Supporting Information

Targeted Strategy to Analyze Anti-Epileptic Drugs in Human Serum by LC-MS/MS and LC-ion mobility-MS

Don E. Davis, Jr.,[†] Stacy D. Sherrod,[†] Randi L. Gant-Branum,[†,ı] Jennifer M. Colby,[‡] and John A. McLean[*,†]

[†]Center for Innovative Technology, Department of Chemistry, Institute of Chemical Biology, Institute for Integrative Biosystems Research and Education, Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN 37235 USA
[‡]Department of Pathology, Microbiology, and Immunology; Vanderbilt University Medical Center, Nashville, TN 37235 USA
[ı]Current location: HQ Air Force Drug Testing Laboratory (AFDTL), Lackland Air Force Base, San Antonio, TX, United States.

*Corresponding Author Email: john.a.mclean@vanderbilt.edu

Comments on LC-MS/MS Data, LC-DTIM-MS Data, and samples presented in this work

In this supporting information, we provide figures for limit of quantitation chromatograms (14 AEDs), patient sample analysis on both analytical platforms, validation studies, and a table for human drug-free 3rd party verified serum quality control material concentrations. We also include additional tables related to linearity study concentrations and calibrator concentrations.

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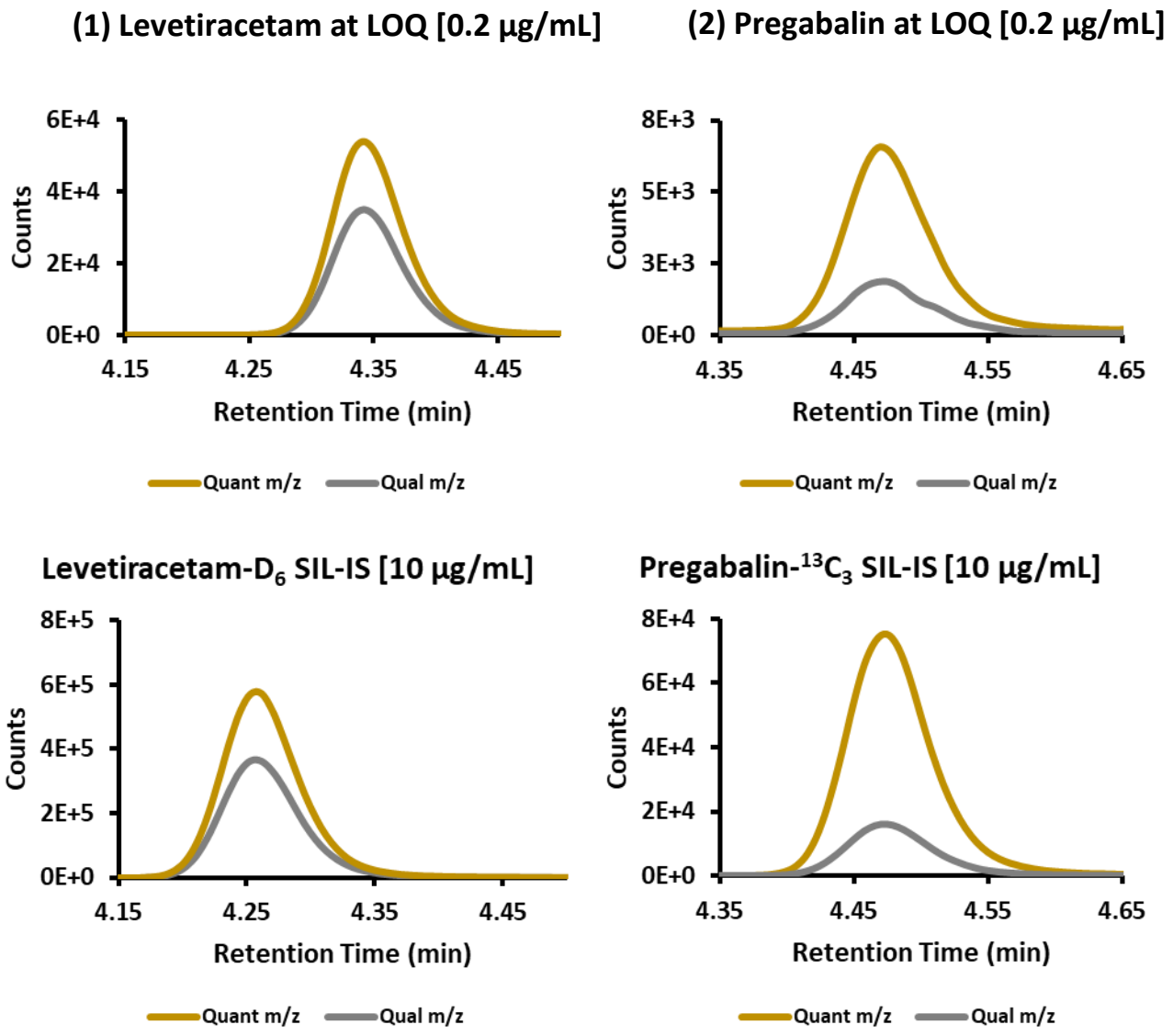
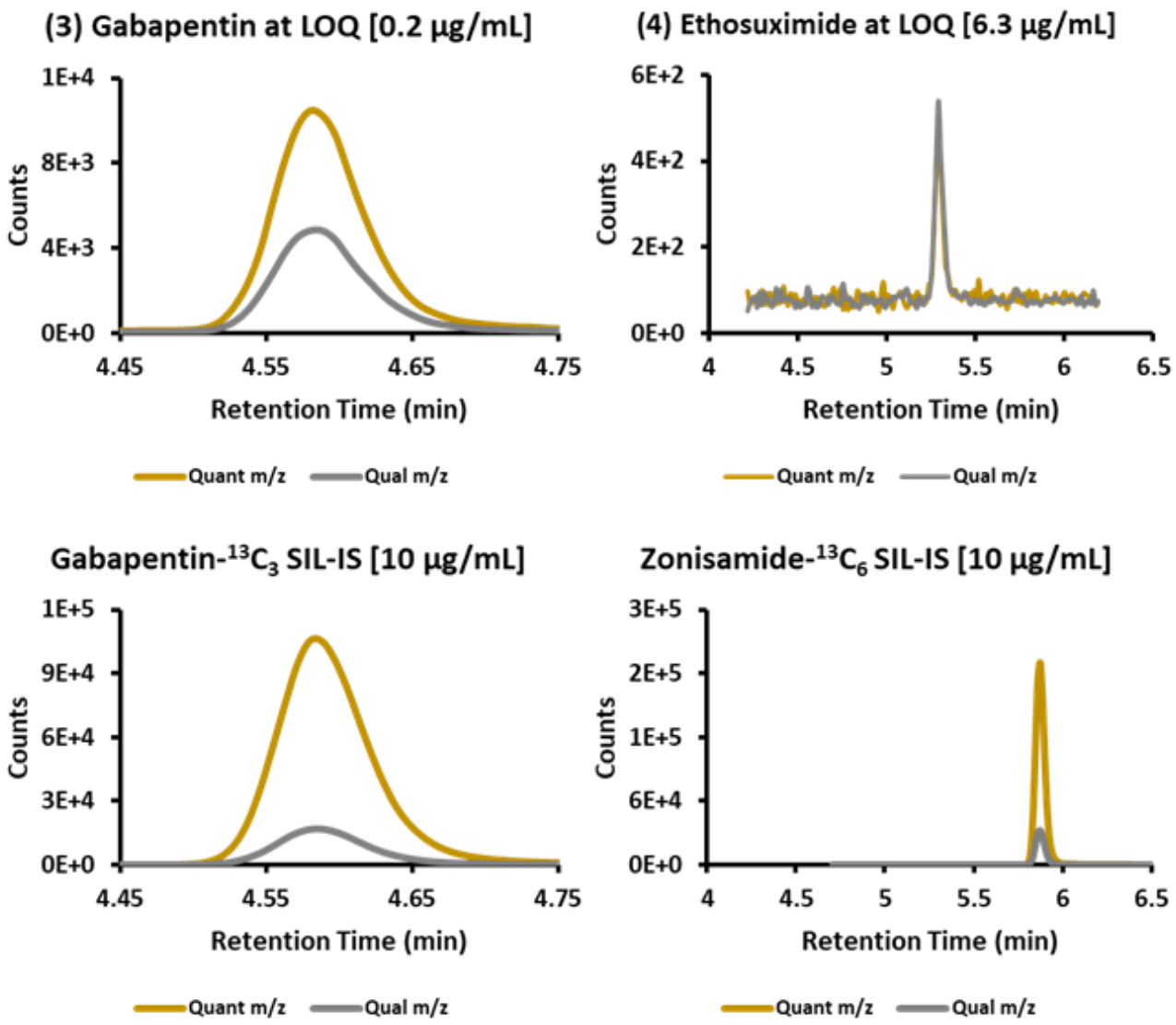
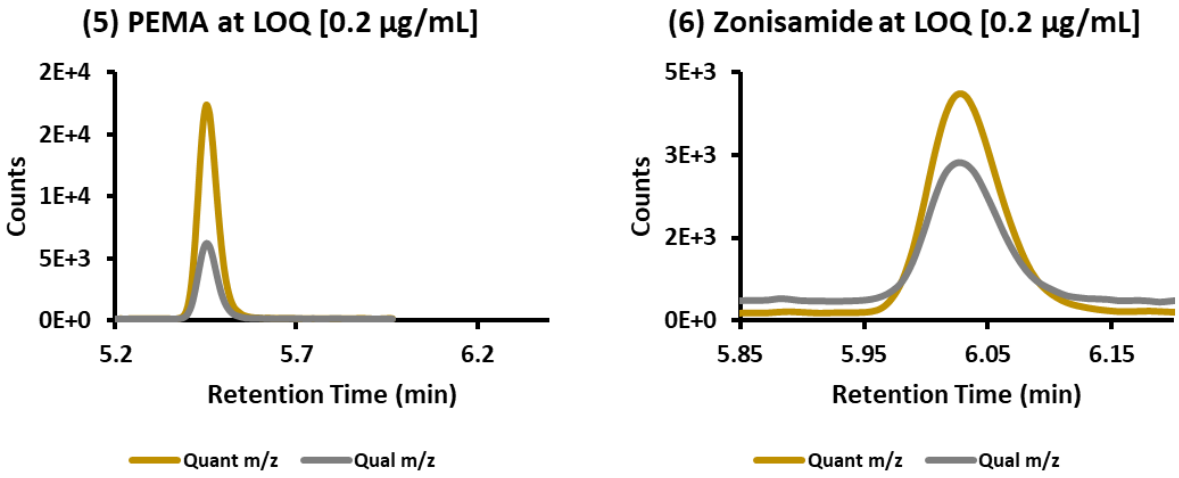
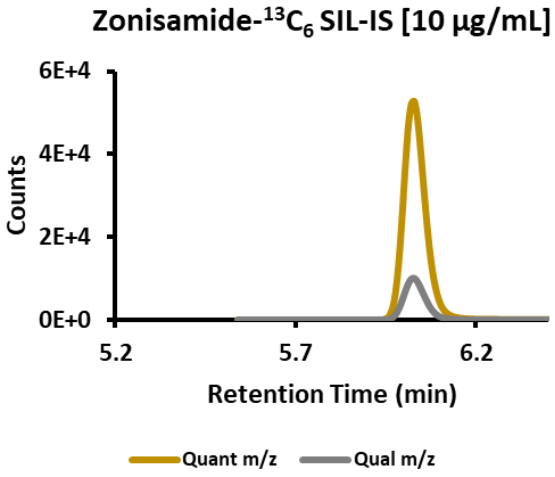
Figure S1. Limit of quantitation (LOQ) chromatograms of 14 AEDs with their given stable isotopically labelled – internal standard (SIL-IS).

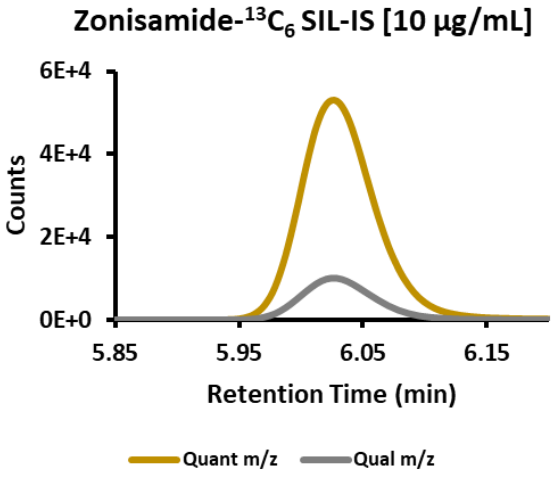



(3) Gabapentin at LOQ [0.2 µg/mL]
1E+4
8E+3
4E+3
0E+0
Counts
4.45
4.55
4.65
4.75
Retention Time (min)
Quant m/z
Qual m/z
(4) Ethosuximide at LOQ [6.3 µg/mL]
6E+2
4E+2
2E+2
0E+0
Counts
4
4.5
5
5.5
6
6.5
Retention Time (min)
Quant m/z
Qual m/z
Gabapentin-13C3 SIL-IS [10 µg/mL]
1E+5
9E+4
6E+4
3E+4
0E+0
Counts
4.45
4.55
4.65
4.75
Retention Time (min)
Quant m/z
Qual m/z
Zonisamide-13C6 SIL-IS [10 µg/mL]
3E+5
2E+5
1E+5
6E+4
0E+0
Counts
4
4.5
5
5.5
6
6.5
Retention Time (min)
Quant m/z
Qual m/z

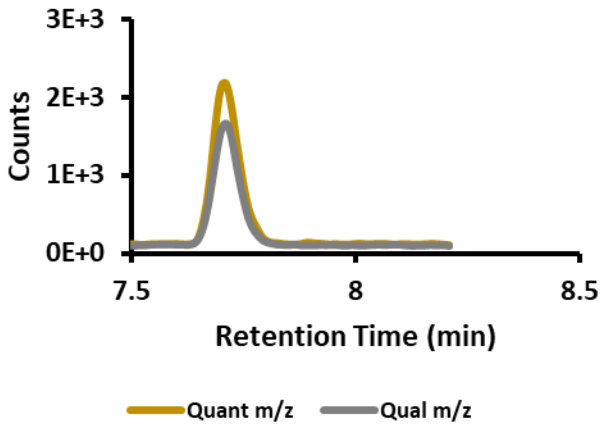

(5) PEMA at LOQ [0.2 µg/mL]
2E+4
2E+4
1E+4
5E+3
0E+0
Counts
5.2
5.7
6.2
Retention Time (min)
Quant m/z
Qual m/z

(6) Zonisamide at LOQ [0.2 µg/mL]
5E+3
3E+3
2E+3
0E+0
Counts
5.85
5.95
6.05
6.15
Retention Time (min)
Quant m/z
Qual m/z

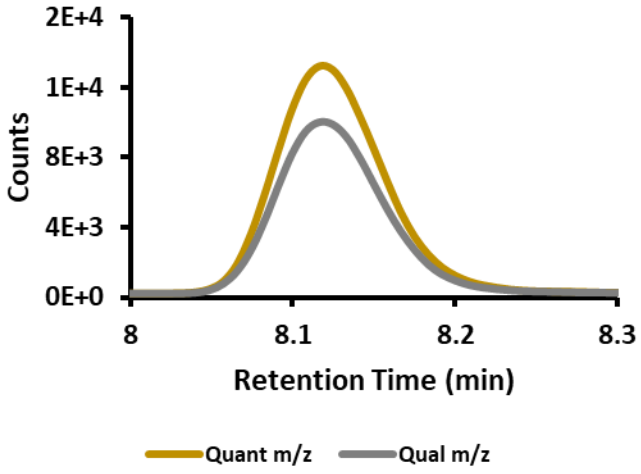

Zonisamide-13C6 SIL-IS [10 µg/mL]
6E+4
4E+4
2E+4
0E+0
Counts
5.2
5.7
6.2
Retention Time (min)
Quant m/z
Qual m/z


Zonisamide-13C6 SIL-IS [10 µg/mL]
6E+4
4E+4
2E+4
0E+0
Counts
5.85
5.95
6.05
6.15
Retention Time (min)
Quant m/z
Qual m/z

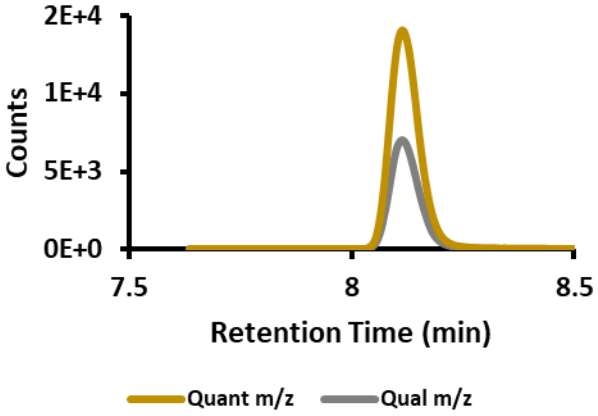
(7) Primidone at LOQ [0.2 µg/mL]



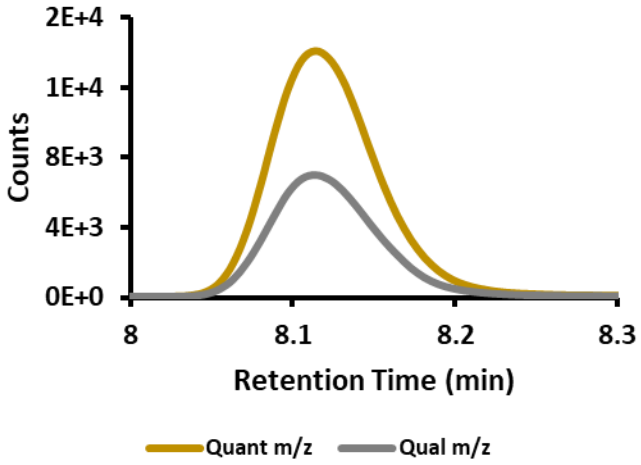
(8) Lamotrigine at LOQ [0.2 µg/mL]

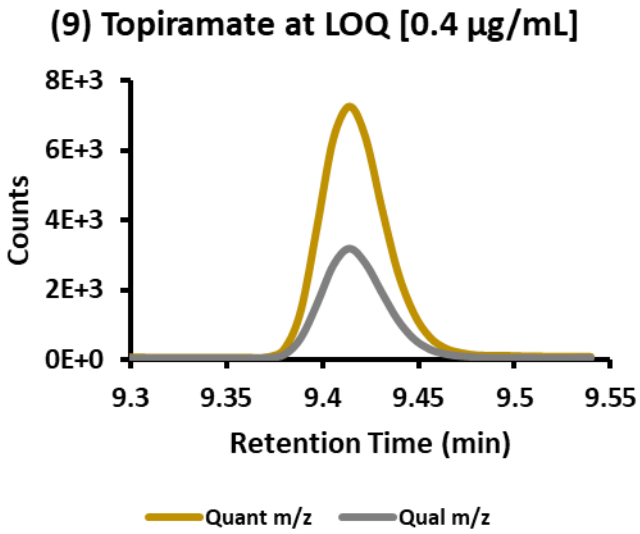


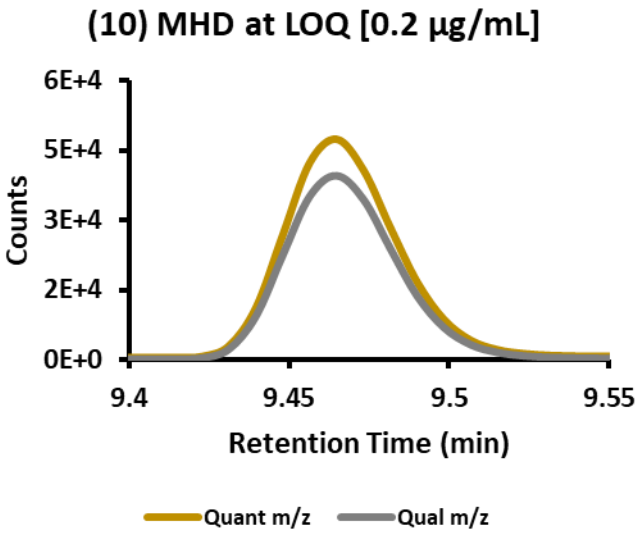
Lamotrigine-^{13}C, $^{15}N_4$ SIL-IS [10 µg/mL]

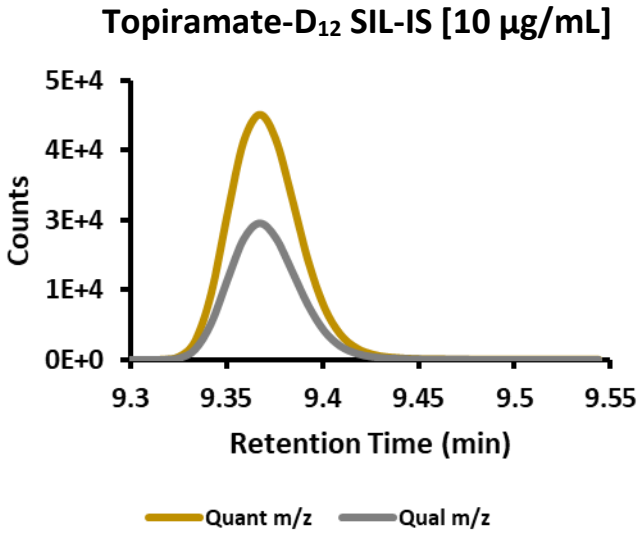


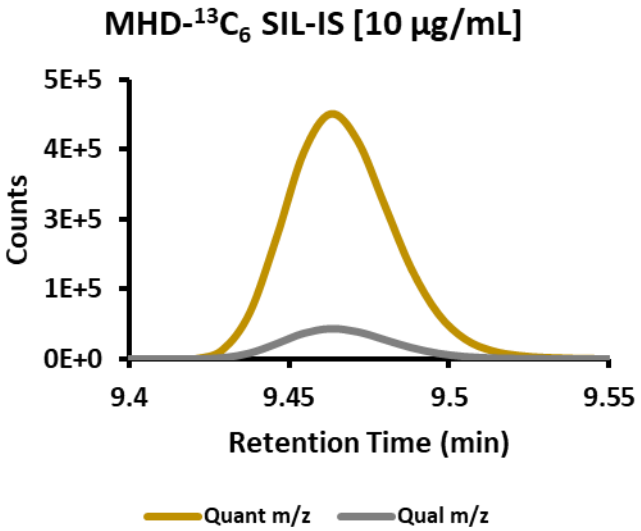
Lamotrigine-^{13}C, $^{15}N_4$ SIL-IS [10 µg/mL]

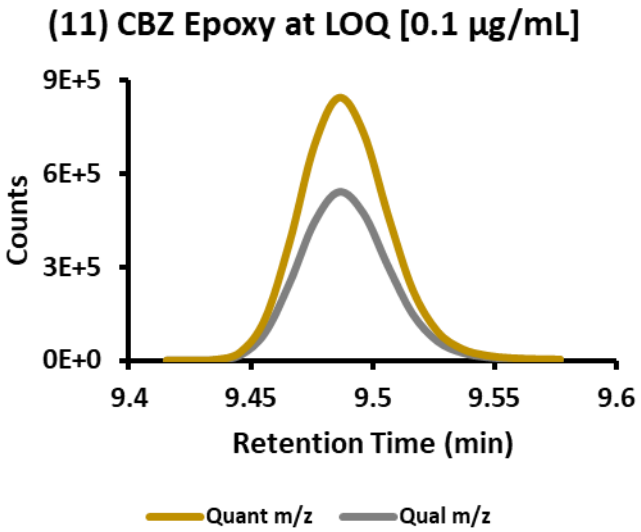


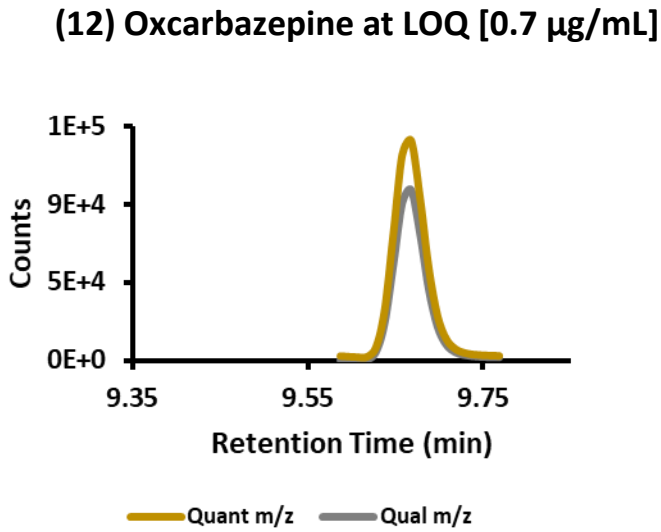

(9) Topiramate at LOQ [0.4 µg/mL]
8E+3
6E+3
4E+3
2E+3
0E+0
Counts
9.3
9.35
9.4
9.45
9.5
9.55
Retention Time (min)
Quant m/z
Qual m/z

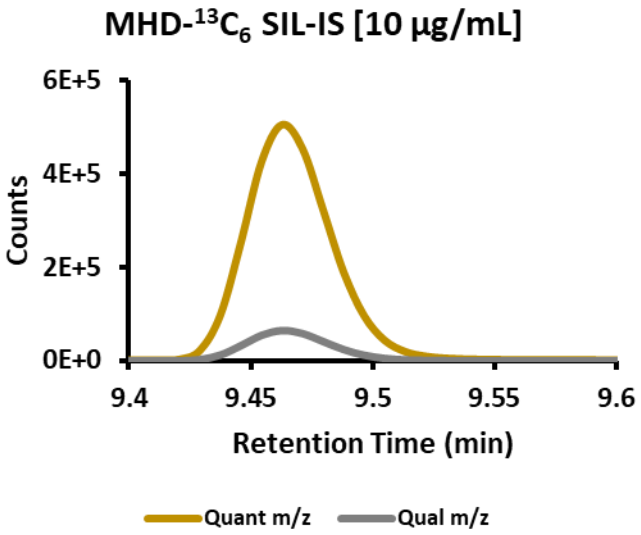

(10) MHD at LOQ [0.2 µg/mL]
6E+4
5E+4
4E+4
3E+4
2E+4
0E+0
Counts
9.4
9.45
9.5
9.55
Retention Time (min)
Quant m/z
Qual m/z

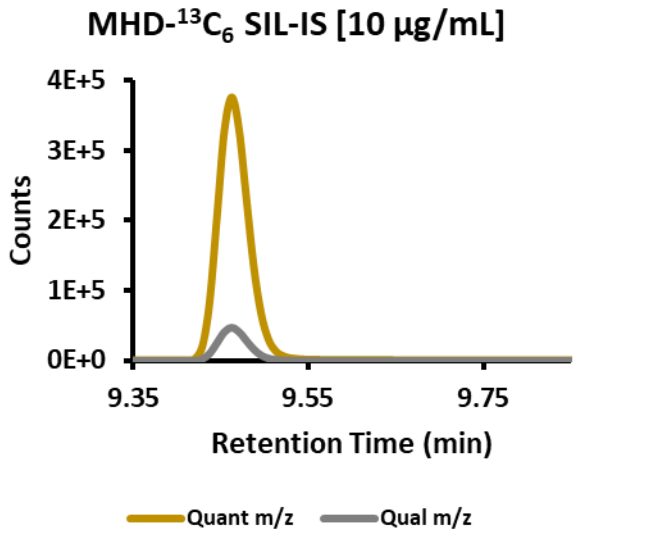

Topiramate-D12 SIL-IS [10 µg/mL]
5E+4
4E+4
3E+4
1E+4
0E+0
Counts
9.3
9.35
9.4
9.45
9.5
9.55
Retention Time (min)
Quant m/z
Qual m/z

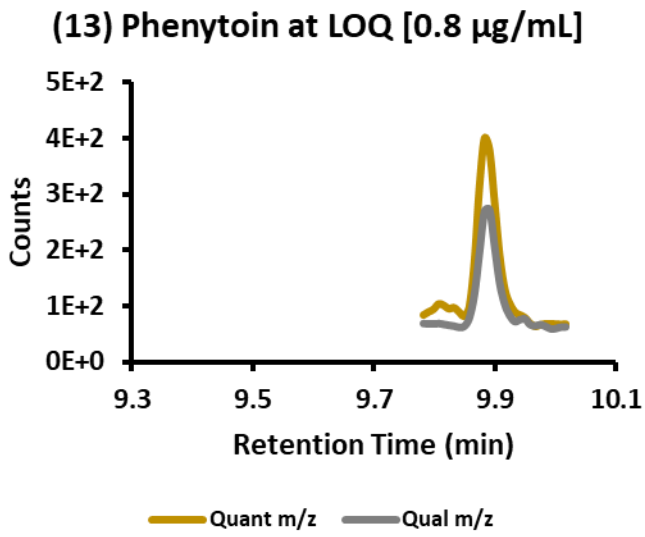

MHD-13C6 SIL-IS [10 µg/mL]
5E+5
4E+5
3E+5
1E+5
0E+0
Counts
9.4
9.45
9.5
9.55
Retention Time (min)
Quant m/z
Qual m/z

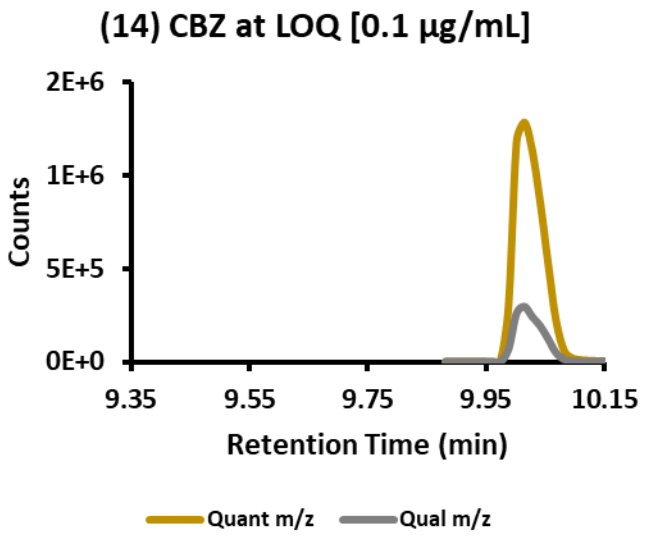

(11) CBZ Epoxy at LOQ [0.1 µg/mL]
9E+5
6E+5
3E+5
0E+0
Counts
9.4
9.45
9.5
9.55
9.6
Retention Time (min)
Quant m/z
Qual m/z

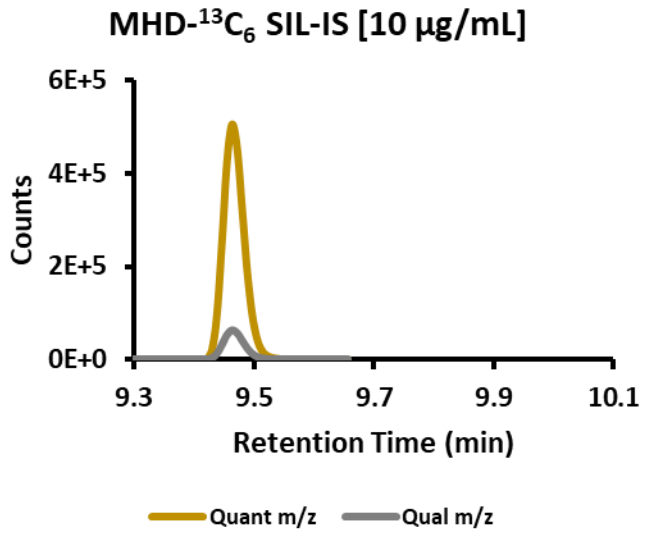

(12) Oxcarbazepine at LOQ [0.7 µg/mL]
1E+5
9E+4
5E+4
0E+0
Counts
9.35
9.55
9.75
Retention Time (min)
Quant m/z
Qual m/z


MHD-13C6 SIL-IS [10 µg/mL]
6E+5
4E+5
2E+5
0E+0
Counts
9.4
9.45
9.5
9.55
9.6
Retention Time (min)
Quant m/z
Qual m/z


MHD-13C6 SIL-IS [10 µg/mL]
4E+5
3E+5
2E+5
1E+5
0E+0
Counts
9.35
9.55
9.75
Retention Time (min)
Quant m/z
Qual m/z


(13) Phenytoin at LOQ [0.8 µg/mL]
5E+2
4E+2
3E+2
2E+2
1E+2
0E+0
Counts
9.3
9.5
9.7
9.9
10.1
Retention Time (min)
Quant m/z
Qual m/z


(14) CBZ at LOQ [0.1 µg/mL]
2E+6
1E+6
5E+5
0E+0
Counts
9.35
9.55
9.75
9.95
10.15
Retention Time (min)
Quant m/z
Qual m/z


MHD-13C6 SIL-IS [10 µg/mL]
6E+5
4E+5
2E+5
0E+0
Counts
9.3
9.5
9.7
9.9
10.1
Retention Time (min)
Quant m/z
Qual m/z

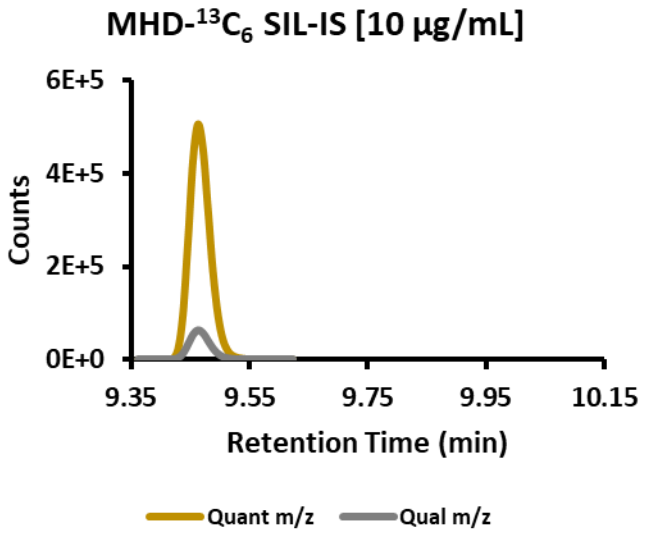

MHD-13C6 SIL-IS [10 µg/mL]
6E+5
4E+5
2E+5
0E+0
Counts
9.35
9.55
9.75
9.95
10.15
Retention Time (min)
Quant m/z
Qual m/z

Figure S2. AED between-day precision (A) and accuracy (B) analyses using LC-MS/MS. Samples (n = 5 process replicates) were extracted and analyzed on 3 separate days. All data/replicates were combined for statistical analysis (n=15 process replicates). (A) Precision is representative of experimental random error where the low, medium, and high averages for all AEDs were 5%, 3%, and 4%, respectively. The total average %CV for all AEDs was 4%. (B) Accuracy is representative of experimental systematic error where the low, medium, and high aver-ages for all AEDs were 7%, 7%, and 9%, respectively. The total average %bias for all AEDs was 8%. Both random and systematic error is within the margin of error (15% as outlined in the FDA's bioanalytical method validation guidelines) demonstrating the method's precision and accuracy.

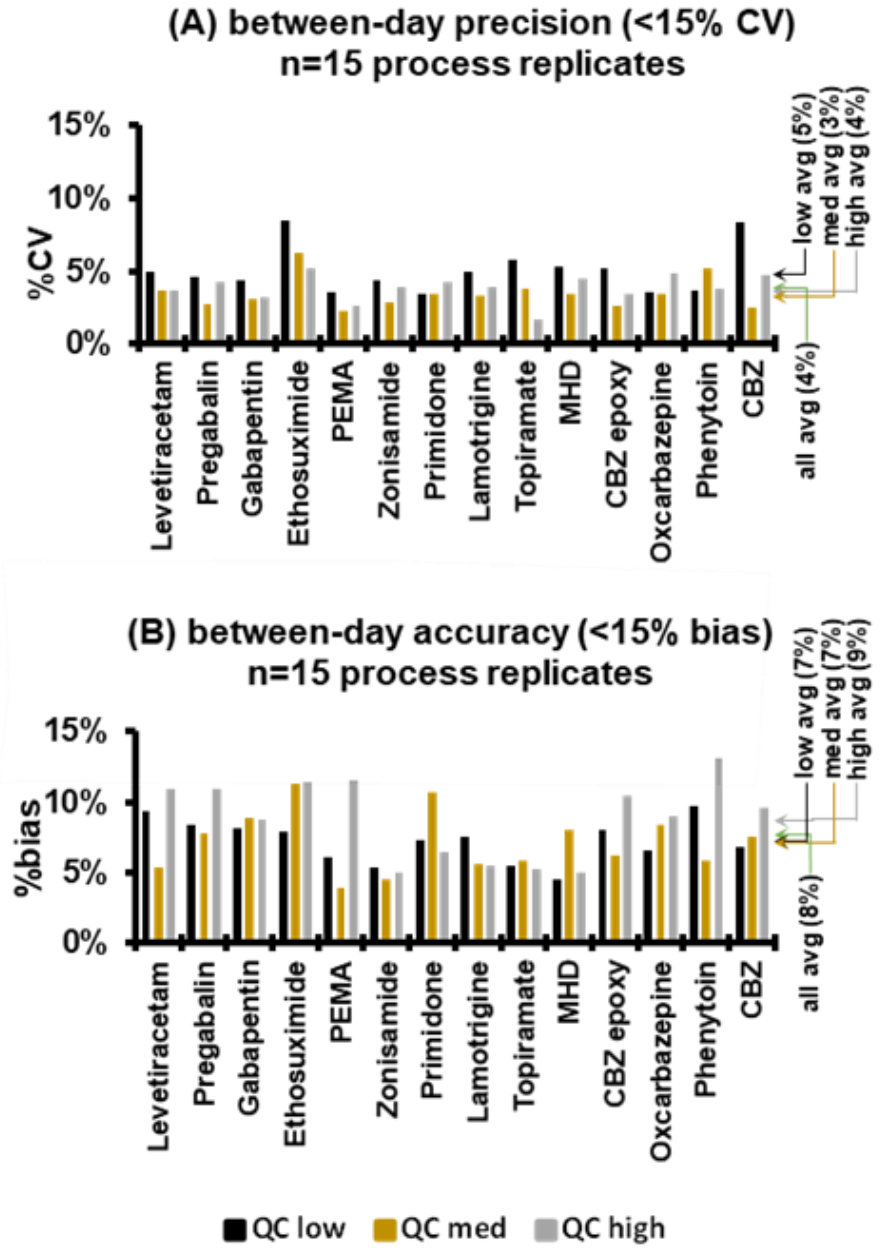


Figure S3. (A) This was a blind study whereas the concentrations of the 21 patient samples received from the clinic were not given until LC-MS/MS analysis was finished. All patients underwent polypharmacy with multiple AEDs. LC-UV analysis was compared to LC-MS/MS analysis. (B) As an example of how LC-DTIM-MS increases confidence in identifications, patient samples 3, 9, and 11 was highlighted to show CCS and drift time alignments of levetiracetam, lamotrigine, zonisamide, pregabalin, and MHD where the QCs (n = 3) confirms the identifications of the patient samples (n = 1). Standard error bars were used for QCs to demonstrate overlapping drift times where all 5 AEDs exhibit statistically significant drift times.

(A) LC-MS/MS patient sample analysis

Patient Sample #	AEDs	LC-UV (µg/mL)	LC-MS/MS (µg/mL)
Patient 1	Lamotrigine	12.2	10.22
	Gabapentin	NR	6.47
Patient 2	Lamotrigine	10.6	4.96
	Levetiracetam	32.3	20.8
Patient 3	Lamotrigine	5.80	4.49
	Levetiracetam	NR	20.7
Patient 4	Lamotrigine	10.6	8.02
Patient 5	Lamotrigine	10.0	8.04
	Levetiracetam	61.5	71.0
Patient 6	Lamotrigine	14.2	8.58
	Levetiracetam	39.8	52.1
Patient 7	Lamotrigine	14.2	11.7
	Levetiracetam	39.8	40.3
Patient 8	Ethosuximide	145	134.8
Patient 9	Zonisamide	11.0	5.94
	Levetiracetam	NR	3.2
	Pregabalin	NR	1.75
Patient 10	Ethosuximide	46.0	44.2
	Lamotrigine	NR	5.18
Patient 11	Zonisamide	51.0	37.9
	Levetiracetam	NR	43.9
	MHD	NR	19.3
Patient 12	Lamotrigine	11.8	6.66
Patient 13	Lamotrigine	8.00	5.74
	Zonisamide	14.0	9.20
Patient 14	Zonisamide	10.0	6.35
	Carbamazepine	4.00	1.53
Patient 15	MHD	24.0	20.7
	Topiramate	17.2	22.1
Patient 16	Levetiracetam	< 3.00	23.8
Patient 17	Lamotrigine	32.7	22.6
	Levetiracetam	46.4	39.7
Patient 18	Lamotrigine	< 1.00	< 1.00
	Levetiracetam	37.7	26.7
	Gabapentin	6.70	4.03
Patient 19	Ethosuximide	75.0	73.9
Patient 20	MHD	13.0	10.5
	Gabapentin	NR	1.03
Patient 21	MHD	39.0	27.6

(B) LC-DTIM-MS patient sample analysis

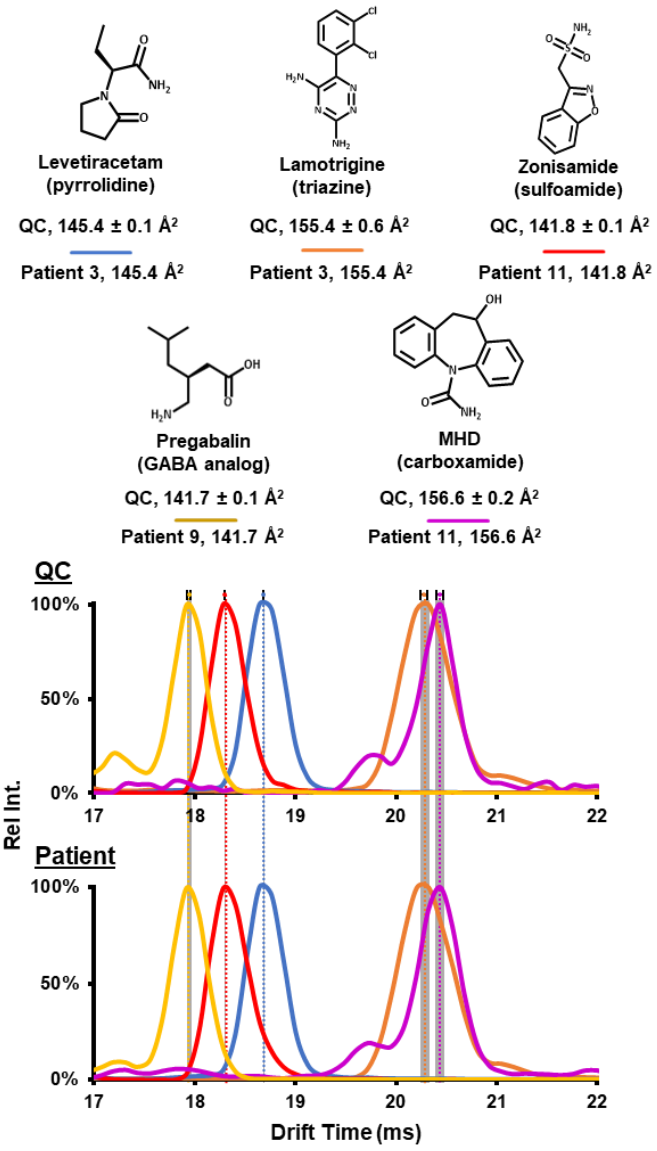


Figure S4. Stability of AEDs in extracted serum at 4°C after 48 idle hours in autosampler (n = 3 technical replicates).

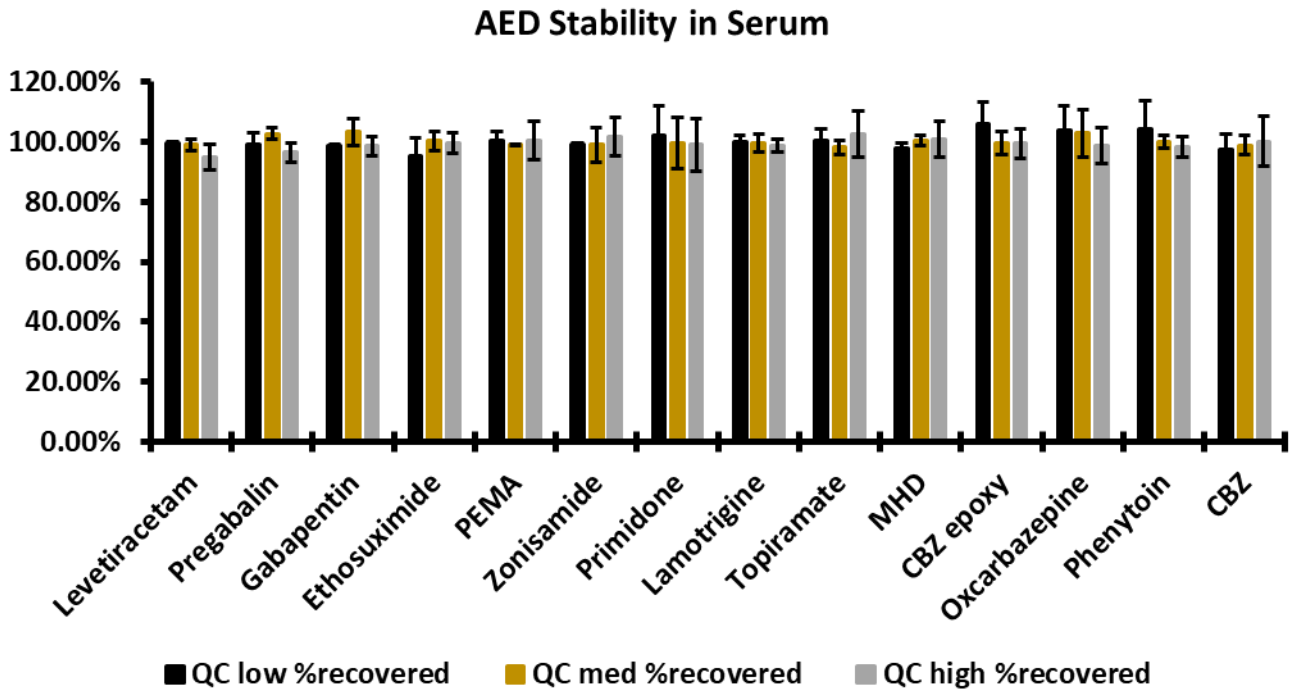


Figure S5. Carry over of AEDs in extracted serum (n = 5 technical replicates). Carry over was determined by injecting an extracted negative sample after the QC high sample. The CLSI C62A recommendation is that the carryover limit should be the highest concentration that does not carry over to the negative sample at or above 25% of the QC low sample.[1,2] The FDA recommendation is 20%.[3]

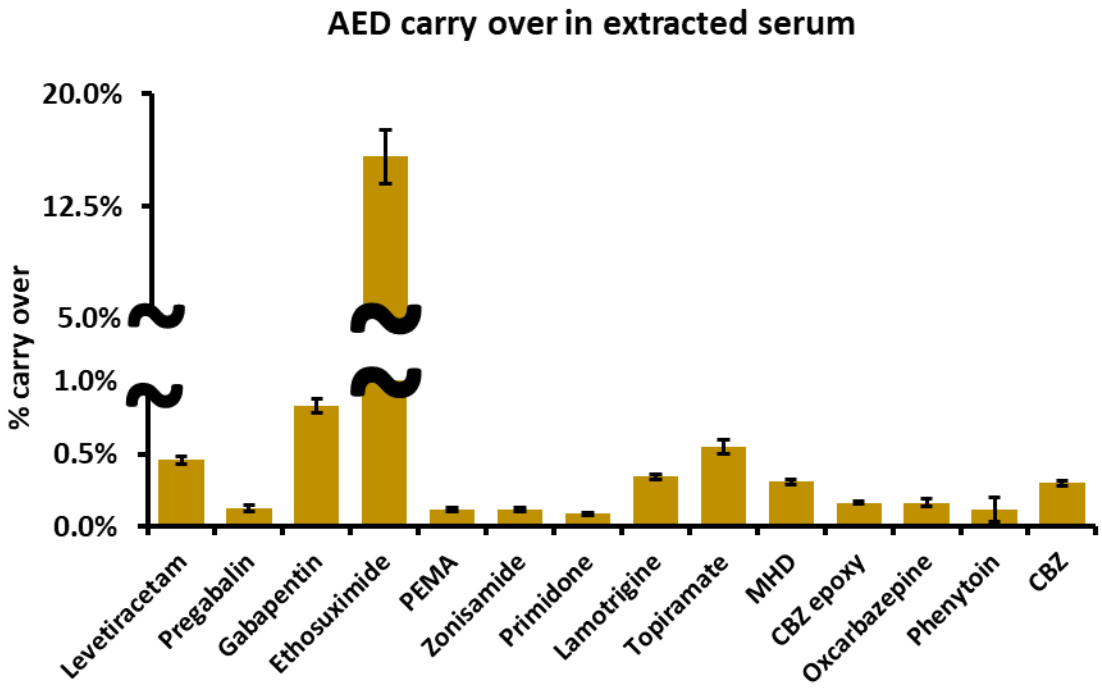


Figure S6: Recovery and Matrix Effects Equations.

$$1)\ \%Recovery = \frac{Peak\ Area\ (spike\ pre - extraction)}{Peak\ Area\ (spike\ post - extraction)}\ x\ 100$$

$$2)\ \%\ Matrix\ effect = \frac{Peak\ Area\ (spike\ post - extraction)}{Peak\ Area\ (neat)}\ x\ 100$$

Table S1. Serum 3rd party verified quality control (QC) material at low, medium, and high AED concentrations were purchased from UTAK Laboratories Inc, (Valencia, CA, USA).

AED	(µg/mL) Low QC	Med QC	High QC
Levetiracetam	5.00	25.0	50.0
Zonisamide	5.00	25.0	50.0
Topiramate	5.00	15.0	30.0
Lamotrigine	5.00	15.0	30.0
MHD	5.00	25.0	50.0
Ethosuximide	15.0	70.0	125
Gabapentin	5.00	15.0	30.0
Pregabalin	5.00	15.0	30.0
Primidone	5.00	15.0	30.0
PEMA	5.00	15.0	30.0
Carbamazepine	5.00	15.0	30.0
Carbamazepine Epoxide	5.00	15.0	30.0
Oxcarbazepine	5.00	15.0	30.0
Phenytoin	5.00	15.0	30.0

Table S2. Linearity study concentrations and correlation coefficients (R^2). These linearity calibrators were prepared in human drug free serum purchased from UTAK Laboratories Inc, (Valencia, CA, USA).

AED	Calibrator Concentration (µg/mL)												R^2
	12	11	10	9	8	7	6	5	4	3	2	1	
Levetiracetam	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
Zonisamide	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
Topiramate	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	>0.985
Lamotrigine	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
MHD	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
Ethosuximide	300	150	75.0	37.5	18.8	9.38	4.69	2.34	1.17	0.59	0.29	0.15	>0.985
Gabapentin	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
Pregabalin	200	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	>0.985
Primidone	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	>0.985
PEMA	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	>0.985
Carbamazepine	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	>0.985
CBZ Epoxy	50.0	25.0	12.5	6.3	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.02	>0.985
Oxcarbazepine	100	50.0	25.0	12.5	6.25	3.13	1.56	0.78	0.39	0.20	0.10	0.05	>0.985
Phenytoin	50.0	25.0	12.5	6.3	3.13	1.56	0.78	0.39	0.20	0.10	0.05	0.02	>0.985

Table S3. Calibrator concentrations and correlation coefficients (R^2). Calibrators are samples used for linearity and/or the calibration curve when analyzing patient samples. These calibrators were prepared in human drug free serum purchased from UTAK Laboratories Inc, (Valencia, CA, USA).

AED	Calibrator Concentration (µg/mL)						R^2
	6	5	4	3	2	1	
Levetiracetam	60	30	15	7.5	3.8	1.9	>0.985
Zonisamide	60	30	15	7.5	3.8	1.9	>0.985
Topiramate	60	30	15	7.5	3.8	1.9	>0.985
Lamotrigine	60	30	15	7.5	3.8	1.9	>0.985
MHD	60	30	15	7.5	3.8	1.9	>0.985
Ethosuximide	200	100	50	25	12.5	6.3	>0.985
Gabapentin	60	30	15	7.5	3.8	1.9	>0.985
Pregabalin	60	30	15	7.5	3.8	1.9	>0.985
Primidone	60	30	15	7.5	3.8	1.9	>0.985
PEMA	60	30	15	7.5	3.8	1.9	>0.985
Carbamazepine	60	30	15	7.5	3.8	1.9	>0.985
CBZ epoxy	60	30	15	7.5	3.8	1.9	>0.985
Oxcarbazepine	60	30	15	7.5	3.8	1.9	>0.985
Phenytoin	60	30	15	7.5	3.8	1.9	>0.985

Table S4. The developed and validated RPLC-UV method was compared to VUMC's pre-existing RPLC-UV method by parallel analysis of samples. The (-) indicates that the sample was not analyzed for the given AED.

AED Parallel Samples Sample #	AED	Expected (µg/mL)	LC - UV (µg/mL)	LC - MS/MS (µg/mL)	LC - UV (%bias)	LC - MS/MS (%bias)
Parallel 1	Pregabalin	3.00	-	2.50	-	-17%
	Gabapentin	1.00	-	1.18	-	18%
Parallel 2	Zonisamide	11.0	0.00	11.0	-100%	0%
	Lamotrigine	2.00	2.40	2.30	20%	15%
	Levetiracetam	6.00	5.80	7.00	-3%	17%
Parallel 3	MHD	9.00	9.00	8.50	0%	-6%
Parallel 4	Pregabalin	11.0	-	11.2	-	2%
	Gabapentin	15.0	-	13.8	-	-8%
Parallel 5	Zonisamide	26.0	26.0	25.0	0%	-4%
	Lamotrigine	11.0	11.0	11.0	0%	0%
	Levetiracetam	22.0	22.8	25.2	4%	15%
Parallel 6	MHD	24.0	23.0	26.0	-4%	8%
Parallel 7	Pregabalin	30.0	-	29.6	-	-1%
	Gabapentin	22.0	-	21.9	-	0%
Parallel 8	Zonisamide	50.0	50.2	52.7	0%	5%
	Lamotrigine	26.0	22.7	27.3	-13%	5%
	Levetiracetam	40.0	37.1	42.0	-7%	5%
Parallel 9	MHD	45.0	45.0	50.0	0%	11%

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