TECHNICAL NOTE 000110

Quantification of 27 antiepileptics in human plasma by LC-HRAM-MS for clinical research

Author: Mariana Barcenas
Thermo Fisher Scientific, Les Ulis, France

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Application benefits

- Accurate and confident results, simple sample preparation, and rapid quantitation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive LC and MS platforms enable increased confidence in data

Goal

Implementation of an analytical method for the quantification of 27 antiepileptics in human plasma on a Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer coupled to a Thermo Scientific™ Vanquish™ Flex Binary UHPLC.

Introduction

Antiepileptic drugs (AED) are used to help alleviate the symptoms associated with epilepsy, a chronic neurological disorder characterized by recurrent epileptic seizures.

Analysis and quantitation of antiepileptic drugs can pose significant challenges due to the large number of analytes in this class as well as the varied concentrations at which these drugs can be found in human plasma.

In this study, an analytical method for clinical research for the quantification of 27 antiepileptics in human plasma is reported. Plasma samples were extracted by internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. Two injections were performed for each analysis batch, one for positive and one for negative ion mode.

Method performance was evaluated using calibrators, controls, and internal standards from RECIPE® Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, and intra- and inter-assay precision for the entire panel of analytes.

Experimental

Target analytes

The list of analytes and their corresponding internal standards are summarized in Table 1. Concentration ranges covered by the calibrators used are also reported in Table 1.

Sample preparation

Reagents included four calibrators (including blank) and two controls from RECIPE, as well as internal standards for quantification. Samples of 50 µL of plasma were protein precipitated using 100 µL of acetonitrile containing the internal standards. Precipitated samples were vortex-mixed and centrifuged. The supernatant was transferred to a clean vial and diluted 10-fold using mobile phase A prior to injection.

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

Analyte	Internal standard	Retention time (min)	Concentration (ng/mL)		
			L1	L2	L3
10-OH-Carbamazepine	10-OH-Carbamazepine-d_4	3.16	2.56	14.1	40.9
Brivaracetam	Brivaracetam-d_7	3.33	0.299	1.49	40.9
Carbamazepine	Carbamazepine-d_{10}	3.56	1.46	7.56	21.3
Carbamazepine-diol	10-OH-Carbamazepine-d_4	3.09	0.506	2.57	8.03
Carbamazepine-epoxide	Carbamazepine-d_{10}	3.17	0.458	2.51	7.27
Ethosuximide	Ethosuximide-d_3	2.34	8.34	39.2	120
Felbamate	Felbamate-d_4	2.91	6.92	35.5	104
Gabapentine	Gabapentine-d_4	2.37	1.72	8.46	26.7
Lacosamide	Lacosamide-d_3	2.87	0.924	5.03	14.8
Lamotrigine	Lamotrigine-$^{13}C_3$	2.88	1.37	7.22	20.9
Levetiracetam	Levetiracetam-d_6	2.26	4.19	21.9	67.1
N-Desmethylmethsuximide	Ethosuximide-d_3	3.12	3.44	15.4	45.0
Oxcarbazepine	Oxcarbamazepine-d_4	3.28	0.258	1.49	4.64
Perampanel	Perampanel-d_5	3.93	0.0936	0.481	1.43
Phenobarbital	Phenobarbital-d_5	3.12	3.61	18.0	53.7
Phenylethylmalonamide	Phenylethylmalonamide-d_5	2.49	0.713	3.93	11.6
Phenytoin	Phenytoin-d_{10}	3.49	1.92	9.00	28.9
Pregabaline	Pregabaline-d_4	2.36	0.571	3.13	9.95
Primidone	Primidone-d_5	2.91	1.60	9.16	28.2
Retigabine	Retigabine-d_4	3.24	0.126	0.715	2.21
Rufinamide	Rufinamide-$^{15}N_2$	2.86	3.03	15.7	46.8
Stiripentol	Stiripentol-d_9	4.29	1.12	5.36	15.8
Sulthiame	Sulthiame-d_4	2.51	0.944	4.24	12.4
Tiagabine	Tiagabine-d_6	3.76	0.0221	0.114	0.345
Topiramate	Topiramate-d_{12}	3.14	1.22	5.95	17.7
Valproic acid	Valproic acid-d_6	4.24	8.56	38.1	112
Zonisamide	Zonisamide-^{15}N,d_4	2.62	3.06	14.3	43.8

Liquid chromatography

LC separation was performed on a Vanquish Flex Binary UHPLC system using the following mobile phases:

- Mobile phase A: 0.1% formic acid in water
- Mobile phase B: 0.1% formic acid in methanol

Chromatographic separation was achieved by gradient elution on a Thermo Scientific™ Hypersil GOLD™ 2.1 × 50 mm (1.9 µm) analytical column (P/N 25002-052130) run at 40 °C at a flow rate of 0.4 mL/min. The injection volume was 5 µL in positive ion mode and 10 µL in negative ion mode. The total run time was 5.5 minutes. The chromatographic conditions used for both injections are given in Table 2.

Table 2. LC method description

Time (min)	Flow (mL/min)	B (%)
0.00	0.4	2
0.80	0.4	2
2.50	0.4	55
3.40	0.4	55
3.50	0.4	98
4.50	0.4	98
4.51	0.4	2
5.50	0.4	2
Other parameters		
Column temp.	40 °C	
Injection volume	5 µL (Positive ion mode)/ 10 µL(Negative ion mode)	

Mass spectrometry

Detection was performed on an Orbitrap Exploris 120 mass spectrometer, equipped with a Heated Electrospray Ionization (HESI) ion source operated in positive and negative ionization mode. Data was acquired in Full MS-ddMS^2 mode using a resolution of 60,000 (FWHM) at *m/z* 200 on a scan range of *m/z* 40 to 400. The ion source conditions and the mass spectrometer settings are presented in Table 3 and Table 4, respectively.

Method evaluation

The parameters used to evaluate the performance of the method included linearity of response, LLOQ, intra- and inter-assay accuracy and precision, and carryover for all the analytes. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at two different levels provided by RECIPE (MS9282 batch #1388) prepared and analyzed in replicates of five on three different days. 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). The LLOQ was investigated by dilution of the lowest calibrator with blank matrix and was established as the lowest concentration with a mean accuracy and precision better than 20%.

Table 3. Ion source settings

	Polarity	
	Positive	Negative
Source type	Heated Electrospray Ionization (HESI)	
Vaporizer temperature	375 °C	
Ion transfer tube temperature	325 °C	
Ion spray voltage	3,500 V	2,800 V
Sheath gas	45 AU	
Sweep gas	0 AU	
Auxiliary gas	8 AU	12 AU

Table 4. MS settings

	Polarity	
	Positive	Negative
Resolution	60,000	
Scan range	40–400	
AGC target	Standard	
RF lens	85%	100%
Maximum injection time mode	Auto	
Data type	Profile	
Source fragmentation	Off	
Mild trapping	On	

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A summary of the LLOQs obtained for all the analytes is presented in Table 5. A linear regression with 1/× weighting down to the LLOQ value was obtained for all the analytes. The percentage bias between nominal and back-calculated concentration was always within ±15% for all the calibrators in all the runs.

Representative chromatograms for the LLOQ for gabapentine, lamotrigine, and sulthiame and their corresponding internal standards are reported in Figure 1, while the respective calibration curves are reported in Figure 2.

A maximum carryover of 0.2% was observed for gabapentine and pregabaline; no significant carryover was observed for the rest of the analytes.

Table 5. Lower limit of quantitation

Analyte	Concentration (ng/mL)	
	Lowest calibrator	LLOQ
10-OH-Carbamazepine	2.56	0.512
Brivaracetam	0.299	0.0598
Carbamazepine	1.46	0.730
Carbamazepine-diol	0.506	0.169
Carbamazepine-epoxide	0.458	0.153
Ethosuximide	8.34	8.34
Felbamate	6.92	1.38
Gabapentine	1.72	0.344
Lacosamide	0.924	0.308
Lamotrigine	1.37	0.274
Levetiracetam	4.19	2.10
N-Desmethylmethsuximide	3.44	1.72
Oxcarbazepine	0.258	0.086
Perampanel	0.0936	0.0312
Phenobarbital	3.61	1.20
Phenylethylmalonamide	0.713	0.238
Phenytoin	1.92	0.640
Pregabaline	0.571	0.114
Primidone	1.60	0.533
Retigabine	0.126	0.042
Rufinamide	3.03	0.303
Stiripentol	1.12	0.373
Sulthiame	0.944	0.315
Tiagabine	0.0221	0.0111
Topiramate	1.22	0.610
Valproic acid	8.56	8.56
Zonisamide	3.06	0.612

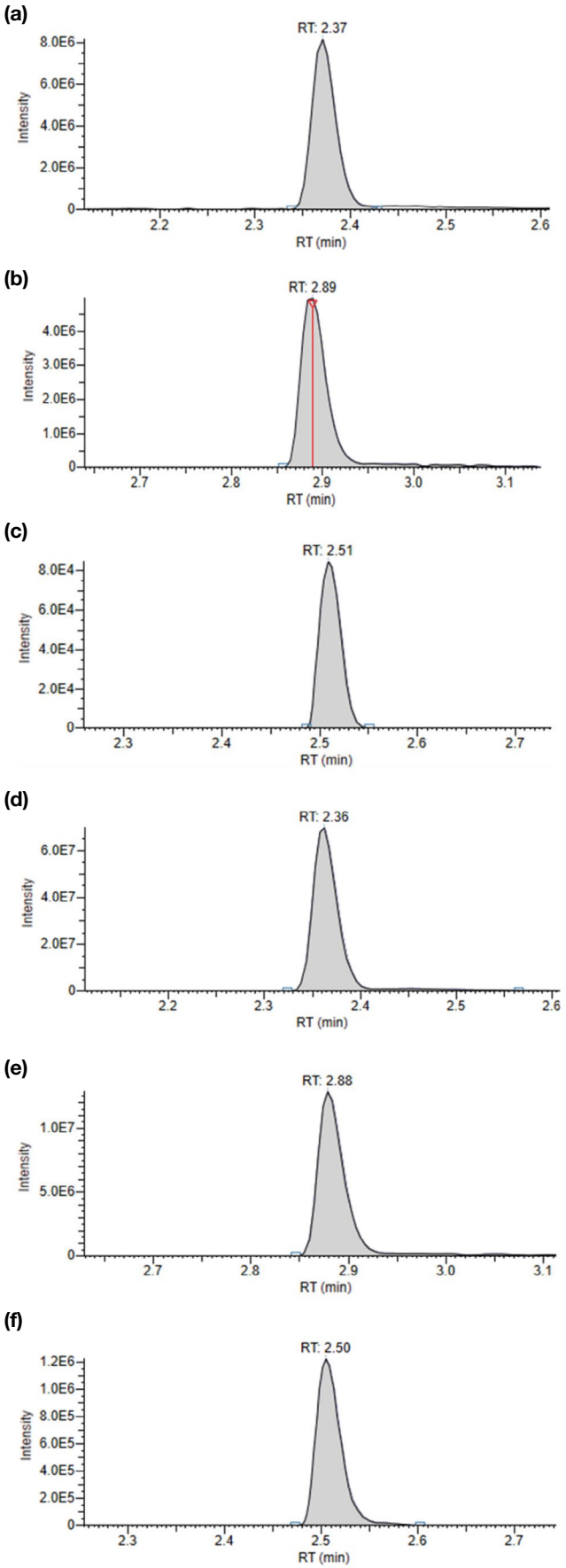

Figure 1. Representative chromatograms of the LLOQ for (a) gabapentine, (b) lamotrigine, (c) sulthiame, (d) gabapentine-d_4, (e) lamotrigine-$^{13}C_3$, and (f) sulthiame-d_4

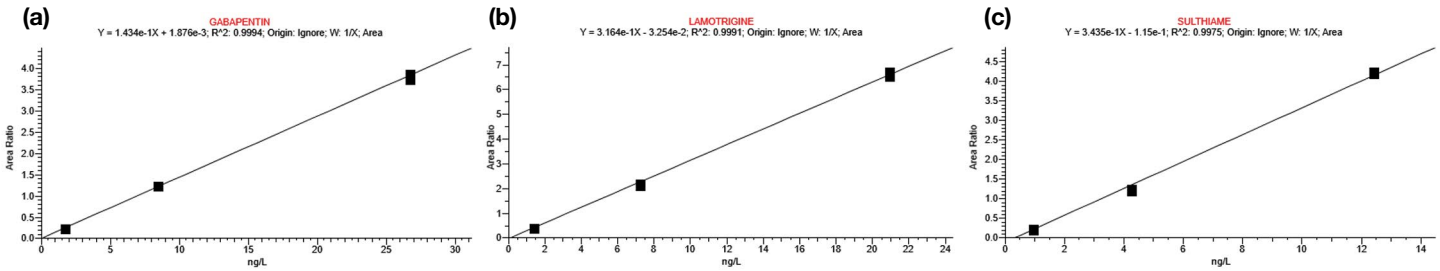

Figure 2. Representative calibration curves for (a) gabapentine, (b) lamotrigine, and (c) sulthiame

The percentage bias between nominal and average back-calculated concentration for the used control samples ranged between -8.8 % and 11 % (Table 6). The %CV for intra-assay precision was always below 9.1% for all the analytes (Table 7). The maximum %CV for inter-assay precision including all the analytes was 7.7% (Table 8).

Table 6. Analytical accuracy results for control MS9282 batch #1388

Analyte	CTRL1			CTRL2		
	Nominal concentration (ng/L)	Average calculated concentration (ng/L)	Bias %	Nominal concentration (ng/L)	Average calculated concentration (ng/L)	Bias %
10-OH-Carbamazepine	7.89	8.11	2.8	18.9	20.0	5.9
Brivaracetam	0.75	0.73	-2.2	1.71	1.81	5.9
Carbamazepine	4.53	4.73	4.5	9.95	10.5	5.6
Carbamazepine-diol	1.74	1.62	-6.7	4.19	4.26	1.6
Carbamazepine-epoxide	1.74	1.71	-1.8	4.03	4.27	5.9
Ethosuximide	23.2	22.9	-1.3	53.3	54.7	2.6
Felbamate	19.9	19.2	-3.6	49.0	50.6	3.3
Gabapentine	5.30	5.54	4.5	12.5	13.8	10.1
Lacosamide	7.89	8.11	2.8	18.9	20.0	5.9
Lamotrigine	4.60	4.58	-0.4	10.7	11.4	6.9
Levetiracetam	13.2	13.5	2.5	29.7	33.0	11.0
N-Desmethylmethsuximide	9.01	8.48	-5.8	20.8	21.6	3.8
Oxcarbazepine	0.451	0.454	0.6	0.881	0.929	5.4
Perampanel	0.266	0.275	3.3	0.638	0.677	6.1
Phenobarbital	10.3	10.8	5.1	24.5	26.6	8.4
Phenylethylmalonamide	2.22	2.25	1.4	5.30	5.68	7.1
Phenytoin	5.30	5.25	-0.9	12.8	13.1	2.2
Pregabaline	1.71	1.81	6.0	4.18	4.58	9.7
Primidone	5.06	5.18	2.4	12.5	13.4	6.9
Retigabine	0.411	0.406	-1.1	1.02	1.06	3.5
Rufinamide	8.88	9.03	1.6	21.5	22.5	4.4
Stiripentol	3.38	3.46	2.5	8.11	8.50	4.8
Sulthiame	2.59	2.44	-5.8	6.07	6.03	-0.7
Tiagabine	0.0600	0.0584	-2.7	0.143	0.151	5.8
Topiramate	3.47	3.17	-8.8	8.23	8.12	-1.3
Valproic acid	23.4	23.5	0.2	52.7	55.9	6.0
Zonisamide	8.37	8.51	1.6	19.7	20.7	5.0

Table 7 (part 1). Analytical intra-assay precision results for control MS9282 (CTRL1) batch #1388

Analyte	CTRL1					
	Day 1		Day 2		Day 3	
	Average calculated concentration (ng/L)	CV %	Average calculated concentration (ng/L)	CV %	Average calculated concentration (ng/L)	CV %
10-OH-Carbamazepine	8.04	2.5	7.95	3.9	8.35	3.8
Brivaracetam	0.729	6.4	0.737	4.0	0.731	5.1
Carbamazepine	4.66	2.9	4.69	3.3	4.85	4.1
Carbamazepine-diol	1.60	1.6	1.60	1.3	1.68	6.3
Carbamazepine-epoxide	1.75	3.0	1.66	2.3	1.72	6.6
Ethosuximide	21.7	3.6	22.2	4.6	24.8	3.7
Felbamate	19.2	4.9	18.8	3.4	19.6	7.7
Gabapentine	5.56	2.9	5.43	1.4	5.63	3.8
Lacosamide	8.04	2.5	7.95	3.9	8.35	3.8
Lamotrigine	4.55	2.6	4.50	3.1	4.70	4.9
Levetiracetam	13.3	1.7	13.3	4.3	14.0	3.8
N-Desmethylmethsuximide	8.07	1.7	8.48	2.0	8.91	7.0
Oxcarbazepine	0.430	2.4	0.451	7.3	0.480	2.3
Perampanel	0.268	3.2	0.281	3.2	0.275	4.9
Phenobarbital	11.1	2.0	10.7	2.6	10.7	4.6
Phenylethylmalonamide	2.25	2.3	2.18	2.2	2.33	4.4
Phenytoin	5.30	5.9	5.11	5.5	5.34	8.1
Pregabaline	1.84	3.2	1.79	2.6	1.82	3.4
Primidone	5.09	4.1	5.12	0.6	5.33	3.6
Retigabine	0.403	3.7	0.396	1.6	0.423	4.8
Rufinamide	8.96	2.4	8.89	3.4	9.23	4.2
Stiripentol	3.48	4.2	3.42	2.6	3.49	5.1
Sulthiame	2.44	2.7	2.40	1.7	2.48	6.8
Tiagabine	0.0606	6.0	0.055	1.3	0.060	6.3
Topiramate	3.12	6.0	3.29	3.2	3.09	7.4
Valproic acid	22.2	9.1	23.2	5.3	25.0	2.9
Zonisamide	8.62	3.2	8.47	2.1	8.43	5.9

Table 7 (part 2). Analytical intra-assay precision results for control MS9282 (CTRL2) batch #1388

Analyte	CTRL2					
	Day 1		Day 2		Day 3	
	Average calculated concentration (ng/L)	CV %	Average calculated concentration (ng/L)	CV %	Average calculated concentration (ng/L)	CV %
10-OH-Carbamazepine	20.2	3.0	19.6	2.7	20.3	1.8
Brivaracetam	1.81	1.3	1.82	2.4	1.80	3.6
Carbamazepine	10.5	2.0	10.5	1.5	10.6	2.0
Carbamazepine-diol	4.10	5.5	4.14	1.8	4.50	2.4
Carbamazepine-epoxide	4.28	1.6	4.15	3.8	4.38	0.9
Ethosuximide	53.2	5.4	55.8	1.7	55.1	6.1
Felbamate	51.0	2.7	49.1	5.0	51.7	4.5
Gabapentine	14.4	2.5	13.5	2.0	13.4	2.1
Lacosamide	20.2	3.0	19.6	2.7	20.3	1.8
Lamotrigine	11.8	3.5	11.3	2.4	11.2	3.4
Levetiracetam	34.9	4.9	31.9	2.4	32.1	1.1
N-Desmethylmethsuximide	20.8	8.3	21.8	2.1	22.1	3.0
Oxcarbazepine	0.930	1.5	0.921	4.8	0.935	1.7
Perampanel	0.677	4.3	0.662	4.1	0.692	0.7
Phenobarbital	26.8	2.0	26.3	1.9	26.6	0.5
Phenylethylmalonamide	5.84	3.1	5.51	2.7	5.67	1.3
Phenytoin	13.5	2.9	12.8	6.6	12.9	2.3
Pregabaline	4.93	3.5	4.41	2.3	4.41	2.2
Primidone	13.5	5.9	13.1	0.7	13.4	1.4
Retigabine	1.10	2.2	1.02	2.7	1.05	3.5
Rufinamide	23.0	2.2	21.8	3.2	22.6	4.0
Stiripentol	8.65	1.9	8.40	1.9	8.43	2.8
Sulthiame	6.24	4.1	5.88	5.1	5.97	5.0
Tiagabine	0.153	2.3	0.148	3.7	0.153	1.5
Topiramate	8.16	7.2	8.12	2.5	8.08	5.0
Valproic acid	57.4	8.3	53.8	5.3	56.4	1.4
Zonisamide	21.0	3.7	20.6	4.1	20.4	3.2

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

Analyte	CTRL1		CTRL2	
	Average calculated concentration (ng/L)	CV %	Average calculated concentration (ng/L)	CV %
10-OH-Carbamazepine	8.11	3.9	20.0	2.9
Brivaracetam	0.732	4.9	1.81	2.5
Carbamazepine	4.73	3.7	10.5	1.8
Carbamazepine-diol	1.62	4.4	4.26	5.3
Carbamazepine-epoxide	1.71	4.7	4.27	3.1
Ethosuximide	22.9	7.2	54.7	4.9
Felbamate	19.2	5.5	50.6	4.5
Gabapentine	5.54	3.1	13.8	3.9
Lacosamide	8.11	3.9	20.0	2.9
Lamotrigine	4.58	3.9	11.4	3.9
Levetiracetam	13.5	4.1	33.0	5.4
N-Desmethylmethsuximide	8.48	5.9	21.6	5.5
Oxcarbazepine	0.454	6.4	0.929	2.9
Perampanel	0.275	4.0	0.677	3.7
Phenobarbital	10.8	3.4	26.6	1.8
Phenylethylmalonamide	2.25	4.1	5.68	3.4
Phenytoin	5.25	6.5	13.1	4.7
Pregabaline	1.81	3.1	4.58	6.1
Primidone	5.18	3.7	13.4	3.6
Retigabine	0.406	4.3	1.06	4.2
Rufinamide	9.03	3.6	22.5	3.8
Stiripentol	3.46	3.9	8.50	2.4
Sulthiame	2.44	4.4	6.03	5.1
Tiagabine	0.0584	6.6	0.151	3.0
Topiramate	3.17	6.1	8.12	4.9
Valproic acid	23.5	7.7	55.9	6.1
Zonisamide	8.51	3.9	20.7	3.6

Conclusions

A robust, reproducible, and sensitive liquid chromatography-high resolution mass spectrometry method for clinical research for quantification of 27 antiepileptics in human plasma was developed, implemented, and analytically validated. Sample preparation is based on a rapid and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy and precision.

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