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Automated Preparation and Analysis of a Large Range of Antiepilpetics for Therapeutic Drug Monitoring by LC-MS/MS

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Introduction

Because of their complicated pharmacokinetics, as well as narrow therapeutic ranges that cause significant differences in individuals' therapeutic dosages, antiepileptic drugs (AEDs) are today among the most common medications for which clinical laboratories perform therapeutic drug monitoring (TDM). In addition, some benzodiazepines (BZDs) are also important drugs used in management of epilepsy. While AEDs are effective within few tens of mg/L, BZDs have a therapeutic range at the µg/L level making them challenging to assay simultaneously.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become a major tool in TDM. It offers high specificity, speed of analysis, multiplexing capabilities and automation associated to a low cost per sample.

Here we present a method to assay a large panel of AEDs and BZDs using a fully automated platform in a single assay without the need for manual pretreatment.

Materials and Method

The system used was comprised of a CLAM-2000 directly coupled to a Nexera X2 UHPLC and a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corp.)

From the sample tube, 20 µL of plasma were aspirated and precipitated with 280 µL of methanol containing internal standards. After filtration, extracts were automatically transferred to the autosampler for LC-MS/MS analysis. The overall process time was 3.5 minutes.

LC parameters

System	: Shimadzu Nexera X2 with binary high pressure gradient (mixer 20µL),
Column	: Shim-Pack GIS C18 5 µm 50*2.1mm (227-30103-03),
Column Temperature	: 40°C,
Mobile phases	: A: Ammonium Formate buffer 3mM pH3.6 / MeOH 90/10 v/v, B: Ammonium Formate buffer 3mM pH3.6 / MeOH 10/90 v/v,
Flow-rate	: 0.6 mL/min,
Gradient	: 100%A to 100%B in 2 min. back to 100%A in 0.1 min,
Total run time	: 3.5 min,
Injection volume	: 0.5 µL,
Rinsing	: External with R0 (Methanol).

MS/MS parameters

System	: Shimadzu LCMS-8050,
Ionization	: Heated ESI in positive and negative,
Acquisition mode	: MRM (see table 1),
Interface voltage	: Tuning values,
Temperatures	: Interface: 300°C DL: 150 °C Heater block: 400°C,
Gas flows	: Heating: 10 L/min Nebulizing: 3 L/min Drying: 10 L/min.
Loop time	: 0.19 s.

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Table 1: MRM Parameters.

Name	Ionization	Quantification		Confirmation		Dwell Time (ms)
		MRM	CE(V)	MRM	CE(V)	
Carbamazepine	pos	237 > 192	-21	237 > 165	-43	8
Carbamazepine-10,11-Epoxide	pos	253 > 210	-12	253 > 167	-35	8
Gabapentin	pos	172 > 154	-15	172 > 137	-16	19
Lamotrigine	pos	256 > 211	-26	256 > 43	-53	14
Levetiracetam	pos	171 > 126	-15	171 > 69	-27	19
Pregabalin	pos	160 > 55	-22	160 > 97	-14	24
Zonisamide	pos	213 > 132	-14	213 > 77	-33	12
13c6-Zonisamide	pos	219 > 138	-15	---	---	26
Lacosamide	pos	251 > 108	-20	251 > 91	-50	12
D5-Phenobarbital	neg	236 > 42	24	---	---	18
Phenobarbital	neg	231 > 42	22	231 > 188	11	8
Phenytoin	neg	251 > 102	23	251 > 208	16	8
Topiramate	neg	338 > 78	31	338 > 96	25	8
Valproic Acid	neg	143 > 143	11	---	---	20
Clonazepam	pos	316 > 214	-38	316 > 270	-25	8
Diazepam	pos	285 > 193	-31	285 > 154	-27	9
N-Desmethylclobazam	pos	287 > 245	-18	287 > 210	-31	8
Nitrazepam	pos	282 > 236	-24	282 > 180	-38	8
Nordiazepam	pos	271 > 140	-27	271 > 165	-27	9
Clobazam	pos	301 > 259	-21	301 > 224	-32	8

Results

Linearity

Calibration standard levels were prepared by spiking a blank plasma pool (EDTA-K3, 6 donors, mixed gender, BioreclamationIVT, USA). Seven levels were prepared. For each compound, the calibration range was determined using therapeutic reference range. The lower limit of quantification (LLOQ) was set as 5 times lower than the low reference concentration. The upper limit of quantification (ULOQ) was set as 1.5 times higher than the high reference concentration. Targeted calibration ranges can be found in table 2.
Linearity of the method was assessed by calculating the relative deviation of calibration standards against the calculated linear regression model. In all cases, deviation was inferior to ±15%, fulfilling acceptance criteria. Typical calibration curves are showed in figure 1.

Recovery

Recovery of the method was evaluated by comparing peak areas measured in QC samples prepared in two different plasma pools (n=3 per pool) to the ones measured in QC samples made in neat solution (n=5). Therefore, total recovery, combining extraction and matrix effect, was measured. Results are presented in table 3.

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Table 2: Calibration ranges.

Compound Name	Acronym	Class	Calibration Range (mg/L)	
			LLOQ	ULOQ
Carbamazepine	CBZ	Carboxamide	1	20
Carbamazepine-10,11-epoxide	EPO-CBZ	Carboxamide	2	45
Gabapentin	GBP	GABA analog	1	30
Lacosamide	LCA	Modified amino acid	0.8	15
Lamotrigine	LMT	Triazine	1	25
Levetiracetam	LVT	Pyrrolidine	4	70
Phenobarbital	PBR	Barbiturate	3	50
Phenytoin	PNT	Hydantoin	1	30
Pregabalin	PGB	GABA analog	0.5	10
Topiramate	TPA	Fructose derivative	1	20
Valproic acid	VPA	Fatty acid	8	120
Zonisamide	ZNA	Sulfonamide	3	45
Clobazam	CLBZ	Benzodiazepine	0.05	0.7
Clonazepam	CZP	Benzodiazepine	0.005	0.07
Diazepam	DIA	Benzodiazepine	0.1	2.5
N-Desmethylclobazam	DM-CLBZ	Benzodiazepine	0.06	4.5
Nitrazepam	NTZ	Benzodiazepine	0.02	0.3
Nordiazepam	NDIA	Benzodiazepine	0.08	1.2

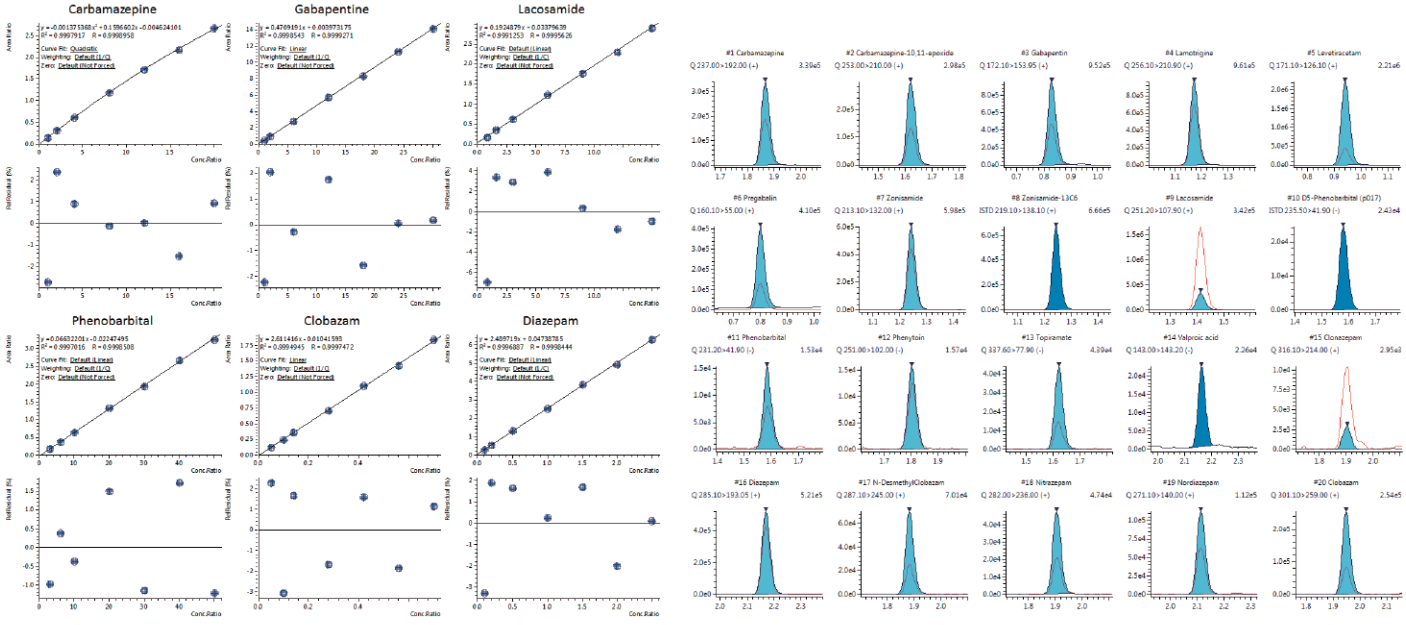

Figure 1: Typical calibration curves and mass chromatograms at the QC A level.

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No significant difference was found between plasma pools, indicating the matrix effect has low or no impact. It must be noted that in pool B, significant level of clonazepam and nordiazepam were found in the blank. Therefore, the recovery measured in QC LOQ was not accurate. These values have been excluded to calculate mean recovery and %RSD. For all compounds, recovery was consistent across the concentration range and the average never inferior to 80 %. The method was suitable for quantitative analysis.

Table 3: MRM Parameters.

		Total Recovery (%)								
		CBZ	EPO-CBZ	GBP	LMT	LVT	PGB	ZNA	LCA	PBR
QC LOQ	Pool A	101	103	92.0	84.2	109	89.4	82.6	86.9	81.8
	Pool B	108	109	97.3	92.2	119	96.4	91.3	95.4	90.9
QC A	Pool A	98.7	103	91.9	83.9	107	90.0	82.2	88.1	83.0
	Pool B	101	104	91.8	85.9	109	90.9	84.8	90.4	77.5
QC B	Pool A	114	110	102	94.9	118	102	94.1	99.6	90.7
	Pool B	113	112	98.9	94.6	120	99.9	93.1	98.9	88.6
QC C	Pool A	104	105	94.3	91.7	110	95.5	88.8	94.5	85.0
	Pool B	97.6	99.6	87.2	86.4	106	88.4	82.2	88.7	78.8
Mean		105	106	94.4	89.2	112	94.1	87.4	92.8	84.5
%RSD		6%	4%	5%	5%	5%	5%	6%	5%	6%

		PNT	TPA	VPA	CZP	DIA	DM-CLBZ	NTZ	NDIA	CLBZ
QC LOQ	Pool A	78.0	75.9	81.1	105	80.3	77.3	82.7	84.2	84.4
	Pool B	85.8	86.8	87.3	125	96.1	84.2	93.9	121*	93.5
QC A	Pool A	82.1	77.5	83.1	83.8	77.1	83.2	85.0	85.4	84.0
	Pool B	80.0	79.4	85.0	94.9	82.4	88.1	86.0	94.0	84.9
QC B	Pool A	89.6	88.1	93.6	92.3	88.0	95.5	92.9	93.0	95.0
	Pool B	89.2	86.9	91.7	89.1	89.0	93.7	91.3	97.2	94.1
QC C	Pool A	83.8	79.3	88.0	89.2	86.0	90.2	89.2	90.1	89.0
	Pool B	77.2	73.7	80.4	84.3	79.1	83.2	82.5	84.4	81.5
Mean		83.2	81.0	86.3	91.2	84.8	86.9	87.9	89.8	88.3
%RSD		6%	7%	6%	8%	7%	7%	5%	6%	6%

Accuracy and Precision

Two sets of quality control samples (QC) were prepared. One in same plasma pool than calibration standards, one in another pool. Concentration levels were LLOQ (QCLOQ), 3 times the LLOQ (QC A), 50% of the concentration range (QC B) and 90% of the concentration range (QC C). Five individual replicates were prepared per level. Accuracy and precision of the QCs were calculated across 5 replicates per concentration level. Results are reported in table 4. A typical chromatogram of target compounds is shown in figure 1. All QCs were within the acceptance criteria for accuracy and precision.

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Table 4: Accuracy and Precision of QC.

		CBZ	EPO-CBZ	GBP	LMT	LVT	PGB	ZNA	LCA	PBR
QC LOQ	Mean Accuracy	103	99.6	110	105	100	110	115	100	109
	%RSD	2%	2%	0.8%	0.5%	3%	3%	0.7%	1%	8%
QC A	Mean Accuracy	103	98.1	102	99.6	98.1	108	94.4	106	97.2
	%RSD	3%	3%	0.5%	0.8%	3%	0.5%	0.3%	1%	5%
QC B	Mean Accuracy	100	103	98.9	98.1	100	98.1	95.1	100	99.1
	%RSD	3%	2%	0.2%	0.9%	2%	1%	0.8%	1%	3%
QC C	Mean Accuracy	110	111	103	103	107	103	104	102	98.5
	%RSD	3%	4%	0.3%	1%	3%	0.3%	0.6%	1%	1%

		PNT	TPA	VPA	CZP	DIA	DM-CLBZ	NTZ	NDIA	CLBZ
QC LOQ	Mean Accuracy	105	111	102	112	90.3	102	102	89.5	107
	%RSD	12%	3%	3%	16%	2%	4%	6%	3%	2%
QC A	Mean Accuracy	104	98.5	97.0	93.3	94.9	100	94.2	93.3	97.7
	%RSD	4%	2%	2%	11%	0.6%	2%	2%	1%	2%
QC B	Mean Accuracy	100	96.1	98.0	95.7	93.4	97.6	98.3	92.0	97.7
	%RSD	3%	2%	1%	7%	0.7%	1%	1%	0.7%	0.7%
QC C	Mean Accuracy	98.6	99.7	100	102	104	103	104	102	107
	%RSD	3%	2%	2%	3%	2%	1%	0.5%	2%	2%

Conclusion

A method for simultaneous measurement of AEDs and BZDs was developed and validated using a fully automated LC-MS/MS platform making it suitable for routine analysis.

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