Quantification of 17 Antiepileptics and Their Metabolites in Human Plasma by LC-MS/MS for Research

Claudio De Nardi, Thermo Fisher Scientific, Dreieich, Germany
Anna Morando, Anna Del Plato, Ospedale "La Colletta", Arenzano, Italy

Application Note 608

Key Words
Antiepileptic drugs, antiepileptics, AEDs, anticonvulsants, antiseizure drugs, liquid chromatography, triple quadrupole mass spectrometry, TSQ Quantum Access MAX

Goal
Implement an LC-MS/MS method for the quantification of 17 antiepileptics and their metabolites in human plasma.

Introduction
Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a valuable tool that can help clinical researchers to monitor that antiepileptic drugs remain within the desired range. Here, an LC-MS/MS method on the Thermo Scientific™ TSQ Quantum Access MAX™ triple quadrupole mass spectrometer was used with the MassTox® TDM Series A kit for antiepileptics from Chromsystems™ to quantify a panel of 17 antiepileptics and their metabolites in human plasma. The MassTox TDM Series A kit includes levetiracetam, theophylline, felbamate, lacosamide, rufinamide, carbamazepine, oxcarbazepine, carbamazepine diol, carbamazepine-10,11-epoxide, 10-hydroxycarbamazepine, phenylethylmalonamide (PEMA), primidone, phenytoin, stiripentol, zonisamide, phenobarbital, valproic acid, and 12 internal standards.

Experimental
Sample Preparation
The MassTox TDM Series A kit for antiepileptics was used. The 17 evaluated analytes were divided into three groups. Each group required a different extraction procedure and analytical method. The kit included dried calibrators at three different concentration levels and dried controls at two different levels. Concentrations of calibrators and controls are reported in Table 1.

The kit also included an extraction buffer, a precipitating agent containing all the internal standards (IS), and two different dilution buffers (dilution buffer 1 and dilution buffer 2).

Dry calibrators and controls were resuspended using 1 mL of distilled water and let rest for 15 minutes at room temperature. Blanks, calibrators, controls, and samples were protein precipitated as follows:

- 100 µL of blank, calibrator, control, or sample
- 50 µL of extraction buffer
- 500 µL of precipitating agent containing the internal standards

Calibrators and controls were extracted in duplicate. Precipitated samples were vortex-mixed and centrifuged for 10 minutes at 4 °C at 3200*g*. Supernatant was diluted using different dilution schemes depending on the group prior to injection onto the LC-MS/MS system:

- Group 1: dilution 1:10 (20 µL + 180 µL) with dilution buffer 1 / dilution buffer 2, 50:50 (v/v)
- Group 2: dilution 1:5 (100 µL + 400 µL) with dilution buffer 1
- Group 3: no dilution

Thermo SCIENTIFIC

Table 1. Concentrations (ng/mL) of calibrators and controls

Group	Analyte	CAL 1	CAL 2	CAL 3	CTRL1	CTRL2
1	Carbamazepine	1.62	8.77	15.1	3.25	10.6
	Oxcarbazepine	0.14	1.89	3.64	0.46	2.75
	Carbamazepine diol	0.16	5.77	11.5	1.11	8.21
	Carbamazepine-10,11-epoxide	0.16	5.61	11.0	1.07	8.1
	10-hydroxycarbamazepine	3.83	26.6	48.3	8.48	36.1
	Felbamate	14.3	70.8	130	27.2	92.9
	Lacosamide	0.78	6.13	11.4	1.91	8.5
	Levetiracetam	4.90	28.5	84.0	16.0	62.8
	Rufinamide	3.98	21.0	35.3	7.54	28.0
	Theophylline	6.11	15.9	24.6	9.78	18.9
2	Phenytoin	3.49	13.3	23.3	5.86	16.9
	Primidone	3.07	10.7	18.3	5.11	13.6
	Phenylethylmalonamide (PEMA)	1.07	6.68	12.7	2.21	9.03
	Stiripentol	2.62	14.3	27.3	5.01	20.3
3	Phenobarbital	7.38	34.2	60.1	13.8	44.7
	Valproic Acid	31.6	79.1	125	47.2	97.7
	Zonisamide	3.97	27.4	50.4	9.04	36.8

Liquid Chromatography

Liquid chromatography analysis was performed using a Thermo Scientific™ Transcend™ TLX-1 system. The LC conditions were as follows:

LC column	Provided with the kit
Mobile phase A	Provided with the kit
Mobile phase B	Provided with the kit
Injection volume	Group 1 – 20 µL
	Group 2 – 100 µL
	Group 3 – 30 µL
LC gradient	See Table 2

Table 2. LC gradient

Group	Time (min)	Flow Rate (mL/min)	A (%)	B (%)
1	0.0	0.8	100	0
	0.1	0.8	100	0
	0.5	0.8	60	40
	2.5	0.8	60	40
	3.0	1.0	0	100
	4.0	1.0	0	100
	4.1	0.8	100	0
2	0.0	1.0	100	0
	0.1	1.0	100	0
	1.0	1.0	0	100
	3.0	1.0	0	100
	3.1	1.0	100	0
3	0.0	1.0	100	0
	0.1	1.0	100	0
	1.0	1.0	0	100
	3.0	1.0	0	100
	3.1	1.0	100	0

Mass Spectrometry

The LC system was connected to a TSQ Quantum Access MAX triple quadrupole mass spectrometer. Acquisition time ranges were used for each analyte and the following MS conditions were used:

Source type	Heated electrospray ionization (HESI)
Vaporizer temp	350 °C
Capillary temp	350 °C
Spray voltage	3500 V
Sheath gas	70 AU
Sweep gas	0 AU
Auxiliary gas	40 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Chrom filter peak width	5.0 s
Collision gas pressure	1.5 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7
SRM settings	See Tables 3, 4 and 5

Table 3. SRM settings – Group 1

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
Levetiracetam	0.9	1.8	+	171.1	69.3	28	70
				171.1	126.2	13	
				171.1	154.1	5	
IS7	0.9	1.8	+	174.1	69.3	29	70
Theophylline	0.9	1.9	+	181.0	69.3	26	110
				181.0	96.2	24	
				181.0	124.1	17	
IS8	0.9	1.9	+	187.0	127.1	19	110
Felbamate	1.3	2.3	+	239.0	91.2	34	100
				239.0	117.1	16	
				239.0	178.0	5	
Rufinamide	1.3	2.3	+	239.0	127.1	23	100
				239.0	211.0	5	
				239.0	222.0	11	
IS5	1.3	2.3	+	243.0	182.1	5	100
Lacosamide	1.3	2.3	+	251.1	91.2	27	100
				251.1	108.2	7	
				251.1	116.1	13	
IS6	1.3	2.3	+	254.1	108.2	6	100
Carbamazepine diol	1.7	2.7	+	271.0	180.0	27	100
				271.0	236.0	11	
				271.0	253.1	5	
Carbamazepine-10,11-epoxide	1.9	2.8	+	253.0	180.1	29	100
				253.0	210.0	13	
				253.0	236.0	12	
10-hydroxycarbamazepine	1.9	2.9	+	255.0	179.0	36	100
				255.0	194.0	19	
				255.0	237.0	5	
IS3	1.9	2.9	+	259.0	198.0	19	100
IS2	1.9	2.9	+	263.1	190.1	25	100
IS4	2.4	3.4	+	257.0	184.0	25	100
				257.0	212.0	19	
				257.0	240.0	13	
Oxcarbazepine	2.5	3.5	+	253.0	180.0	25	100
				253.0	208.0	19	
				253.0	236.0	11	
Carbamazepine	2.9	3.9	+	237.0	165.0	42	100
				237.0	179.1	32	
				237.0	194.0	19	
IS1	2.9	3.9	+	247.1	204.1	20	100

Table 4. SRM settings – Group 2

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
PEMA	0.6	1.6	+	207.1	91.2	25	70
				207.1	119.2	15	
				207.1	162.1	10	
Primidone	0.8	1.8	+	219.0	91.2	25	90
				219.0	119.2	15	
				219.0	162.1	10	
IS13	0.8	1.8	+	224.0	167.1	20	90
IS5	0.8	1.8	+	243.0	182.1	5	100
Phenytoin	1.1	2.1	+	253.0	104.1	20	100
				253.0	182.0	15	
				253.0	225.0	10	
Stiripentol	1.3	2.3	+	217.0	145.1	16	55
				217.0	159.1	13	
				217.0	187.1	10	

Table 5. SRM settings – Group 3

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
Zonisamide	0.7	1.7	-	211.1	119.1	18	70
				211.1	147.1	12	
IS18	0.7	1.7	-	216.0	123.2	15	70
Phenobarbital	0.9	1.9	-	231.0	85.3	15	70
				231.0	144.2	15	
				231.0	188.0	10	
IS16	0.9	1.9	-	236.0	193.1	10	70
Valproic Acid	1.2	2.2	-	143.1	143.1	10	70
IS17	1.2	2.2	-	147.1	147.1	10	70

Data Acquisition and Processing

Data were quantitated using a linear regression, and 1/x weighting was used to build the calibration curves. Maximum percentage bias between nominal and calculated concentration of 15% and 20% was set as acceptance criterion for calibrators and controls, respectively.

Results and Discussion

Linear calibration curves were obtained for all the analytes in the evaluated concentration ranges, and correlation factors (R^2) were always above 0.99. The percentage bias between nominal and experimental concentration for all calibrators and controls was always within the set acceptance criteria (15% for calibrators and 20% for controls). A summary of calibration range, intercept, slope, and correlation factor (R^2) for each analyte is reported in Table 6.

Table 6. Calibration range, intercept, slope, and correlation factor (R^2)

Analyte	Calibration Range (ng/mL)	Intercept	Slope	R^2
Carbamazepine	1.62 – 15.1	-0.068	0.610	0.998
Oxcarbazepine	0.14 – 3.64	-0.054	1.585	0.998
Carbamazepine diol	0.16 – 11.5	0.000	0.115	0.996
Carbamazepine-10,11-epoxide	0.16 – 11.0	-0.056	7.099	0.995
10-hydroxycarbamazepine	3.83 – 48.3	0.072	0.347	0.999
Felbamate	14.3 – 130	-0.119	0.484	0.995
Lacosamide	0.78 – 11.4	-0.064	1.145	0.996
Levetiracetam	4.90 – 84.0	-0.451	0.954	0.995
Rufinamide	3.98 – 35.3	-0.449	1.026	0.996
Theophylline	6.11 – 24.6	0.117	0.198	0.991
Phenytoin	3.49 – 23.3	-2.185	0.667	0.996
Primidone	3.07 – 18.3	2.570	1.375	0.997
PEMA	1.07 – 12.7	-1.964	6.885	0.996
Stiripentol	2.62 – 27.3	-2.298	1.922	0.994
Phenobarbital	7.38 – 60.1	-0.027	0.083	0.998
Valproic Acid	31.6 – 125	-0.041	0.011	0.998
Zonisamide	3.97 – 50.4	-0.157	0.286	0.999

Representative calibration curves for carbamazepine and phenobarbital are shown in Figure 1. Representative chromatograms at the limit of quantitation (LOQ) for each analyte, including the internal standards, are shown in Figures 2, 3, and 4.

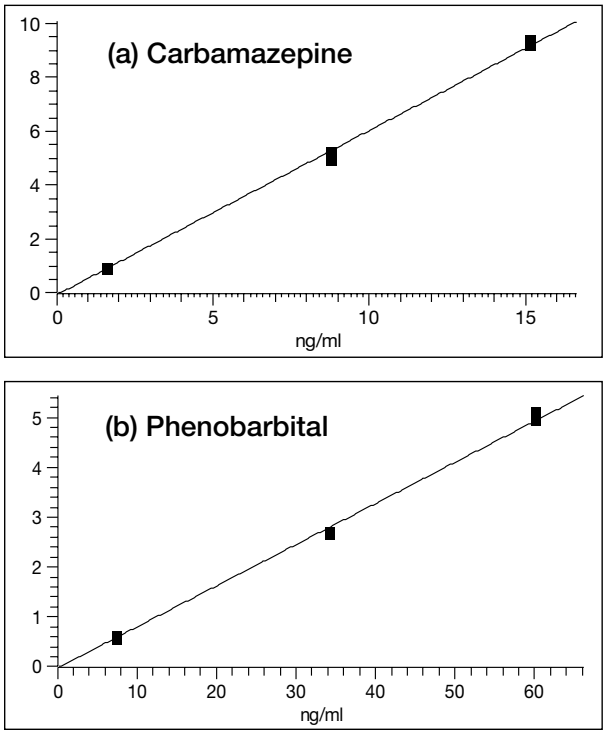

Figure 1. Calibration curves for (a) carbamazepine and (b) phenobarbital

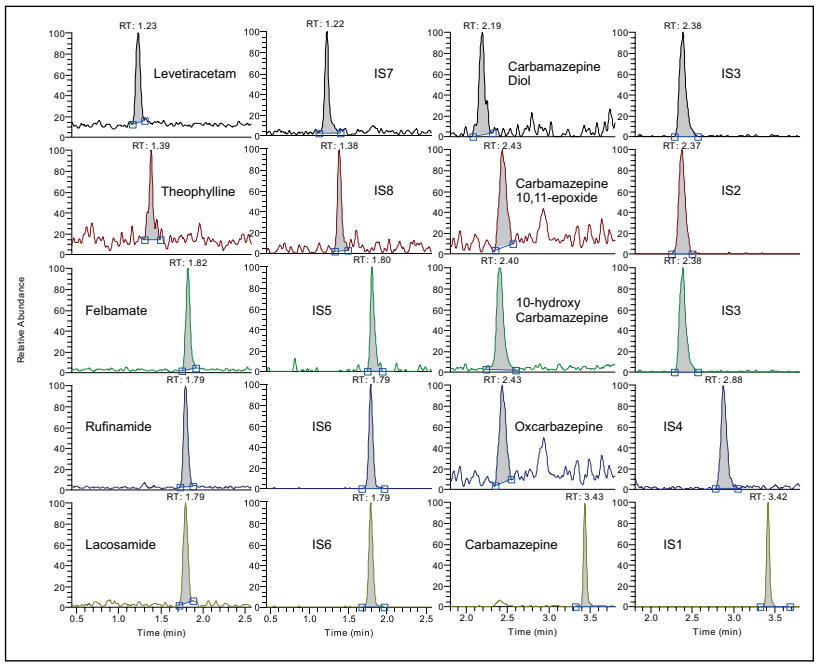

Figure 2. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 1

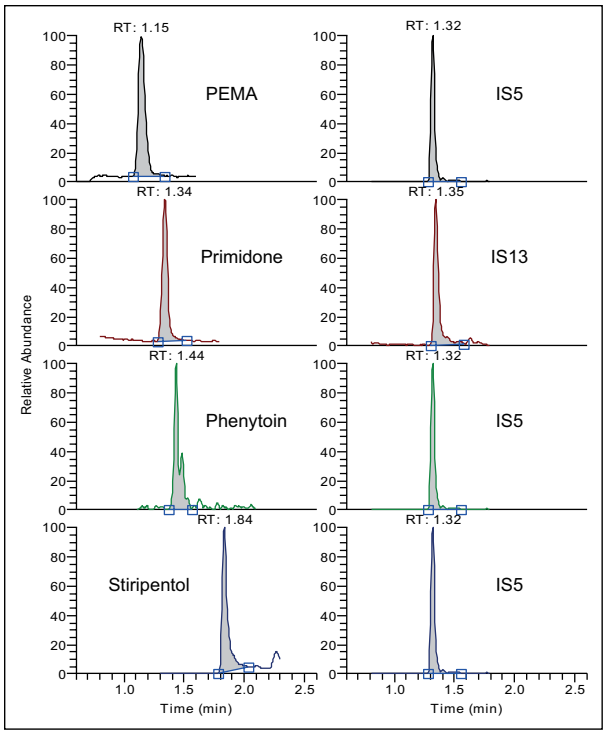

Figure 3. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 2

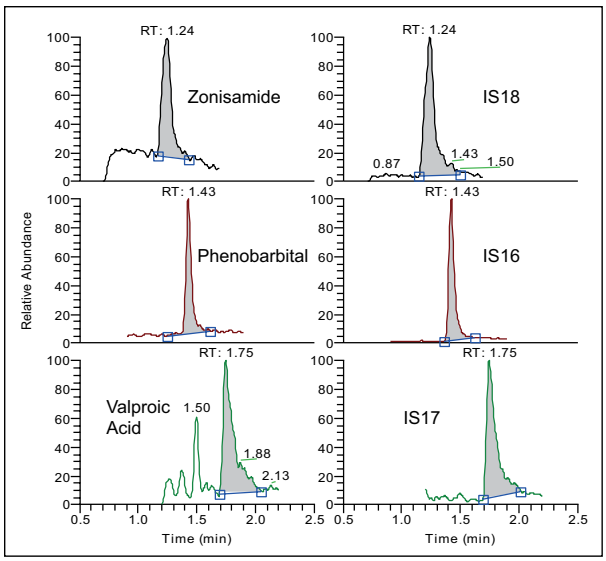

Figure 4. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 3

Conclusion

The quantification of a panel of antiepileptic drugs in human plasma has been implemented and analytically validated on a TSQ Quantum Access MAX mass spectrometer using the MassTox TDM Series A kit for antiepileptics from Chromsystems. The TSQ Quantum Access MAX mass spectrometer proved to have the proper sensitivity, accuracy, and precision for the application of this analytical method to clinical research.

For Research Use Only. Not for use in diagnostic procedures.

AN64066-EN 0616S