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TECHNICAL NOTE 65139

An LC-MS/MS method for the quantification of 19 antiepileptic drugs in human plasma for clinical research use

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Keywords

Antiepileptics, antiepileptic drugs, AEDs, drug monitoring research, offline sample preparation, plasma, TSQ Endura

Goal

Implementation of an analytical method for the quantification of 19 antiepileptics and their metabolites in human plasma using a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer for clinical research use

Application benefits

- One quantitative method for 19 antiepileptics
- Chromatographic separation of isobaric analytes
- Universal sample preparation method for all the analytes tested
- Rapid analytical method

Introduction

Analysis and quantitation of antiepileptic drugs can pose significant challenges owing to the large size of this drug type, as well as the varied concentrations at which these drugs can be found in human plasma. A quantitative workflow that can offer robust, sensitive, reproducible, and reliable data is expected to monitor many compounds simultaneously, over several orders of magnitude. Liquid chromatography-mass spectrometry (LC-MS/MS) technology is widely accepted for this type of analysis.

In this report, a robust assay for quantification of 19 different antiepileptics in human plasma is reported. Fourteen stable isotope labeled internal standards are used for the quantification. Regardless of the different analytical ranges, from tens of µg/L to hundreds of mg/L, a unique and simple sample preparation protocol was applied to the entire panel of analytes. Sample preparation is based on a protein precipitation step followed by dilution of

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the supernatant and injection onto Thermo Scientific™ UltiMate™ 3000 RS LC system. Two different injection volumes were used for positively and negatively ionizing compounds. Detection was performed using a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization (HESI) operated in selected reaction monitoring (SRM) mode.

Linearity ranges of 500× were tested. Method performance was evaluated in terms of lower limit of quantification (LLOQ), linearity range, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

The list of analytes and corresponding concentration ranges tested for the analytical validation process are reported in Table 1.

Sample preparation

To prepare the samples, 100 µL of internal standard mix in acetonitrile were added to 50 µL of calibrators, controls, or plasma samples in centrifuge tubes. Precipitated samples were vortexed and centrifuged. The supernatant was diluted to a 1:10 ratio using mobile phase A in LC micro vials for analysis.

Liquid chromatography

An injection volume of 5 µL was used for positively ionizing compounds and 10 µL for the negatively ionizing compounds. A 5.5-minute gradient elution was performed using an UltiMate 3000 RS LC system. Mobile phases consisted of 0.1% formic acid in water and methanol (Fisher Chemical™ Optima™ grade) for mobile phases A and B, respectively. Chromatographic separation was achieved using a 50 × 2.1 mm (1.9 µm) Thermo Scientific™ Hypersil GOLD™ analytical column. Details of the chromatographic method are reported in Table 2.

Table 1. Commonly analyzed ranges and concentration ranges covered by the calibrators.

Compound	Internal Standards	Commonly Analyzed Ranges (mg/L)	Ref.	Calibration Range (mg/L)
Vigabatrin	Pregabalin-$^{2}H_{6}$	1–36	c	0.10–50
Pregabalin	Pregabalin-$^{2}H_{6}$	0.15–10	a	0.03–15
Levetiracetam	Levetiracetam-$^{2}H_{6}$	10–43	a	0.20–100
Gabapentin	Gabapentin-$^{2}H10$	2–20	b, c	0.05–25
Theophylline	Theophylline-$^{2}H_{6}$	6–25	a	0.05–25
Primidone	Gabapentin-$^{2}H_{10}$	5–15	b	0.05–25
Carbamazepine	Carbamazepine-$^{2}H_{10}$	2–10	a	0.03–15
Lacosamide	Lacosamide-$^{13}C,^{2}H_{3}$	1–10	b	0.03–15
Felbamate	Levetiracetam-$^{2}H_{6}$	2–100	b	0.29–150
Phenytoin	Phenytoin-$^{2}H_{10}$	5–20	a, b	0.05–25
Oxcarbazepine	Oxcarbazepine-$^{13}C_{6}$	0.4–2	a	0.01–5
Carbamazepine-10,11-epoxide	Oxcarbazepine-$^{13}C_{6}$	0.2–9	c	0.03–15
10,11 Dihydro-10-hydroxycarbazepine	Oxcarbazepine-$^{13}C_{6}$	3–40	c	0.10–50
Lamotrigine	Lamotrigine-$^{13}C,^{15}N_{4}$	3–14	c	0.05–25
Valproic Acid	Valproic Acid-$^{2}H_{6}$	40–100	a, b	0.29–150
Tiagabine	Tiagabine-$^{2}H_{6}$	0.02–0.2	a	0.01–5
Zonisamide	Zonisamide-$^{13}C_{6}$	10–38	c	0.10–50
Topiramate	Topiramate-$^{2}H_{12}$	5–25	c	0.10–50
Phenobarbital	Phenobarbital-$^{2}H_{5}$	10–40	b	0.20–100

[a] R. Ludewig und R. Regenthal (Hrsg.), Akute Vergiftungen und Arzneimittelüberdosierungen, 10. Auflage, 2007, WVG Stuttgart.

[b] Gesellschaft für Epilepsieforschung e.V., Serumkonzentrationen von Antiepileptika (mittlere Wirkungsbereiche), Homepage – Stand August 2011.

[c] Neels H.M. et al. Clin Chem Lab Med 42: 1228–1255 (2004).

Table 2. HPLC settings.

LC system:	UltiMate 3000 RS Binary Gradient LC		
Analytical column:	Hypersil GOLD 50 × 2.1 mm (1.9 µm), held at 30 °C		
Mobile phases:	A: Water + 0.1 % formic acid B: Methanol + 0.1 % formic acid		
Gradient profile:			
Time (min)	*Flow Rate (mL/min)*	*A (%)*	*B (%)*
0.0	0.35	98	2
0.8	0.35	98	2
2.5	0.35	45	55
3.4	0.35	45	55
3.5	0.35	2	98
4.5	0.35	98	2
5.5	0.35	98	2
Injection volume:	Positives, 5 µL Negatives, 10 µL		

Table 3. MS settings.

Source type:	Heated electrospray ionization (HESI)
Vaporizer temperature:	350 °C
Capillary temperature:	350 °C
Spray voltage positive ionization:	3500 V
Spray voltage negative ionization:	2800 V
Sheath gas:	42 AU
Sweep gas:	1 AU
Auxiliary gas:	12 AU
Data acquisition mode:	Selected reaction monitoring (SRM)
Chrom filter peak width:	3.0 s
Collision gas pressure:	1.5 mTorr
Cycle time:	0.500 s
Q1 mass resolution (FWMH):	0.7
Q3 mass resolution (FWMH):	0.7

Mass spectrometry

Target analytes and internal standards were detected by scheduled SRM on a TSQ Endura triple quadrupole mass spectrometer with HESI. MS settings are reported in Table 3.

A 0.5-minute acquisition window was used for each analyte, and two SRM transitions were included in the acquisition method for quantification and confirmation, except for valproic acid, for which only a pseudo SRM was used. SRM transitions together with the corresponding RF lens and collision energy values are reported in Table 4.

Method evaluation

Method performance was evaluated by obtaining limit of quantification (LOQ), linearity range, accuracy, and intra- and inter-assay precision for each analyte. Ten calibration levels containing all analytes were prepared in triplicate to evaluate sensitivity and linearity. A maximum percentage bias between nominal and back-calculated concentration of 15% was set as the acceptance criterion for all the calibrators (20% for the LOQs). Control samples containing all the analytes at four different levels were prepared in replicates of five to evaluate the precision of the analytical method. A maximum percentage bias between nominal and back-calculated concentration of 20% was set as the acceptance criterion for all the control samples. Accuracy for the assay was evaluated in terms of trueness of measurement measuring the percentage bias between nominal and average back-calculated concentration for the calibrators. Intra-assay precision was evaluated in terms of percentage RSD using the control samples in replicates of five (n=5) analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of five prepared and analyzed on three different days (n=15).

Matrix effect for the assay was measured in terms of recovery for each analyte: it was evaluated in triplicate in terms of percentage ratio between the analyte / internal standard peak area ratio in calibrator 5 and the same ratio in water at the same concentration.

Data analysis

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

Table 4. SRM settings for target compounds.

Compound	Ionization Mode	Precursor Ion (*m/z*)	RF Lens (V)	Product Ion (*m/z*)	Collision Energy (V)	Quan. Ion	Qual. Ion
Vigabatrin	Positive	130.1	73	113.1	10	•	
				71.2	15		•
Levetiracetam	Positive	171.1	79	126.1	15	•	
				69.2	28		•
Theofylline	Positive	181.0	130	124.1	19	•	
				96.1	23		•
Pregabalin	Positive	160.2	101	142.1	10	•	
				97.2	15		•
Gabapentin	Positive	172.2	112	91.2	26	•	
				137.1	17		•
Lacosamide	Positive	251.1	75	108.1	10	•	
				91.2	20		•
Lamotrigine	Positive	255.9	164	211.0	27	•	
				145.0	38		•
Pirimidone	Positive	219.2	113	162.1	13	•	
				106.1	19		•
Topiramate	Positive	357.0	119	264.0	15	•	
				282.0	12		•
Felbamate	Positive	261.1	115	200.0	13	•	
				115.0	40		•
10,11 Dihydro-10-hydroxycarbazepine	Positive	255.1	99	194.0	21	•	
				237.1	10		•
Carbamazepine-10,11-epoxide	Positive	253.0	97	180.1	28	•	
				210.0	14		•
Oxcarbazepine	Positive	253.1	113	180.1	31	•	
				208.1	20		•
Carbamazepine	Positive	237.0	130	194.0	19	•	
				179.1	35		•
Tiagabine	Positive	376.0	132	247.3	17	•	
				149.1	25		•
Zonisamide	Negative	211.0	80	119.0	14	•	
				147.0	10		•
Phenobarbital	Negative	230.9	106	188.0	18	•	
				144.0	14		•
Phenytoine	Negative	250.9	140	208.1	17	•	
				102.0	21		•
Valproic Acid	Negative	143.0	95	143.0	8	•	

Results and discussion

The method proved to meet research laboratory requirements in terms of linearity, being linear not only in the commonly analyzed ranges for all the compounds (Table 1) but also in wider ranges covered by the calibrators obtained by serial dilutions. The obtained correlation factors (R^2) were always above 0.99. A summary of calibration performance and the obtained linearity ranges for all the target analytes are reported in Tables 5 and 6, respectively. The quantitative performances of the method show that it is robust and reliable even if more than four magnitude orders of concentration are covered. Moreover, its overall sensitivity is enough to meet standard requirements of clinical research laboratories. In fact, the LLOQs obtained for all the compounds are always lower than the inferior limits of the reference ranges for the drugs monitoring research.

Table 5. Calibration performance summary.

Compound	Retention Time (min)	Internal Standard	Curve Type	Origin	Weighting	R^2	LOQ (mg/L)
Vigabatrin	0.54	Pregabalin-$^{2}H_{6}$	Linear	Ignore	1/X	0.9966	0.10
Levetiracetam	2.71	Levetiracetam-$^{2}H_{6}$	Linear	Ignore	1/X	0.9987	0.20
Theophylline	2.76	Theophylline-$^{2}H_{6}$	Linear	Ignore	1/X	0.9992	0.10
Pregabalin	2.86	Pregabalin-$^{2}H_{6}$	Linear	Ignore	1/X	0.9979	0.03
Gabapentin	2.87	Gabapentin-$^{2}H_{10}$	Linear	Ignore	1/X	0.9991	0.05
Zonisamide	3.08	Zonisamide-$^{13}C_{6}$	Linear	Ignore	1/X	0.9983	0.20
Lacosamide	3.34	Lacosamide-$^{13}C,^{2}H_{3}$	Linear	Ignore	1/X	0.9973	0.03
Lamotrigine	3.35	Lamotrigine-$^{13}C,_{15}N_{4}$	Linear	Ignore	1/X	0.9965	0.10
Felbamate	3.36	Levetiracetam-$^{2}H_{6}$	Quadratic	Ignore	Equal	0.9938	4.69
Primidone	3.39	Gabapentin-$^{2}H_{10}$	Quadratic	Ignore	1/X	0.9983	0.10
Phenobarbital	3.57	Phenobarbital-$^{2}H_{5}$	Linear	Ignore	1/X	0.9942	0.78
Topiramate	3.59	Topiramate-$^{2}H_{12}$	Quadratic	Ignore	1/X	0.9941	0.20
10,11 Dihydro-10-hydroxycarbazepine	3.63	Oxcarbazepine-$^{13}C_{6}$	Quadratic	Ignore	1/X	0.9966	0.20
Carbamazepine-10,11-epoxide	3.65	Oxcarbazepine-$^{13}C_{6}$	Quadratic	Ignore	1/X	0.9973	0.06
Oxcarbazepine	3.83	Oxcarbazepine-$^{13}C_{6}$	Quadratic	Ignore	1/X	0.9986	0.01
Phenytoin	4.07	Phenytoin-$^{2}H_{10}$	Linear	Ignore	1/X	0.9976	0.39
Carbamazepine	4.17	Carbamazepine-$^{2}H_{10}$	Quadratic	Ignore	1/X	0.9952	0.06
Tiagabine	4.39	Tiagabine-$^{2}H_{6}$	Quadratic	Ignore	1/X	0.9991	0.02
Valproic Acid	4.57	Valproic Acid-$^{2}H_{6}$	Linear	Ignore	1/X	0.9976	4.69

Table 6. Concentration of the calibrators with reported therapeutic ranges and LLOQ.

Compound	Concentration (mg/L)									
	Cal 10	Cal 9	Cal 8	Cal 7	Cal 6	Cal 5	Cal 4	Cal 3	Cal 2	Cal 1
Vigabatrin	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	0.39	0.20	*0.10*
Levetiracetam	**100**	**50**	**25**	**12.5**	**6.25**	**3.13**	1.56	0.78	0.39	*0.20*
Theophylline	**25**	**12.5**	**6.25**	**3.13**	1.56	0.78	0.39	0.20	*0.10*	0.049
Pregabalin	**15**	**7.50**	**3.75**	**1.88**	**0.94**	**0.47**	**0.23**	**0.12**	0.06	*0.029*
Gabapentin	**25**	**12.5**	**6.25**	**3.13**	**1.56**	0.78	0.39	0.20	0.10	*0.049*
Zonisamide	**50**	**25**	**12.5**	**6.25**	**3.13**	1.56	0.78	0.39	*0.20*	0.098
Lacosamide	**15**	**7.50**	**3.75**	**1.88**	**0.94**	0.47	0.23	0.12	0.06	*0.029*
Lamotrigine	**25**	**12.5**	**6.25**	**3.13**	1.56	0.78	0.39	0.20	*0.10*	0.049
Felbamate	**150**	**75**	**37.5**	**18.75**	**9.38**	**4.69**	2.34	1.17	0.59	0.29
Primidone	**25**	**12.5**	**6.25**	**3.13**	**1.56**	0.78	0.39	0.20	*0.10*	0.049
Phenobarbital	**100**	**50**	**25**	**12.5**	**6.25**	3.13	1.56	*0.78*	0.39	0.20
Topiramate	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	0.39	*0.20*	0.10
10,11 Dihydro-10-hydroxycarbazepine	**50**	**25**	**12.5**	**6.25**	**3.13**	1.56	0.78	0.39	*0.20*	0.10
Carbamazepine-10,11-epoxide	**15**	**7.50**	**3.75**	**1.88**	**0.94**	**0.47**	**0.23**	**0.12**	*0.06*	0.029
Oxcarbazepine	**5**	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	0.08	0.04	0.02	*0.01*
Phenytoin	**25**	**12.5**	**6.25**	**3.125**	1.563	0.781	*0.391*	0.195	0.10	0.049
Carbamazepine	**15**	**7.50**	**3.75**	**1.88**	0.94	0.47	0.23	0.12	*0.06*	0.029
Tiagabine	**5**	**2.50**	**1.25**	**0.63**	**0.31**	**0.16**	**0.078**	**0.04**	***0.02***	0.01
Valproic Acid	**150**	**75**	**37.5**	**18.75**	9.38	*4.69*	2.34	1.17	0.59	0.29

In bold: laboratory reference range
In italics: LLOQ
In white: below LLOQ

Representative chromatograms for the LLOQ for carbamazepine-10,11-epoxide, topiramate, pirimidone, lacosamide, zonisamide, gabapentin, carbamazepine, and phenobarbital are reported in Figure 1, together with the respective calibration curves.

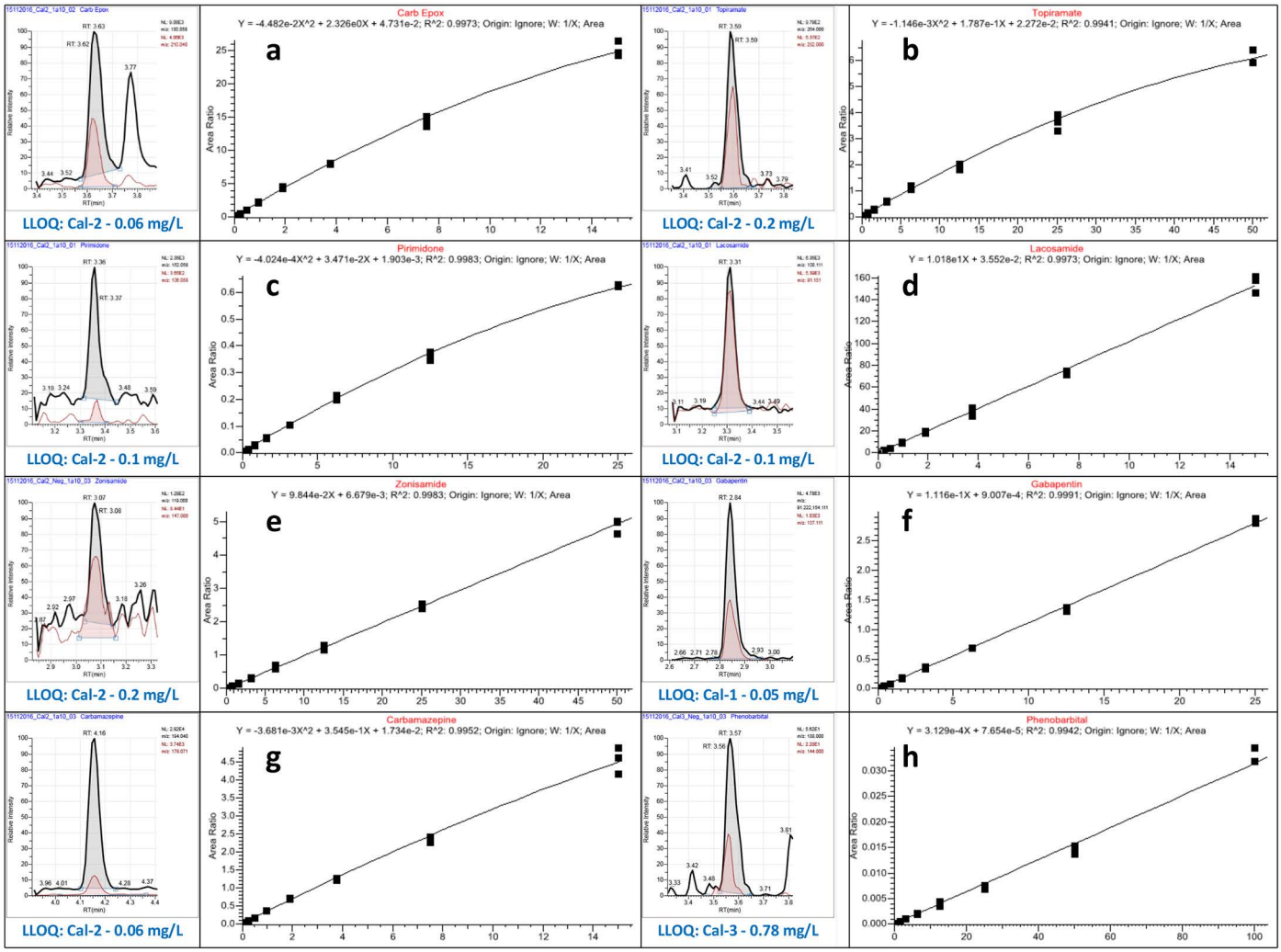

Figure 1. Chromatograms for the LLOQ for carbamazepine-10,11-epoxide (a), topiramate (b), pirimidone (c), lacosamide (d), zonisamide (e), gabapentin (f), carbamazepine (g), and phenobarbital (h).

Results for intra- (n=5) and inter-assay (n=15) precision for each analyte are reported in Table 7, together with relative recovery. %RSD was always below 15% for inter-assay precision and 19% for intra-assay precision. A minimum value of 82% was obtained for recovery.

Table 7. Intra- (n=5) and inter-assay (n=15) precision results.

Compound	QC1			QC2			QC3			QC4			Recovery
	Conc (µg/mL)	Intra-%RSD	Inter-%RSD	Conc (µg/mL)	Intra-%RSD	Inter-%RSD	Conc (µg/mL)	Intra-%RSD	Inter-%RSD	Conc (µg/mL)	Intra-%RSD	Inter-%RSD	
Vigabatrin	0.24	6.9	12.6	1.2	2.6	13.0	6	1.9	12.9	30	1.1	12.0	84.0
Levetiracetam	0.6	11.3	8.1	3	6.5	7.1	15	5.2	4.3	75	1.4	2.8	95.2
Theophylline	0.12	8.2	8.0	0.6	7.6	6.6	3	1.5	3.4	15	2.1	2.5	90.2
Pregabalin	0.06	7.0	6.2	0.28	4.5	4.4	1.4	4.2	3.5	7	2.3	4.1	103.4
Gabapentin	0.14	2.5	7.8	0.7	2.1	2.7	3.5	2.3	2.7	17.5	2.0	2.1	93.8
Zonisamide	0.24	8.1	6.4	1.2	4.2	4.7	6	2.7	3.2	30	3.3	2.8	102.8
Lacosamide	0.064	4.7	7.1	0.32	5.5	6.2	1.6	5.9	6.6	8	6.7	7.7	83.9
Lamotrigine	0.16	0.5	5.2	0.8	2.3	3.1	4	2.9	5.2	20	4.2	3.9	84.8
Felbamate	NA	NA	NA	4	6.3	6.5	20	2.8	3.4	100	3.9	4.6	87.4
Pirimidone	0.12	6.1	7.4	0.6	8.1	6.7	3	3.7	4.1	15	4.7	3.6	82.5
Topiramate	0.24	18.6	14.3	1.2	8.2	9.0	6	5.6	6.5	30	3.1	7.5	92.5
10,11 Dihydro-10-hydroxycarbazepine	0.24	9.2	8.4	1.2	4.5	4.2	6	3.1	4.0	30	3.2	5.4	110.9
Carbamazepine-10,11-epoxide	0.06	9.4	12.6	0.3	8.7	9.6	1.5	2.9	7.4	7.5	2.5	3.8	98.4
Oxcarbazepine	0.02	4.4	7.6	0.1	4.8	4.7	0.5	4.6	3.5	2.5	0.9	3.0	89.0
Carbamazepine	NA	NA	NA	0.28	4.2	3.0	1.4	1.7	2.2	7	1.2	2.1	106.5
Tiagabine	0.02	9.6	7.2	0.1	4.7	3.7	0.5	3.2	2.8	2.5	0.8	3.5	86.0
Phenytoin	NA	NA	NA	0.6	12.8	10.4	3	7.6	10.3	15	4.2	6.7	94.3
Valproic Acid	NA	NA	NA	4	6.5	6.0	20	4.3	5.1	100	2.2	4.3	82.0
Phenobarbital	NA	NA	NA	2.4	11.0	10.5	12	5.6	5.0	60	2.8	2.9	103.6

Conclusions

A liquid chromatography-tandem mass spectrometry method for the quantification of 19 different antiepileptics in human plasma was implemented for clinical research. A single, quick sample preparation protocol was applied to the entire panel of analytes. It consisted of a simple protein precipitation step followed by the dilution of supernatant prior to the injection onto the HPLC system. The method was analytically validated on an UltiMate 3000 RS LC system connected to a TSQ Endura triple quadrupole mass spectrometer. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision and is also able to separate and properly quantify two isobaric compounds, namely carbamazepine-10,11-epoxide and oxcarbazepine.

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