

Quantification of antiepileptics in human plasma or serum by LC-HRAM(MS) for clinical research

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Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass[®] kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive hardware enables increased confidence in data
- Simple offline sample preparation by protein precipitation

Goal

Implementation of an analytical method for the quantification of 26 different antiepileptic drugs in human plasma or serum on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer

Introduction

Antiepileptic drugs (AED) are used for the treatment of epilepsy, a chronic neurological disorder, which is characterized by recurrent epileptic seizures.

An analytical method for clinical research for the quantification of 26 antiepileptic drugs in human plasma or serum is reported in this study. While most reported LC-MS analyses of the above-mentioned antiepileptic drugs involve triple quadrupole mass spectrometers, traditionally used for targeted, sensitive quantitation assays, in this report we present LC-MS data acquired high-resolution accurate-mass (HRAM) mass spectrometry leveraging Orbitrap technology. This report demonstrates the capability of HRAM mass spectrometry for routine quantitation analyses in addition to its use for performing in-depth qualitative investigations.

Plasma or serum samples were extracted by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization. Two injections were performed for each batch of analysis, using the same LC method, but one for positively and one for negatively ionizing compounds. Detection was performed in both cases by full scan coupled to data-dependent fragmentation (fullMS-ddMS²) using 23 isotopically labelled internal standards. Method performance was evaluated using the ClinMass® TDM Platform with the ClinMass Add-On Set for Antiepileptics from RECIPE® Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

A description of the panel of analytes and corresponding internal standards, together with the concentration ranges covered by the calibrators (MS9213 batch #1428) used, are reported in Table 1.

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE (MS9282 batch #1388), as well as 23 deuterated internal standards for the quantification. Samples of 50 µL of plasma or serum were protein precipitated using 100 µL of precipitating solution containing the internal standards. Precipitated samples were vortex-mixed and centrifuged. The supernatant was diluted 10-fold with the dilution solution provided with the kit and transferred to a clean plate or vial.

Table 1. Analytes, internal standards, and concentration ranges covered by calibrators

Analyte	Internal standard	Concentration range (ng/mL)
10-OH-carbamazepine	d ₄ -10-OH-carbamazepine	2.8–42.9
Brivaracetam	d ₇ -brivaracetam	0.300–4.25
Carbamazepine	d ₁₀ -carbamazepine	1.44–20.6
Carbamazepine-diol	d ₅ -phenylethylmalonamide	0.542–8.74
Carbamazepine-epoxide	d ₄ -oxcarbazepine	0.625–9.57
Ethosuximide	d ₃ -ethosuximide	8.33–119
Felbamate	d ₄ -felbamate	7.04–106
Gabapentine	d ₄ -gabapentine	1.87–27.8
Lacosamide	d ₃ -lacosamide	0.879–13.9
Lamotrigine	¹³ C ₃ -lamotrigine	1.55–23.1
Levetiracetam	d ₆ -levetiracetam	4.32–67.3
N-desmethysuximide	d ₅ -phenobarbital	3.44–50.9
Oxcarbazepine	d ₄ -oxcarbazepine	0.249–3.99
Perampanel	d ₅ -perampanel	0.0970–1.46
Phenobarbital	d ₅ -phenobarbital	3.54–51.3
Phenylethylmalonamide	d ₅ -phenylethylmalonamide	0.711–11.5
Pregabalin	d ₄ -pregabalin	0.565–9.56
Primidone	d ₅ -primidone	1.63–28.8
Retigabine	d ₄ -retigabine	0.134–2.30
Rufinamide	¹⁵ N,d ₂ -rufinamide	3.11–47.4
Stiripentol	d ₉ -stiripentol	1.24–17.8
Sulthiame	d ₄ -sulthiame	0.946–12.7
Tiagabine	d ₆ -tiagabine	0.020–0.316
Topiramate	d ₁₂ -topiramate	1.23–17.7
Valproic acid	d ₆ -valproic acid	8.59–114
Zonisamide	¹⁵ N,d ₄ -zonisamide	3.05–42.5

Liquid chromatography

A Vanquish Duo UHPLC system, a dual-channel instrument configured for both LC-only and online SPE applications (Figure 1), was used for chromatographic separation. The LC-only channel was used in this case, utilizing mobile phases and analytical column provided by RECIPE. Details of the analytical method used for both injections are reported in Table 2. Total runtime was 4.5 minutes.

Table 2. Liquid chromatography method description

Gradient Profile		
Time (min)	Flow Rate (mL/min)	B (%)
0.00	0.6	0
0.03	0.6	0
0.04	0.6	13
1.30	0.6	13
1.31	0.6	21
2.30	0.6	21
2.80	0.6	50
3.30	0.6	50
3.40	0.6	80
3.60	0.6	80
3.70	0.6	0
4.50	0.6	0
Other Parameters		
Injection volume (μL)		10
Column temperature ($^{\circ}\text{C}$)		40

Mass spectrometry

Analytes and internal standards were detected by FullMS-ddMS² mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (H-ESI II) operated in positive and negative ion mode. FullMS data were used for quantification, ddMS² fragments for confirmation. A summary of the MS conditions is reported in Table 3.

Table 3. MS settings

	Polarity	
	Positive	Negative
Source type	Heated electrospray ionization (H-ESI II)	
Vaporizer temperature	350 $^{\circ}\text{C}$	437.50 $^{\circ}\text{C}$
Capillary temperature	275 $^{\circ}\text{C}$	268.75 $^{\circ}\text{C}$
Spray voltage	3000 V	2500 V
Sheath gas	55 AU	52.5 AU
Sweep gas	0 AU	2.75 AU
Auxiliary gas	15 AU	13.75 AU
S-Lens RF level	60	
Data acquisition mode	FullMS-ddMS ²	
FullMS resolution @ m/z 200	70,000	
FullMS scan range	50–400 m/z	
ddMS ² resolution @ m/z 200	17,500	
ddMS ² isolation window	2.0 m/z	
Collision energy (NCE)	Individual values in inclusion list	

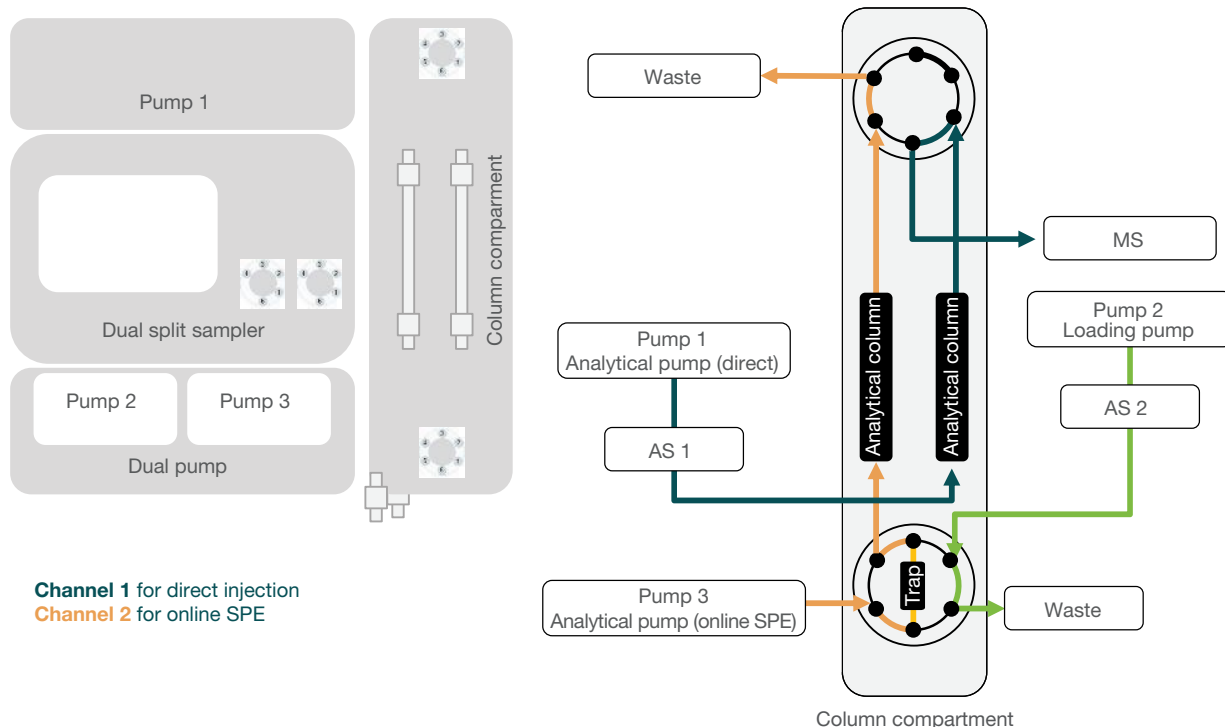


Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, and intra- and inter-assay precision for all the analytes. To determine the LLOQ, the lowest calibrator was diluted down to 20-fold with blank matrix; a full set of calibrators (three levels, excluding blank), diluted calibrators (four levels), and controls (two levels) were extracted in replicates of five (n=5), injected in a single batch and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV <20% across the entire batch of samples. Carryover was calculated in terms of the percentage ratio between the peak area of the highest calibrator and a blank sample injected just after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using the quality control samples at two different levels provided by RECIPE, prepared and analyzed in replicates of five on three different days.

Additional accuracy evaluation was based on external certified quality controls from different proficiency tests, prepared and analyzed in replicates of five on a single day. Quality controls from INSTAND e.V. (Düsseldorf, Germany) and from GTFCh (Jena, Germany) were used. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

The LLOQ achieved for each analyte is reported in Table 4. A linear response with 1/x weighting was obtained for all the analytes down to the individual LLOQs. The percentage bias between nominal and back-calculated concentration was always within $\pm 15\%$ for all the calibrators ($\pm 20\%$ for the lowest calibrator) in all the runs. Representative chromatograms for the LLOQ for lacosamide, phenobarbital, and the corresponding internal standards are depicted in Figure 2. Representative calibration curves for the same analytes in the concentration range covered by the kit (three calibrators) are shown in Figure 3.

Table 4. Analytes and LLOQs

Analyte	LLOQ (ng/mL)
10-OH-carbamazepine	2.8
Brivaracetam	0.300
Carbamazepine	0.220
Carbamazepine-diol	0.0542
Carbamazepine-epoxide	0.625
Ethosuximide	8.33
Felbamate	7.04
Gabapentine	1.87
Lacosamide	0.879
Lamotrigine	1.55
Levetiracetam	4.32
N-desmethyloxuximide	3.44
Oxcarbazepine	0.249
Perampanel	0.0970
Phenobarbital	3.54
Phenylethylmalonamide	0.0711
Pregabalin	0.565
Primidone	1.63
Retigabine	0.0670
Rufinamide	3.11
Stiripentol	0.620
Sulthiame	0.946
Tiagabine	0.020
Topiramate	1.23
Valproic acid	0.859
Zonisamide	3.05

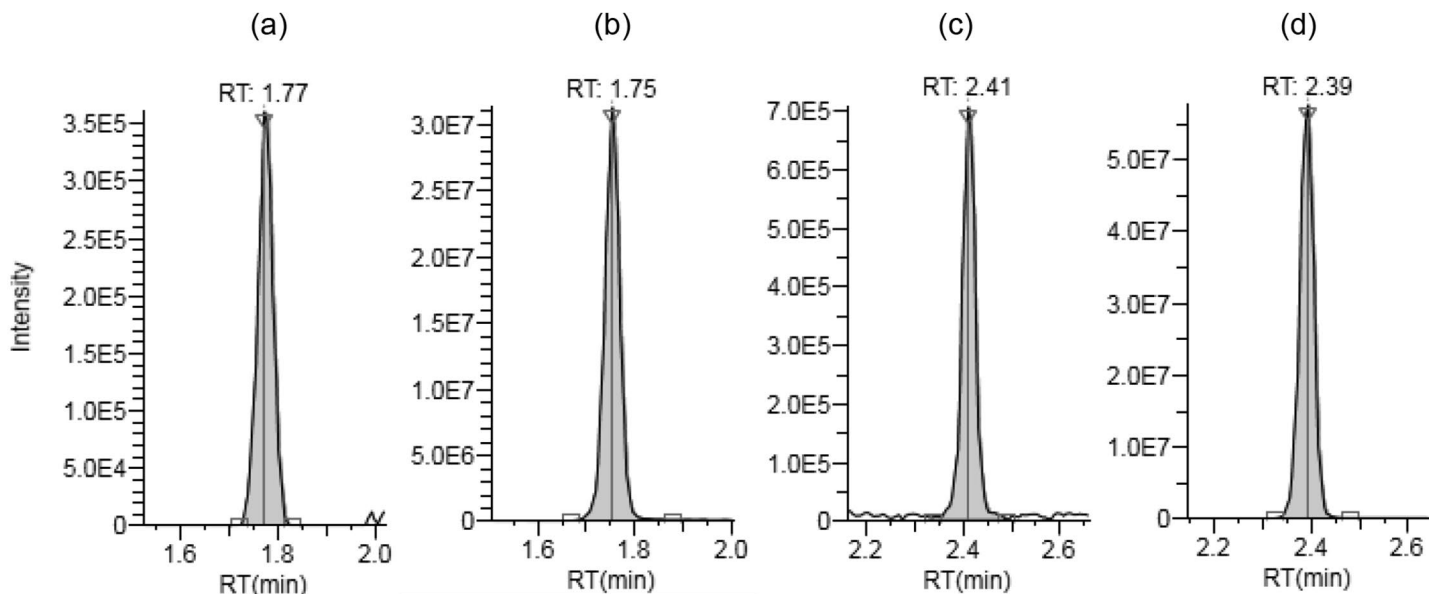


Figure 2. Representative chromatograms of the LLOQ for (a) lacosamide, (b) d_3 -lacosamide, (c) phenobarbital, and (d) d_5 -phenobarbital

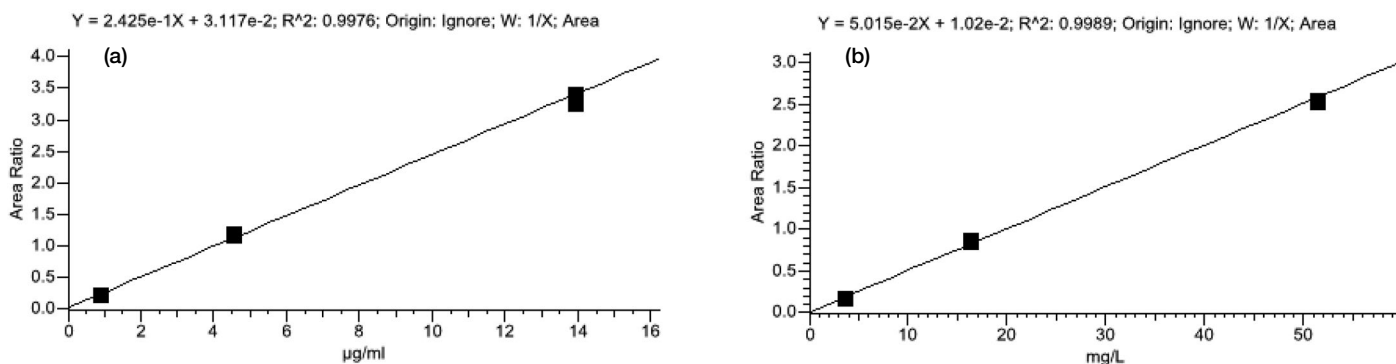


Figure 3. Representative calibration curves for (a) lacosamide and (b) phenobarbital – day 1

A maximum carryover of 0.1% was reported for gabapentine and pregabalin; no significant carryover was observed for the remaining analytes.

The data presented in this report demonstrate outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for RECIPE control samples ranging between -8.6% and

9.6% (Table 5). The use of external certified controls confirmed the good accuracy for the reported method, with a percentage bias always between -14.5% and 15.1% (Table 6). The %CV for intra-assay precision was always below 9.3%. The maximum %CV for inter-assay precision was 7.9%. Results for intra- and inter-assay precision are reported in Table 7 and Table 8, respectively.

Table 5. Analytical accuracy results for control MS9282 batch #1388

Analyte	Control 1			Control 2		
	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Nominal concentration (ng/mL)	Average Calculated concentration (ng/mL)	Bias (%)
10-OH-Carbamazepine	7.89	8.38	6.2	18.9	20.1	6.6
Brivaracetam	0.749	0.731	-2.3	1.71	1.71	0.1
Carbamazepine	4.16	4.47	7.4	9.95	10.2	2.8
Carbamazepine-diol	1.74	1.86	7.0	4.19	4.55	8.6
Carbamazepine-epoxide	1.93	2.06	6.6	4.47	4.80	7.5
Ethosuximide	23.20	23.39	0.8	53.3	55.3	3.7
Felbamate	19.9	20.2	1.7	49.0	47.5	-3.2
Gabapentine	5.30	5.79	9.2	12.5	13.7	9.6
Lacosamide	2.72	2.62	-3.5	6.66	6.21	-6.7
Lamotrigine	4.60	4.99	8.4	10.7	11.7	9.1
Levetiracetam	13.2	13.0	-1.5	29.7	31.2	5.2
NDMS	9.01	9.53	5.8	21.9	22.1	1.0
Oxcarbazepine	0.451	0.412	-8.6	0.881	0.821	-6.8
PEMA	2.22	2.19	-1.3	5.30	5.21	-1.7
Perampanel	0.266	0.277	4.0	0.638	0.638	0.0
Phenobarbital	10.30	10.33	0.3	24.5	23.6	-3.8
Pregabalin	1.71	1.72	0.3	4.18	4.17	-0.2
Primidone	5.06	5.29	4.6	12.5	13.0	3.7
Retigabine	0.411	0.418	1.8	1.02	1.01	-0.6
Rufinamide	8.88	9.23	4.0	21.5	21.6	0.6
Stiripentol	3.38	3.48	2.9	8.11	8.39	3.4
Sulthiame	2.59	2.64	2.1	6.1	6.1	0.2
Tiagabine	0.0600	0.0559	-6.9	0.143	0.133	-7.1
Topiramate	3.47	3.58	3.2	8.2	8.3	0.4
Valproic acid	23.4	23.1	-1.2	52.7	52.4	-0.5
Zonisamide	8.37	8.65	3.4	19.7	19.6	-0.7

Table 6. Analytical accuracy results for external quality controls from INSTAND e.V. and GTFCh

Analyte	Control 1			Control 2		
	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Nominal concentration (ng/mL)	Average Calculated concentration (ng/mL)	Bias (%)
10-OH-Carbamazepine**	33.7	36.9	8.7	8.35	9.24	9.7
10-OH-Carbamazepine****	11.2	12.2	8.1	21.1	23.6	10.4
Carbamazepine***	2.41	2.51	4.0	12.6	12.5	-0.7
Carbamazepine****	5.18	5.36	3.4	9.50	9.67	1.8
Carbamazepine-epoxide**	2.42	2.72	11.1	5.24	5.90	11.2
Ethosuximide**	70.8	73.3	3.4	69.5	74.0	6.1
Felbamate****	40.8	41.3	1.3	88.9	95.7	7.1
Gabapentine****	1.85	1.90	2.5	5.84	6.28	7.0
Lacosamide****	3.61	3.37	-7.2	9.29	8.91	-4.2
Lamotrigine*	4.62	5.44	15.1	9.09	10.7	15.1
Lamotrigine****	5.97	6.69	10.8	12.9	14.4	10.7
Levetiracetam*	16.5	16.1	-2.3	19.5	20.3	4.0
Levetiracetam****	12.4	12.3	-0.5	22.5	22.1	-1.6
NDMS**	23.4	23.2	-0.7	33.4	33.5	0.4
Oxcarbazepine****	7.90	7.15	-10.5	17.5	15.3	-14.5
Phenobarbital***	45.6	44.2	-3.3	10.4	10.3	-1.0
Pregabalin****	3.06	3.01	-1.7	5.75	5.59	-2.8
Primidone***	5.93	6.51	8.8	15.3	16.0	4.2
Rufinamide****	16.9	17.3	2.6	39.5	43.6	9.5
Sulthiame*	6.98	7.48	6.7	8.25	8.55	3.6
Tiagabine****	0.0529	0.0492	-7.5	0.104	0.0952	-9.2
Topiramate****	4.09	4.40	7.0	7.75	8.09	4.2
Valproic acid***	75.2	72.6	-3.6	105	99.3	-5.7
Zonisamide****	13.3	13.5	1.2	27.0	26.9	-0.4

* RV Instand 191 Pr11 & Pr12

** RV Instand 192 Pr11 & Pr12

*** RV Instand 195 Pr41 & Pr42

**** RV GTFCH TDMB PrA & PrB

Table 7. Intra-assay precision results for control MS9282 batch #1388

Analyte	Control 1						Control 2					
	Day 1		Day 2		Day 3		Day 1		Day 2		Day 3	
	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average Calculated concentration (ng/mL)	CV (%)	Average Calculated concentration (ng/mL)	CV (%)	Average Calculated concentration (ng/mL)	CV (%)	Average Calculated concentration (ng/mL)	CV (%)
10-OH-Carbamazepine	8.41	5.1	8.47	2.2	8.27	3.2	20.2	5.6	20.3	4.0	19.9	2.2
Brivaracetam	0.732	6.2	0.748	3.4	0.714	5.1	1.72	1.6	1.74	3.3	1.67	7.3
Carbamazepine	4.59	4.5	4.41	5.3	4.39	4.6	10.4	2.3	9.93	2.2	10.3	2.7
Carbamazepine-diol	1.91	3.5	1.85	4.8	1.82	3.1	4.48	1.7	4.42	3.7	4.75	3.8
Carbamazepine-epoxide	2.20	6.8	1.89	5.2	2.08	1.2	4.85	7.9	4.79	1.7	4.77	2.2
Ethosuximide	23.66	1.2	22.97	3.1	23.54	1.3	55.5	2.1	54.9	2.9	55.5	1.0
Felbamate	20.3	3.3	20.0	2.0	20.4	3.2	48.0	1.4	47.7	3.7	46.7	3.7
Gabapentine	5.91	1.4	5.84	2.0	5.62	1.7	13.7	1.0	13.9	2.3	13.5	1.0
Lacosamide	2.67	2.4	2.63	2.7	2.58	4.7	6.21	1.8	6.46	2.8	5.96	3.3
Lamotrigine	5.11	1.6	4.89	0.7	4.97	1.0	11.8	0.6	11.7	1.1	11.5	1.9
Levetiracetam	13.3	7.2	12.9	3.2	12.8	0.2	31.7	5.8	31.3	1.9	30.7	2.0
NDMS	9.50	2.9	9.52	2.7	9.56	1.9	22.2	1.4	21.9	1.0	22.2	3.4
Oxcarbazepine	0.399	2.0	0.405	4.5	0.432	3.7	0.851	4.4	0.833	3.2	0.779	3.1
PEMA	2.22	2.2	2.19	2.5	2.17	3.0	5.26	1.7	5.20	3.1	5.17	3.7
Perampanel	0.288	3.3	0.274	1.8	0.267	1.6	0.653	2.4	0.640	2.6	0.620	4.7
Phenobarbital	10.19	1.1	10.44	1.6	10.36	1.2	23.5	1.3	23.8	1.7	23.4	2.1
Pregabalin	1.71	3.1	1.75	1.1	1.69	1.5	4.15	0.7	4.25	2.5	4.11	3.6
Primidone	5.44	3.1	5.39	4.6	5.04	8.1	12.7	3.9	13.1	5.7	13.1	6.3
Retigabine	0.425	2.1	0.411	5.8	0.419	4.7	1.05	2.7	1.00	7.8	0.995	4.3
Rufinamide	9.28	1.4	9.47	1.8	8.96	2.2	21.7	1.5	22.4	2.5	20.8	2.9
Stiripentol	3.52	9.3	3.54	8.1	3.38	0.0	8.47	6.9	8.59	9.1	8.11	0.0
Sulthiame	2.67	1.6	2.65	2.7	2.61	1.7	6.0	0.9	6.2	1.9	6.0	1.3
Tiagabine	0.0546	1.0	0.0558	2.3	0.0572	4.4	0.130	3.4	0.137	4.6	0.131	2.6
Topiramate	3.67	2.9	3.61	2.8	3.47	0.0	8.4	2.3	8.2	1.5	8.2	0.0
Valproic acid	23.4	1.7	23.0	1.8	23.0	2.0	53.0	1.8	52.9	1.6	51.5	3.3
Zonisamide	8.63	0.9	8.65	0.9	8.68	1.9	18.9	1.1	20.0	1.8	19.8	2.3

Table 8. Inter-assay precision results for control MS9282 batch #1388

Analyte	Control 1		Control 2	
	Average calculated concentration (ng/mL)	CV (%)	Average Calculated concentration (ng/mL)	CV (%)
10-OH-Carbamazepine	8.38	3.6	20.1	4.0
Brivaracetam	0.731	5.0	1.71	4.6
Carbamazepine	4.47	4.9	10.2	3.1
Carbamazepine-diol	1.86	4.1	4.55	4.4
Carbamazepine-epoxide	2.06	7.9	4.80	4.6
Ethosuximide	23.39	2.3	55.3	2.0
Felbamate	20.2	2.8	47.5	3.1
Gabapentine	5.79	2.7	13.7	1.9
Lacosamide	2.62	3.5	6.21	4.2
Lamotrigine	4.99	2.2	11.7	1.6
Levetiracetam	13.0	4.7	31.2	3.7
NDMS	9.53	2.4	22.1	2.2
Oxcarbazepine	0.412	4.9	0.821	5.1
PEMA	2.19	2.7	5.21	2.8
Perampanel	0.277	3.9	0.638	3.8
Phenobarbital	10.33	1.6	23.6	1.7
Pregabalin	1.72	2.4	4.17	2.8
Primidone	5.29	6.2	13.0	5.3
Retigabine	0.418	4.4	1.01	5.4
Rufinamide	9.23	2.9	21.6	3.9
Stiripentol	3.48	7.0	8.39	6.7
Sulthiame	2.64	2.2	6.1	1.8
Tiagabine	0.0559	3.4	0.133	4.2
Topiramate	3.58	3.2	8.3	1.8
Valproic acid	23.39	2.3	55.3	2.0
Zonisamide	8.65	1.3	19.6	3.0

Conclusions

An HRAM mass spectrometry-based method (a Vanquish Duo system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap MS) is reported here, demonstrating the power of Orbitrap technology in performing accurate qualitative analyses and routine quantitation with high efficiency. Two liquid chromatography-HRAM MS methods for clinical research were developed and implemented for

quantification of 26 different antiepileptic drugs in human plasma or serum. The ClinMass TDM Platform with the ClinMass Add-On Set for Antiepileptics from RECIPE was used. The method incorporates a quick and simple offline protein precipitation step with concomitant internal standard addition. The described analytical method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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