Application Note

Clinical Research

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Analysis of anti-epileptic drugs in human serum using an Agilent Ultivo LC/TQ

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Abstract

Analyzing anti-epileptic drugs can be challenging in clinical research due to the disparate concentrations at which these drugs may be present in human serum. Therefore, a quality assay must be able to analyze many compounds simultaneously, over several orders of magnitude. A highly sensitive and specific analytical method for the quantitation of 15 anti-epileptic drugs in human serum was tested on the Agilent Ultivo triple quadrupole LC/MS (LC/TQ). Samples were prepared through a simple protein precipitation/dilution protocol. Analytes could be quantified over a wide dynamic range; accuracy and reproducibility metrics, as well as R^2 values, were acceptable.

Introduction

Liquid chromatography-tandem mass spectrometry (LC/MS/MS) has long demonstrated its value in analytical research laboratories due to its analytical specificity and sensitivity when analyzing multiple compounds in a single injection. Despite this convenience, many large clinical research laboratories find physical instrumentation space to be at a premium, sometimes driving the need for creative solutions. Minimizing the instrument footprint while maintaining the power of a traditionally sized mass spectrometer may provide an answer for such laboratories. In this context, the analytical capabilities of a miniature Agilent Ultivo LC/TQ was evaluated using a panel of anti-epileptic drugs in human serum. This panel was compared to an identical sample set analyzed on a larger Agilent 6470 LC/TQ MS. Compounds comprising the analytical panel were acetylretigabine, carbamazepine-10,11-epoxide, carbamazepine, 10,11-dihydro-10-hydroxy-carbamazepine, felbamate, gabapentin, lacosamide, lamotrigine, levetiracetam, oxcarbazepine, pregabalin, retigabine, rufinamide, tiagabine, and vigabatrin. The analytical method tested the ability of the instruments to detect compounds over numerous concentration ranges simultaneously, at calibration concentrations ranging from 0.59 ng/mL to 20,000 ng/mL for individual analytes. Top concentrations could range between 0.15 and 20 µg/mL.

Samples were created by spiking drug standards into clean human serum. To prepare samples and controls for analysis, a simple protein precipitation protocol was performed, followed by dilution into water to reduce the sample organic content. The injection-to-injection cycle time was less than 10 minutes, and two transitions were monitored for each of the 15 target compounds. A transition meant to detect phospholipids was also included in the analytical method to verify any minimal interference or suppression from these endogenous molecules.

Calibration curve accuracies were within 20 % of the expected concentration at the lowest calibration level, and reproducibility across all levels was acceptable, with CVs less than 15 %. R^2 values were all greater than 0.994, and two-thirds of the compounds displayed linear responses throughout the concentration range, while the remaining one-third required quadratic fits.

Experimental

LC configuration and parameters

Table 1. UHPLC configuration and settings.

Parameter	Value
Instruments	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	100 % 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 7.5 95 7.51 10
Stop time	8 minutes
Post time	1 minute

Triple quadrupole mass spectrometer configuration and parameters

Table 2. Mass spectrometer instrument configuration and source settings.

Parameter	Value
Instrument	Agilent Ultivo Triple Quadrupole Mass Spectrometer
MS/MS mode	Dynamic MRM
Ion mode	Positive
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
Delta EMV, positive	0 V
MS1/MS2 resolution	0.7/0.7 Unit
Dwell time	Variable

MS/MS compound information for analytes and internal standards

Table 3. Detailed MRM settings in dynamic MRM Mode.

Compound	ISTD	Precursor ion (*m/z*)	Product ion (*m/z*)	Retention time (min)	Retention window (min)	Fragmentor (V)	CAV (V)	Collision energy (V)	Polarity
10,11-Dihydro-10-hydroxycarbamazepine		255.1	237.0	5.70	1.10	80	9	4	+
10,11-Dihydro-10-hydroxycarbamazepine		255.1	194.0	5.70	1.10	80	9	16	+
Carbamazepine		237.1	194.1	6.85	1.12	146	9	12	+
Carbamazepine		237.1	193.3	6.85	1.12	146	9	32	+
Carbamazepine D10	✓	247.2	204.1	6.85	1.00	152	9	14	+
Carbamazepine 10,11 epoxide		253.1	210.0	5.84	0.87	94	9	8	+
Carbamazepine 10,11 epoxide		253.1	180.1	5.84	0.87	94	9	24	+
Carbamazepine 10,11 epoxide ^{13}C6	✓	259.1	186.1	5.84	1.00	97	9	28	+
Felbamate		178.1	117.1	4.81	1.07	71	9	11	+
Felbamate		178.1	91.1	4.81	1.07	71	9	25	+
Gabapentin		172.1	154.1	2.36	1.39	106	9	8	+
Gabapentin		172.1	137.1	2.36	1.39	106	9	12	+
Gabapentin D10	✓	182.2	164.1	2.28	1.00	91	9	8	+
Lacosamide		251.1	108	4.71	1.21	80	9	0	+
Lacosamide		251.1	91.1	4.71	1.21	80	9	16	+
Lacosamide ^{13}C D3	✓	255.3	108	4.82	1.00	88	9	2	+
Lamotrigine		256.0	210.9	5.11	1.22	154	9	24	+
Lamotrigine		256.0	43.0	5.11	1.22	154	9	44	+
Lamotrigine ^{13}C ^{15}N4	✓	261.0	46.0	5.10	1.00	157	9	46	+
Levetiracetam		171.1	154.0	2.85	1.21	71	9	0	+
Levetiracetam		171.1	126.0	2.85	1.21	71	9	8	+
Levetiracetam D6	✓	177.1	132.1	2.83	1.00	71	9	10	+
N-Acetyleretigabine		274.1	256.1	6.00	1.25	120	9	8	+
N-Acetyleretigabine		274.1	109.0	6.00	1.25	120	9	32	+
Oxcarbazepine		253.1	208.0	6.13	1.21	120	9	12	+
Oxcarbazepine		253.1	180.0	6.13	1.21	120	9	28	+
Oxcarbazepine ^{13}C6	✓	259.1	214.0	6.13	1.00	120	9	12	+
Pregabalin		160.1	142.1	2.26	1.39	89	9	4	+
Pregabalin		160.1	55.1	2.26	1.39	89	9	20	+
Pregabalin D6	✓	166.2	148.1	2.21	1.00	88	9	4	+
Retigabine		304.2	230.0	7.05	0.87	123	9	12	+
Retigabine		304.2	109.0	7.05	0.87	123	9	32	+
Retigabine D4	✓	308.2	113.0	7.05	1.00	126	9	32	+
Rufinamide		239.1	127.0	4.73	1.21	100	9	20	+
Rufinamide		239.1	101.0	4.73	1.21	100	9	54	+
Tiagabine		376.1	247.0	7.33	0.75	143	9	12	+
Tiagabine		376.1	111.0	7.33	0.75	143	9	28	+
Tiagabine D6	✓	382.2	253.1	7.33	1.00	149	9	12	+
Vigabatrin		130.1	113.0	0.67	1.19	71	9	4	+
Vigabatrin		130.1	71.1	0.67	1.19	71	9	12	+

Chemicals and reagents

Human serum, used for matrix-matched calibrators, was sourced from Golden West Biologicals (Temecula, 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, clean human serum was spiked with drug standards of the 15 compounds. Eight lower concentrations were created by a serial dilution into clean serum. Each sample was combined with an internal standard solution and extracted by protein precipitation using methanol. Samples were vortexed and centrifuged, and an aliquot of supernatant was diluted 10-fold with water before introduction into the LC system.

Data analysis

Data were acquired and analyzed using Agilent MassHunter software suite B.08.00 for data collection from the 6470 LC/TQ MS, or C.01.00 for data collection from the Ultivo. MS/MS transitions were obtained for both instruments using Agilent MassHunter Acquisition Optimizer software to determine optimal precursor and product ions, fragmentor voltages, and collision energies upon injection of a neat solution of each individual compound or internal standard at a concentration level of 1,000 ng/mL, 2 µL injection volume in flow injection mode.

Results and discussion

Linearity

The calibration concentrations ranged from 0.59 ng/mL to 20,000 ng/mL for some individual analytes. Other analyte concentrations ranged from 0.15 to 20 µg/mL. R^2 values were greater than 0.994 for all analytes, with 10 of the compounds displaying linear responses throughout the concentration range, and five requiring quadratic fits to cover the complete concentration range.

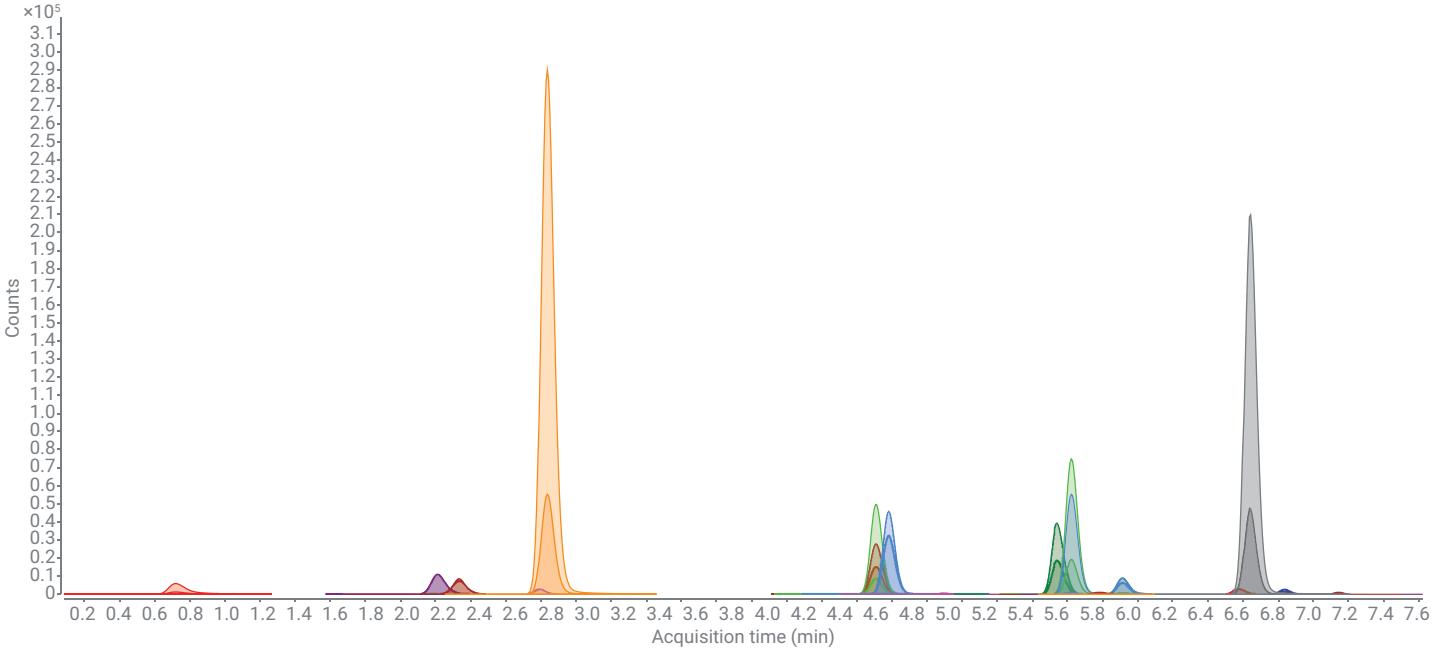

Figure 1. Overlaid dMRM chromatograms, showing elution of the 15 compounds.

Table 4. Results summary comparing the Agilent Ultivo TQ and Agilent 6470 TQ. Data include signal-to-noise (S/N), calculated LOQ on-column, coefficient of determination, and calibration curve fit. Data were calculated from calibration standard 1, except for concentrations denoted with a * or **, which used calibration standards 3 and 2, respectively. All compounds used a 1/x weighted calibration curve.

	Agilent Ultivo TQ					Agilent 6470 TQ				
Compound	Cal 1 (ng/mL)	Cal 1 S/N	Calc. LOQ on col (pg)	R^2	Cal. curve	Cal 1 (ng/mL)	Cal 1 S/N	Calc. LOQ on col (pg)	R^2	Cal. curve
Vigabatrin	293.11*	3.00	23.44	0.9977	Linear	84.14	17.57	6.73	0.9974	Linear
Pregabalin	6.83	4.32	0.55	0.9977	Linear	6.84	28.04	0.55	0.9971	Linear
Gabapentin	9.75	58.50	0.78	0.9971	Linear	10.63	98.49	0.85	0.9978	Linear
Levetiracetam	40.42	687.31	3.23	0.9997	Quadratic	40.59	1,084.66	3.25	0.9997	Quadratic
Lacosamide	6.92	261.91	0.55	0.9976	Linear	7.75	343.6	0.62	0.9989	Linear
Rufinamide	13.88	350.73	1.11	0.9995	Quadratic	30.50**	856.41	2.44	0.9948	Quadratic
Felbamate	26.44	67.59	2.11	0.9990	Quadratic	24.16	824.09	1.93	0.9977	Quadratic
Lamotrigine	7.07	3.81	0.57	0.9937	Linear	8.12	224.01	0.65	0.9993	Linear
10,11-Dihydro-10-hydroxycarbamazepine	13.69	372.73	1.10	0.9993	Quadratic	12.88	1,020.54	1.03	0.9986	Quadratic
Carbamazepine 10,11 epoxide	9.92	632.08	0.79	0.9992	Linear	9.98	318.23	0.80	0.9995	Linear
N-Acetylretigabine	1.14	5.04	0.09	0.9987	Quadratic	1.20	28.44	0.10	0.9994	Quadratic
Oxcarbazepine	2.02	20.68	0.16	0.9991	Linear	1.98	70.23	0.16	0.9982	Linear
Carbamazepine	16.37	140.18	1.31	0.9969	Linear	16.34	782.22	1.31	0.9963	Linear
Retigabine	1.81	4.27	0.14	0.9944	Linear	2.14	4.44	0.17	0.9960	Linear
Tiagabine	0.61	126.86	0.05	0.9973	Linear	0.59	28.77	0.05	0.9986	Linear

Accuracy and reproducibility

Calibration curves for each of the 15 compounds demonstrated accuracies within 20 % of each expected concentration at the lowest calibration level, and reproducibility across all other levels exhibited CVs less than 15 %. Tables 5 and 6, respectively show a detailed comparison of accuracies and reproducibility between the Ultivo and 6470 instruments.

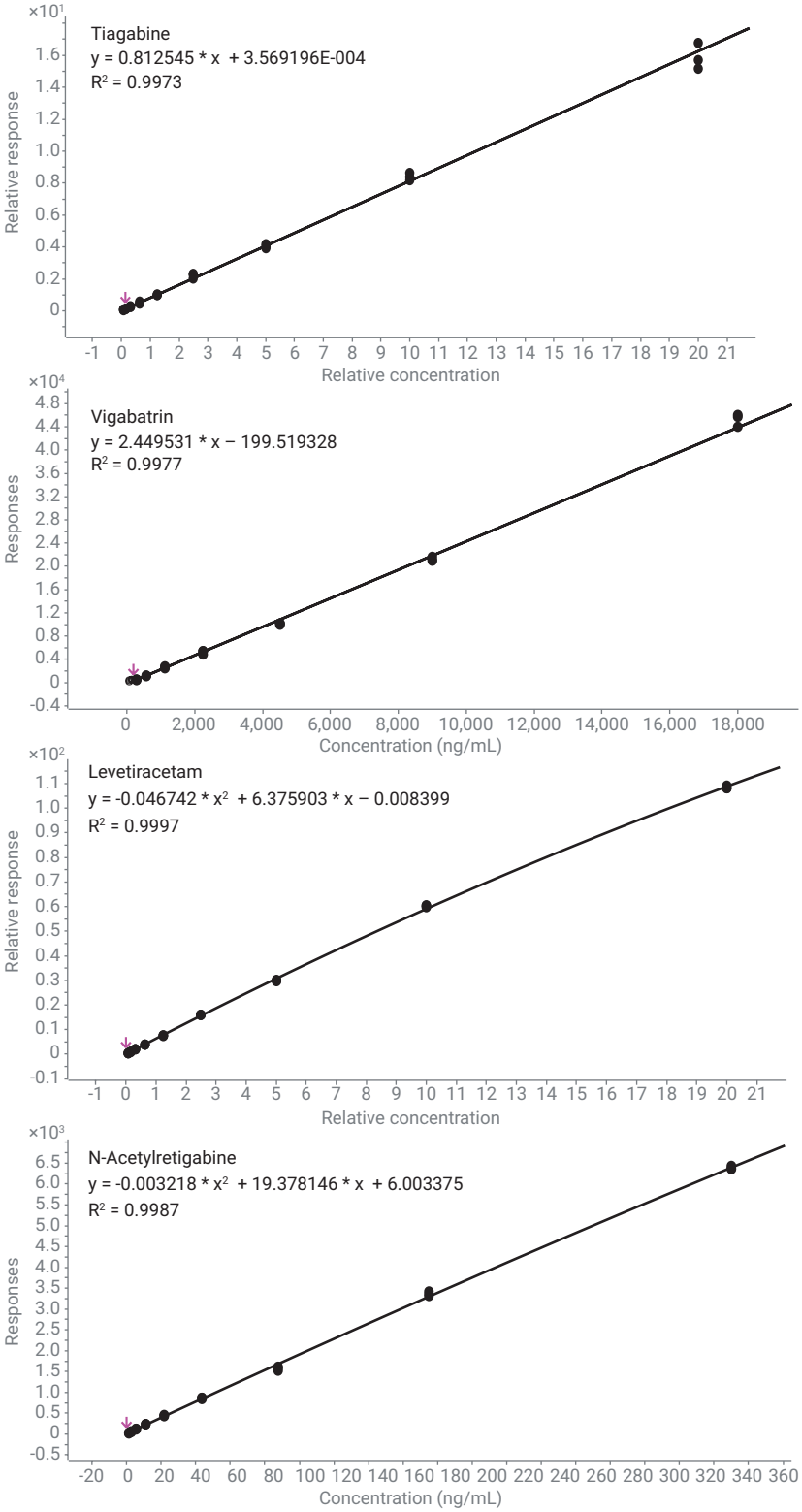

Figure 2. Example calibration curves for compounds dispersed throughout the chromatogram. All calibration curves used a 1/x weighting factor.

Table 5. Accuracy and reproducibility for curves analyzed on the Agilent Ultivo TQ (n = 3).

	10,11-Dihydro-10-hydroxy carbamazepine		Acetylretigabine		Carbamazepine		Carbamazepine 10,11 epoxide		Felbamate		Gabapentin		Lacosamide		Lamotrigine	
Level	Avg	CV	Avg	CV	Avg	CV	Avg	CV	Avg	CV	Avg	CV	Avg	CV	Avg	CV
1	87.6	4.2	96.2	6.7	83.8	1.6	101.6	1.7	84.6	2.5	83.2	8.0	88.6	2.4		
2	100.4	3.1	104.1	13.3	95.1	1.4	98.8	3.9	100.0	1.3	98.6	4.2	93.9	1.4	99.9	6.3
3	102.5	1.2	103.1	3.1	99.8	0.7	99.0	0.4	103.4	2.1	101.8	3.2	101.8	1.8	92.4	1.9
4	104.9	3.0	109.3	1.8	105.0	1.6	98.6	1.4	108.1	1.3	104.1	2.1	102.8	1.7	101.9	7.5
5	104.0	1.2	102.7	4.5	104.2	1.5	98.4	1.3	105.4	1.7	102.9	3.7	101.3	2.0	101.3	7.0
6	104.3	3.2	102.1	2.2	109.2	2.3	102.8	1.1	102.0	0.8	106.5	1.5	109.1	1.4	102.1	1.4
7	97.7	1.9	93.8	3.0	102.0	0.5	99.1	1.1	97.3	1.8	101.5	5.5	102.0	1.6	100.7	2.4
8	97.9	1.0	101.9	1.7	105.6	1.6	103.5	0.2	98.4	2.2	105.6	1.2	104.4	1.9	99.9	0.8
9	100.7	0.9	99.8	0.6	95.2	1.1	98.3	0.8	100.9	2.6	95.8	2.1	96.1	1.6	99.5	1.9

	Levetiracetam		Oxcarbazepine		Pregabalin		Retigabine		Rufinamide		Tiagabine		Vigabatrin	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	103.5	1.5	103.2	5.5	87.4	11.8	92.5	7.4	88.8	1.8	103.9	13.5		
2	99.6	1.4	97.2	1.3	95.9	8.8	94.5	9.8	101.2	2.2	92.4	6.2		
3	98.7	1.6	95.9	1.1	99.8	0.3	98.2	1.2	103.3	2.4	98.1	13.1	104.2	8.5
4	100.6	1.2	98.5	3.5	102.4	1.4	104.3	6.9	106.0	0.2	98.4	5.8	100.6	4.7
5	96.7	0.8	101.0	3.2	102.9	1.1	107.2	3.8	102.2	1.9	98.7	5.6	104.0	8.2
6	101.8	1.7	104.0	2.2	108.7	0.8	105.4	5.9	101.2	0.7	107.9	1.8	97.7	5.9
7	97.5	1.0	100.1	0.5	102.4	1.2	96.1	3.3	96.7	1.0	99.6	2.6	93.3	1.3
8	102.0	1.8	101.0	1.0	104.5	1.1	104.2	3.0	100.5	2.3	103.3	3.5	97.1	1.6
9	99.6	1.5	99.0	1.6	95.9	1.4	97.7	4.0	100.1	1.2	97.7	5.7	103.1	2.3

Table 6. Accuracy and reproducibility for curves analyzed on the Agilent 6470 TQ (n = 3).

	10,11-Dihydro-10-hydroxy carbamazepine		Acetylretigabine		Carbamazepine		Carbamazepine 10,11 epoxide		Felbamate		Gabapentin		Lacosamide		Lamotrigine	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	82.4	3.6	87.8	6.7	83.7	1.9	102.2	3.4	77.3	2.5	90.7	1.2	99.2	3.3	103.9	6.3
2	94.4	1.4	100.0	3.5	91.3	0.5	96.9	1.5	94.3	1.2	92.9	1.4	94.5	0.4	96.5	6.8
3	107.1	0.9	104.2	3.6	99.7	0.4	97.9	1.7	108.0	1.6	99.3	0.6	98.0	0.8	99.6	4.5
4	110.3	2.2	105.8	1.8	104.7	0.9	100.3	1.3	113.3	1.8	103.5	2.0	101.2	1.5	97.1	2.2
5	107.3	1.6	104.0	1.6	105.7	0.8	98.7	0.8	109.4	0.7	102.3	1.2	100.3	0.7	98.5	3.2
6	103.1	0.8	100.8	3.9	110.9	0.7	103.7	1.1	103.6	1.5	108.5	1.4	105.3	0.9	13.4	1.4
7	97.4	1.3	97.3	2.2	104.0	0.9	99.0	0.9	96.9	1.2	102.3	1.0	100.1	0.6	100.0	1.6
8	96.5	1.7	99.8	1.5	105.5	0.7	102.9	1.0	95.3	1.4	104.7	1.2	104.2	1.0	102.7	2.2
9	101.8	1.6	100.2	1.9	94.5	1.2	98.5	1.8	102.9	2.5	95.9	2.6	97.2	1.1	98.4	1.2

	Levetiracetam		Oxcarbazepine		Pregabalin		Retigabine		Rufinamide		Tiagabine		Vigabatrin	
Level	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV	Average	CV
1	103.9	1.3	101.2	2.7	87.6	4.7	109.5	0.7			100.8	2.6	119.7	14.4
2	97.6	1.3	97.0	5.8	91.8	0.8	110.4	12.5	97.6	0.5	97.8	3.0	105.3	12.1
3	98.9	0.3	99.9	5.0	100.0	2.7	87.6	2.7	120.4	2.2	96.8	4.0	96.8	3.2
4	100.3	1.4	98.6	2.5	104.4	2.6	86.4	8.9	122.2	3.9	102.7	2.4	95.1	6.7
5	98.2	0.4	98.3	1.8	103.0	1.4	99.2	14.9	111.6	3.5	97.8	0.8	92.5	6.3
6	102.5	1.4	103.3	1.7	109.7	2.0	101.0	6.6	103.0	3.3	102.5	1.1	96.5	0.1
7	97.2	1.2	98.5	1.9	103.0	1.5	97.8	6.7	94.9	2.1	98.9	0.4	92.3	3.8
8	101.6	0.9	106.0	2.0	105.2	1.4	104.1	5.5	94.6	2.4	105.3	0.8	97.6	0.7
9	99.7	1.7	97.1	1.9	95.2	2.1	98.8	5.6	103.6	3.0	97.4	0.7	104.1	1.2

Conclusion

The miniature Agilent Ultivo LC/TQ delivers comparable results to those achieved on the Agilent 6470 LC/TQ. This demonstrates that downsizing the instrument has not compromised any analytical sensitivity or dynamic range, as determined from a head-to-head comparison under identical analytical conditions.

Future work is required to assess potential matrix interferences to potentially include additional anti-epileptic drugs, creating a more comprehensive or inclusive analytical research method.

Reference

1. Frick, L. E.; Miller, V. P. Simultaneous LC/MS/MS Quantitation of 20 Anti-epileptic Drugs in Human Serum. *Poster presented at MSACL*, January **2017**, 23-26, Palm Springs, CA.

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