4.9
6	97.5	1.6	95.4	5.5	100.7	0.9	102.6	1.6	99.4	0.5	101.9	1.6
7	97.6	1.9	102.9	3.8	99.3	4.0	104.7	2.5	97.7	1.1	102.6	2.1
8	100.9	0.5	99.5	0.9	100.2	1.0	96.1	0.9	100.6	0.3	98.5	0.4

Conclusion

An accurate, reproducible, and robust LC/MS/MS analytical method has been developed to simultaneously quantitate 20 antiepileptic drugs in human serum. Further work is needed to better understand matrix interferences that could impact the quantitation of any drugs in the panel. Additionally, different sources of mobile phase components and samples from an alternate source will be analyzed.

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