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"MAGICAL TECHNOLOGY: MS/MS FOR THERAPEUTIC 28 ANTIEPILEPTIC DRUGS MONITORING IN HUMAN."

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Abstract: The aim of this study was to develop and validate a simple, robust and sensitive ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous analysis of 28 antiepileptic drugs (AEDs) in human serum. Fifty microliters of human serum were extracted using a protein precipitation extraction method and the final extracts were analyzed using reserve chromatography and triple-quadrupole tandem mass spectrometer by multiple reactions monitoring (MRM) mode via Electro spray ionization (ESI). Chromatographic separation was achieved on a JNJ C18 column within 6 min via isocratic elution with an aqueous solution containing 10 mM ammonium acetate and an organic solution containing acetonitrile, at the flow rate of 0.3 mL/min. Method validation consisted of linearity, sensitivity, accuracy and precision, all antiepileptic drugs were detected and quantified within 6 min without endogenous interferences. The correlation coefficient (R2) was >0.99 for all antiepileptic drugs with accuracy ranging from 93 to 107% and precision < 8% for all analytes. No carryover was observed in a blank control injected after the highest standard. The method has been successfully verified using authentic case samples that had previously been quantified using different methods. The assay is suitable for clinical utilization and management of patients on these medications.

KEYWORDS: Antiepileptic; UPLC-MS/MS; human serum

Introduction:

Antiepileptic drugs (AEDs) are those which decrease the frequency and/or severity of seizures in people with epilepsy. During a seizure, the level of nerve cell electrical signals increases to an excessive or abnormal amount, which leads to the signs and symptoms of a seizure. The change in nerve activity can have various causes (Niessen 2020). Sudden unexplained death in epilepsy is a major cause of death among epileptic patients. It is responsible for 18% of epileptic-related deaths (Walczak, T.S et al., 2001). The second most important factor after the frequency of seizures is the number of AEDs taken concomitantly. The risk is almost 10 times higher in patients taking more than two drugs compared with those who are on monotherapy (Walczak T.S et al., 2001, Nilsson L, et al., 1999). Therefore medication monitoring for AEDs is of importance so physicians are aware of what drugs their patients are using, thereby broadly assessing patient adherence and help in avoiding severe side effects, potential drug-drug interactions from poly-drug therapy, and identifying possible misuse.

The use of LC coupled with electrospray tandem mass spectrometry has become the very popular technique in bioavailability studies due to the fast, sensitive, and reliable results generated by its use (Silva *et al*., 2006). UPLC has been evaluated as a faster and more efficient analytical tool compared to current HPLC (Villiers *et al*., 2006). A number of methods for the quantitation of antiepileptic drugs in biological fluids have been reported. Several analytical methods mainly based on high resolution gas chromatography (Abraham, Gresham 1977), high performance liquid chromatography (HPLC) with UV detection (Contin *et al*., 2007, Greiner-Sosanko et al., 2007), fluorescence polarization immunoassay (Berry et al., 2000, Ma et at., 2002), enzyme multiplied immunoassay (Carl *et at*., 1982), high performance liquid chromatography

with Evaporative light scattering detection (Manoj Babu 2004), high performance liquid chromatography (Heideloff *et at*.,2010), application of DBS methods in TDM of AEDS has been investigated (Linder *et at*.,2017, 2018, 2019, Liu *et at*.,2019, Velghe *et at*.,2019), liquid chromatography-electrospray tandem mass spectrometry (Bhatt *et al*., 2011, Don *et al*., 2020, Shaza et al., 2014, Alffenaar *et al*., 2010, Mingming *et al*., 2004), simultaneous LC– MS-MS analysis of a selection of AEDs in one analytical step (Shibata et al., 2012, Subramanian et al., 2008, Kim et al., 2010). These reported methods have higher quantification limit, higher matrix volume and longer chromatographic run time which are normally not preferable of samples.

In the present work, we described validated method for performance of selective determination of 28 AEDs contains combination of protein precipitation extraction, reversed phase LC and tandem mass detection. Protein precipitation extraction was chosen for the minimum time of sample preparation, because this technique is more feasible and less polluting than the traditional liquid-liquid extraction used in other methods.

Experimental

Chemicals and reagents.

The Calibrator & Controls standard set lyophilised of Antiepileptic drugs (Figure 1) 10 hydroxy carbamazepine (10-OH CBZ), Brivaracetam (BVC), Carbamazepine (CBZ), Carbamazepine-diol (CBZ-diol), Carbamazepine-epoxide (CBZ-epoxide), N-Desmethylmethsuximide (N-DMS), Ethosuximide (ETH), Felbamate (FLB), Gabapentine (GBP), Lacosamide (LCM), Lamotrigine (LMT), Levetiracetam (LTC), Oxcarbazepine (OXC), Phenylethylmalonamide (PEMA), Perampanel (PRM), Phenobarbital (PNB), Phenytoin (PNT), Pregabaline (PGB), Primidone (PMD), Retigabine (RTG), Rufinamide (RFM), Stiripentol (SRP), Sulthiame (SLA), Tiagabine (TGB), Topiramate (TPM), Valproic acid (VAL), Vigabatrine (VGB) and Zonisamide (ZNS) were procured from Recipe, Munich, Germany. High purity water used for the LC-MS/MS was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). HPLC methanol and acetonitrile were purchased from. J.T.Baker (USA). All other regents and solvents were obtained from general commercial suppliers.

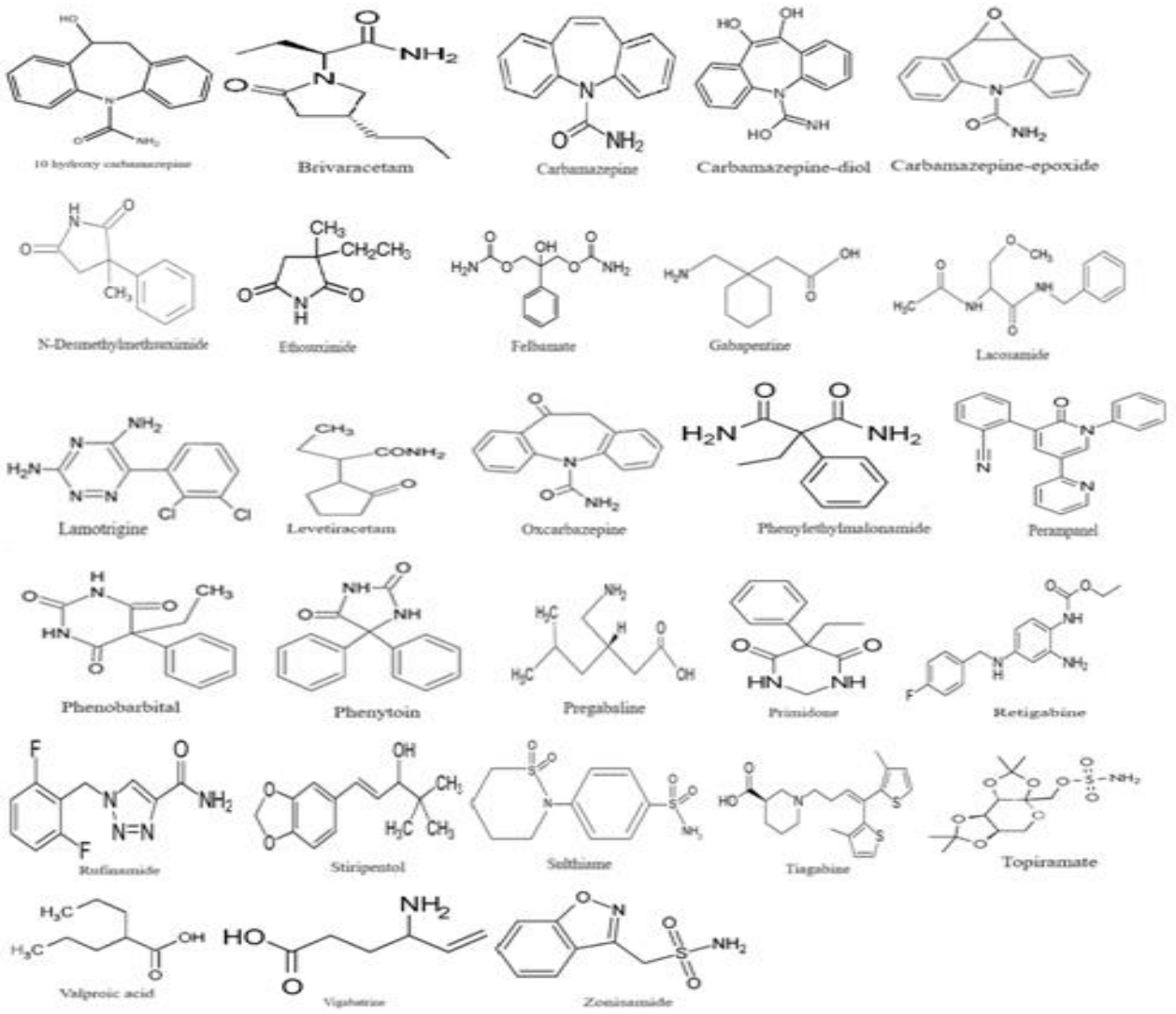

Figure 1. Structure of 28 antiepileptic drugs.

Calibration curves:

The Calibration curve was established using the recipe clinical calibrator set lyophilised for Antiepileptic (Recipe, Munich, Germany). Three levels of calibration curve (CC) and two levels of quality controls (QC) were reconstituted as per company recommendation.

Sample Preparation

An aliquot of 50µL of (CC, QC) or unknown samples were transferred to a 2ml capacity of polypropylene disposable micro centrifuge tube. Then 700µL of reconstituted solution [Acetonitrile: 10 mM ammonium acetate (80:20, V/V) with 0.1% formic acid] was added in each tube. The samples were vortexed before centrifuged (4˚C) at 13000 rpm for 10 min. The organic supernatant layer was transferred to an auto sampler vial for LCMS/MS system.

Chromatographic conditions

Chromatography separation was performed on Nexera UPLC system (Shimadzu, Japan) with cooling auto-sampler and column oven enabling temperature control of the analytical column. Separations were performed on a JNJ C18 column (100 mm × 4.6 mm, 5µm). The column temperature maintained at 40˚C and chromatographic separation was achieved using mobile phase composition, acetonitrile-10mM ammonium acetate (70:30, v/v) was delivered at a flow rate of 0.300mL/min. Mobile phase was used as weak wash and strong wash solvent to avoid any carry over from previous injection. The auto-sampler was maintained at 10˚C and the injection volume was 5µL. Total run time for each sample analysis was 6.0min.

Result and Discussion

The described LCMS/MS method enables the simultaneous quantification of 28 AEDs. Using a 50µL sample volume (for each calibrator & Control) or patient sample and a simple protein precipitation method prepared in reconstituted solution, the total sample preparation time for this LCMS/MS was approximately 20 min. Chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as run time 6 min per injection. Ammonium acetate buffer in the mobile phase improved the detection of the analytes. It was found that mixture of acetonitrile-10mM ammonium acetate (70:30, *v/v*) could achieve this purpose and was finally adopted as the mobile phase. The use of small particles of stationery phase allowed UPLC to push the limits of peak capacity (due to higher efficiency), speed of analysis (due to higher linear velocities) and this met the requirement for a high sample throughput.

Mass Spectrometry

The optimized MS conditions were performed using direct infusion of a methanolic solution of both positive and negative ionization modes for 28 Antiepileptic into the ESI source of the mass spectrometer and parameters such as Q1 pre bias (v), collision energy (V) and Q3 pre bias (v) were adjusted. However, with optimal MS tuning, a more consistent and higher response was achieved in positive and negative ionization mode. The tandem mass spectrometer was operated in electro spray with multiple reactions monitoring acquisition parameters shown in Table 1. The Desolvation and Heat Block Temperature were set at 200°C and 400°C respectively. Nitrogen was used as nebulizing and drying gas, flow was set at 3.0 and 15.0 L/min respectively.

Table 1.Optimization of MRM parameters, Ionization mode Q1 pre bias, Collision energy, Q3 pre bias and retention time (RT) of AEDs.

AEDs	Precursor ion (m/z)	Product ion (m/z)	Ionization mode	Q1 pre bias (v)	Collision energy (v)	Q3 pre bias (v)	Dwell time(msec)	RT (minutes)
10-OH CBZ	255.20	237.10	+	-11.0	-9.0	-26.0	5.0	2.86
BVC	213.12	168.00	+	-14.0	-13.0	-23.0	3.0	3.07
CBZ	237.20	193.10	+	-14.0	-33.0	-20.0	5.0	3.41
CBZ-diol	271.00	180.00	+	-15.0	-13.0	-23.0	5.0	2.74
CBZ-epoxide	253.20	210.15	+	-18.0	-25.0	-20.0	5.0	3.07
N-DMS	190.05	119.82	+	-14.0	-12.0	-22.0	5.0	2.21
ETH	142.10	124.30	+	-22.0	-12.0	-22.0	5.0	2.71
FLB	239.00	117.30	+	-15.0	-13.0	-25.0	5.0	2.77
GBP	171.80	55.10	+	-17.0	-15.0	-19.0	3.0	2.82
LCM	251.10	108.10	+	-14.0	-18.0	-16.0	5.0	2.85
LMT	256.10	211.00	+	-21.0	-15.0	-26.0	5.0	2.88
LTC	171.20	126.10	+	-13.0	-19.0	-24.0	5.0	2.70
OXC	253.10	208.10	+	-10.0	-20.0	-22.0	5.0	3.15
PEMA	207.00	91.10	+	-16.0	-17.0	-23.0	5.0	2.76
PRM	349.70	247.00	+	-19.0	-24.0	-20.0	5.0	4.50
PNB	231.10	42.00	-	-15.0	-16.0	-19.0	5.0	3.01
PNT	253.10	182.10	+	-10.0	-22.0	-19.0	5.0	3.08
PGB	160.20	142.10	+	-17.0	-15.0	-26.0	5.0	2.85
PMD	219.20	162.10	+	-12.0	-14.0	-25.0	3.0	2.82
RTG	304.00	230.00	+	-18.0	-20.0	-22.0	5.0	3.61
RFM	239.00	127.00	+	-19.0	-15.0	-27.0	3.0	2.90
SRP	217.00	187.10	+	-16.0	-15.0	-24.0	5.0	5.41
SLA	290.90	225.30	+	-14.0	-13.0	-23.0	3.0	3.60
TGB	376.20	246.90	+	-20.0	-13.0	-21.0	3.0	3.95
TPM	357.00	282.00	+	-15.0	-12.0	-20.0	3.0	3.17
VAL	143.10	143.20	-	15.0	11.0	24.0	5.0	3.22
VGB	130.10	113.10	+	-14.0	-16.0	-27.0	3.0	2.86
ZNS	211.00	119.00	-	18.0	13.0	26.0	3.0	2.95

Specificity and Selectivity

The specificity and selectivity of this method was evaluated by analyzing 10 different sources of matrix in comparison with lower limit of quantification (LLOQ) samples (Figure 2). No significant direct interference in the blank serum traces were observed from endogenous substances in drug-free human serum at the retention time of the Antiepileptic respectively.

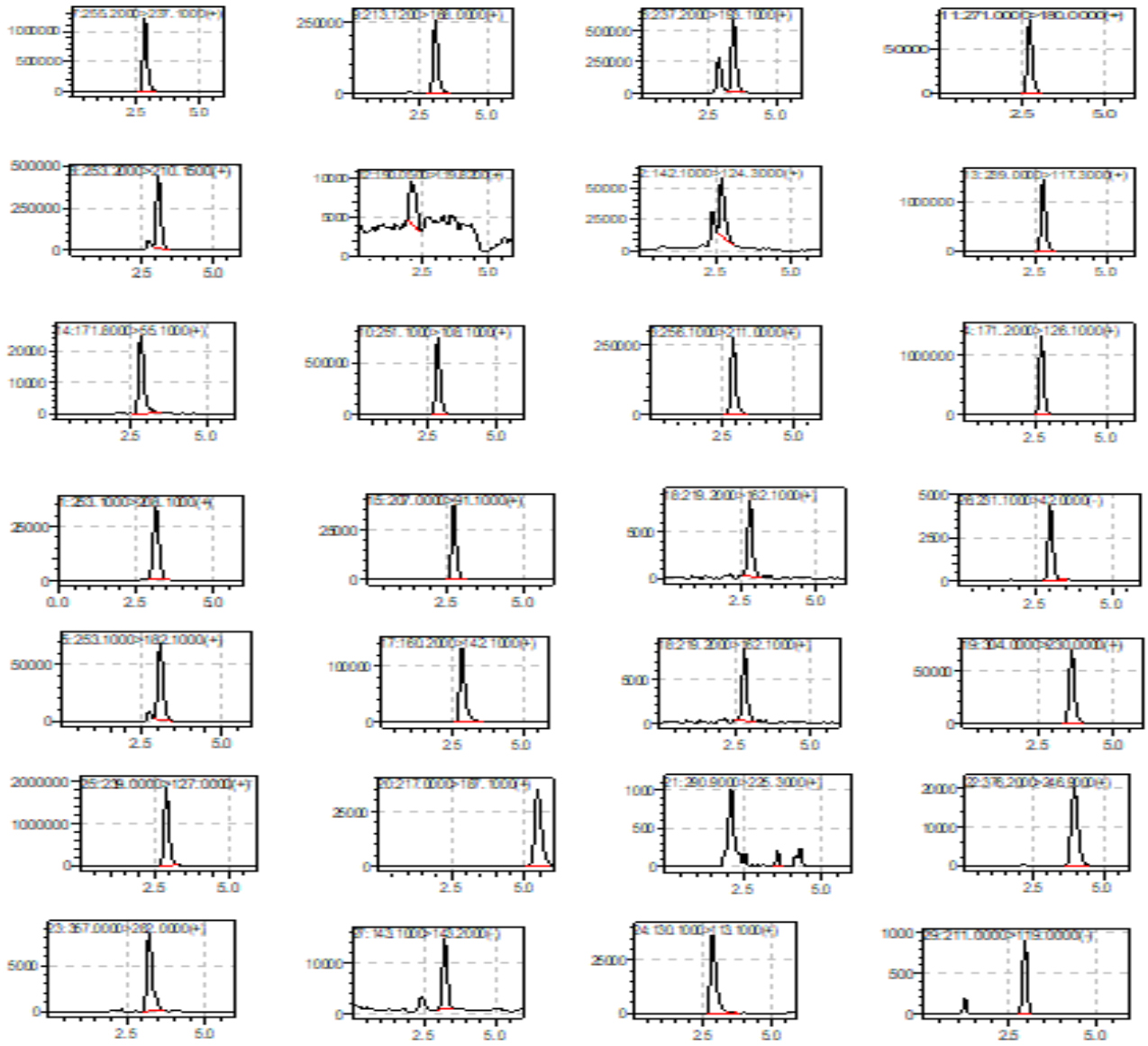

Figure 2: Representative chromatograms of Extracted lower limit of quantification plasma sample.

Linearity and lower limit of detection (LOD)

The linearity of the target compound peak area versus the calculated concentration was verified in human serum using a 1/x2 weighted linear regression and the linearity was conducted by external calibration, for it is a better way using the specific standard AEDs to quantitative themselves. The correlation coefficient (r^2) was > 0.99 for all of the target compounds in human serum. Hence, the method exhibited good linearity. LOD was determined by repeated analyses of spiked samples at decreasing concentrations. Five different sources of matrix samples were spiked at decreasing concentrations and were processed and analyzed by proposed extraction procedure. Table 2 summarizes the calibration range, lower limit of quantification and concentration of LOD.

Table 2. Calibration range, LLOQ and LOD for AEDs in human serum.

AEDs	Calibration range (µg/mL)	Quality Control (µg/mL)		LLOQ (µg/mL)	LOD (µg/mL)
		Level-1	Level-2		
10-OH CBZ	2.670-42.500	8.130	19.400	2.670	1.335
BVC	0.283-4.120	0.874	2.040	0.283	0.142
CBZ	1.480-21.500	4.390	9.930	1.480	0.740
CBZ-diol	0.574-8.880	1.580	3.810	0.574	0.287
CBZ-epoxide	0.585-8.470	1.830	4.180	0.585	0.293
N-DMS	3.270-43.600	8.900	19.900	3.270	1.635
ETH	8.270-121.000	22.000	51.000	8.270	4.135
FLB	7.020-105.000	20.300	48.300	7.020	3.510
GBP	1.790-27.600	4.950	11.700	1.790	0.895
LCM	0.850-13.800	2.710	6.470	0.850	0.425
LMT	1.340-20.400	4.010	9.490	1.340	0.670
LTC	3.900-63.100	12.300	28.600	3.900	1.950
OXC	0.302-4.970	0.958	2.140	0.302	0.151
PEMA	0.714-11.700	2.190	5.170	0.714	0.357
PRM	0.099-1.430	0.292	0.661	0.099	0.050
PNB	3.720-53.100	9.220	22.000	3.720	1.860
PNT	1.650-27.500	5.060	12.600	1.650	0.825
PGB	0.671-11.500	1.930	4.650	0.671	0.336
PMD	1.630-29.100	5.330	13.000	1.630	0.815
RTG	0.133-2.330	0.409	0.974	0.133	0.067
RFM	2.770-42.300	8.310	19.500	2.770	1.385
SRP	1.050-14.600	3.150	7.420	1.050	0.525
SLA	0.941-12.000	2.540	5.740	0.941	0.471
TGB	0.023-0.360	0.063	0.151	0.023	0.012
TPM	1.200-18.100	3.510	8.250	1.200	0.600
VAL	8.240-112.000	22.900	51.800	8.240	4.120
VGB	1.340-21.00	3.850	9.560	1.340	0.670
ZNS	2.930-42.900	8.540	20.200	2.930	1.465

Precision and accuracy

The intra-run precisions and accuracies were estimated by analyzing five replicates containing AEDs at three different QC levels. The inter-run precisions were determinate by analyzing QC samples on three different days (one batch per day). The criteria for acceptability of the data included accuracy within ±15% deviation from the nominal values and a precision of within ±15% relative standard deviation**.** The results for intra-day and inter-day precision and accuracy for AEDs in serum quality control samples are summarized in Table 3.

Table 3. Precision an accuracy of the method for determining AEDs concentration in serum samples.

Analytes	Concentration added (µg/mL)		**Intra-day precision(n=5)**				**Inter-day precision (n=5)**			
	Level-1	Level-2	Precision (%)		Accuracy (%)		Precision (%)		Accuracy (%)	
			Level-1	Level-2	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2
10-OH CBZ	8.130	19.400	5.4	3.7	103.6	104.3	4.7	2.7	104.0	104.9
BVC	0.874	2.040	5.3	4.3	98.8	103.9	5.5	4.1	101.1	103.1
CBZ	4.390	9.930	6.4	1.7	96.1	99.7	7.9	3.8	98.7	98.6
CBZ-diol	1.580	3.810	6.3	5.2	94.3	105.6	6.5	5.9	96.6	101.1
CBZ-epoxide	1.830	4.180	2.8	4.3	100.1	101.8	4.6	3.4	100.4	101.1
N-DMS	8.900	19.900	2.1	2.6	94.6	96.8	2.6	2.3	96.4	96.3
ETH	22.000	51.000	5.7	1.1	99.8	98.0	5.2	1.7	102.3	98.6

FLB	20.300	48.300	2.1	3.3	97.9	103.1	2.6	2.6	97.3	104.3
GBP	4.950	11.700	1.8	2.3	105.1	106.4	2.3	3.8	103.4	104.5
LCM	2.710	6.470	2.5	3.6	99.0	104.2	2.8	3.4	100.5	104.1
LMT	4.010	9.490	3.0	1.6	98.7	103.6	3.4	2.2	98.9	103.3
LTC	12.300	28.600	3.6	1.7	105.0	99.9	4.0	1.8	103.7	99.4
OXC	0.958	2.140	4.6	5.3	93.8	104.1	4.0	5.0	95.7	102.0
PEMA	2.190	5.170	2.6	1.8	93.1	95.7	4.3	4.6	93.8	95.0
PRM	0.292	0.661	3.3	2.0	104.5	105.5	2.8	2.5	104.1	104.3
PNB	9.220	22.000	5.5	1.6	103.3	98.3	3.9	1.7	104.6	97.9
PNT	5.060	12.600	2.0	0.5	102.1	102.5	2.0	1.5	101.6	102.0
PGB	1.930	4.650	3.5	3.9	96.6	97.2	3.6	4.1	97.3	99.8
PMD	5.330	13.000	4.1	2.3	105.0	100.9	4.2	1.9	102.7	100.3
RTG	0.409	0.974	3.5	5.5	97.0	104.7	4.0	6.0	98.3	102.8
RFM	8.310	19.500	3.3	3.9	105.9	104.4	3.1	3.9	105.2	104.4
SRP	3.150	7.420	5.5	5.1	99.5	103.1	5.6	5.4	97.1	102.8
SLA	2.540	5.740	5.1	2.0	98.2	103.4	4.7	1.8	99.9	102.9
TGB	0.063	0.151	6.1	2.5	102.9	99.2	5.0	2.5	101.7	99.5
TPM	3.510	8.250	4.3	2.4	97.2	99.5	5.6	4.0	99.2	101.1
VAL	22.900	51.800	2.2	1.0	97.0	97.3	2.0	0.9	97.2	97.6
VGB	3.850	9.560	2.5	3.6	102.9	99.4	2.2	3.4	102.0	101.2
ZNS	8.540	20.200	4.9	5.4	97.5	100.4	4.7	5.4	99.6	100.3

Carryover effect

Carryover effects must be evaluated during assay validation intended for confirmation and/or quantitation. Carryover effect was assessed by injecting the processed blank sample just after the highest calibrator of CC in triplicate. Carryover in the blank samples following the highest calibration standard should not be greater than 20% of the analyte response at the LLOQ.

Stability

The stability of each analyte in serum was assessed by analyzing two concentration levels of QC samples with five determinations under different conditions, including kept at bench top stability, the results allowed us to conclude that analytes are stable for at least 15 h at room temperature in serum samples. The auto sampler stability was conducted by reanalyzing the extracted QC samples kept under auto sampler conditions (10°C) for 10 hrs. The stability experiments were performed exhaustively to evaluate the AEDs in serum sample under different temperature and timing conditions was evaluated as follows and the results of the stability studies are enumerated in Table 4.

Table 4. Stability samples result for AEDs concentration in serum samples.

Analytes	**Concentration added (µg/mL)**		**Mean calculated comparison sample concentration for BT**		**Mean calculated stability sample concentration for BT**		**Mean percentage change for BT**		**Mean calculated comparison sample concentration for ASS**		**Mean calculated stability sample concentration for ASS**		**Mean percentage change for ASS**	
	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2	Level-1	Level-2
10-OH CBZ	8.130	19.400	8.387	19.619	8.506	20.516	1.4	4.6	8.735	20.230	8.478	20.485	-2.9	1.3
BVC	0.874	2.040	0.838	2.138	0.86	2.088	3.1	-2.3	0.867	2.119	0.833	2.049	-3.9	-3.3
CBZ	4.390	9.930	4.372	9.649	4.220	9.836	-3.5	1.9	4.446	9.670	4.388	9.904	-1.3	2.4
CBZ-diol	1.580	3.810	1.563	3.779	1.490	3.723	-4.7	-1.5	1.579	3.684	1.510	3.934	-4.4	6.8
CBZ-epoxide	1.830	4.180	1.804	4.175	1.845	4.193	2.3	0.4	1.832	4.256	1.860	4.121	1.5	-3.2
N-DMS	8.900	19.900	8.662	19.060	8.415	18.745	-2.8	-1.7	8.752	18.713	8.593	19.260	-1.8	2.9
ETH	22.000	51.000	22.225	49.993	23.054	51.038	3.7	2.1	21.953	49.859	22.843	50.599	4.1	1.5
FLB	20.300	48.300	20.112	49.782	19.874	51.393	-1.2	3.2	19.628	49.418	19.415	50.990	-1.1	3.2
GBP	4.950	11.700	5.193	12.404	4.943	12.038	-4.8	-3.0	5.201	12.453	5.038	12.007	-3.1	-3.6

LCM	2.710	6.470	2.785	6.864	2.683	6.961	-3.7	1.4	2.765	6.742	2.691	6.726	-2.7	-0.2
LMT	4.010	9.490	3.868	9.847	3.975	10.000	2.8	1.5	3.957	9.769	4.002	9.832	1.1	0.6
LTC	12.300	28.600	13.263	18.863	12.611	18.579	-4.9	-1.5	12.911	18.403	13.335	18.308	3.3	0.5
OXC	0.958	2.140	0.910	2.192	0.899	2.142	-1.2	-2.3	0.934	2.225	0.886	2.157	-5.2	-3.0
PEMA	2.190	5.170	2.124	4.964	2.068	4.871	-2.6	-1.9	2.039	4.949	2.127	5.133	4.3	3.7
PRM	0.292	0.661	0.298	0.659	0.305	0.697	2.4	5.8	0.303	0.681	0.293	0.691	-3.4	1.4
PNB	9.220	22.000	9.231	21.468	9.760	21.626	5.7	0.7	9.526	21.990	9.733	21.970	2.2	-0.1
PNT	5.060	12.600	5.249	12.910	5.132	12.722	-2.2	-1.5	5.112	12.919	5.026	13.253	-1.7	2.6
PGB	1.930	4.650	1.835	4.670	1.891	4.518	3.1	-3.3	1.864	4.763	1.923	4.518	3.2	-5.2
PMD	5.330	13.000	5.462	12.820	5.355	13.116	-2.0	2.3	5.594	12.971	5.522	13.157	-1.3	1.4
RTG	0.409	0.974	0.399	0.988	0.407	1.020	2.2	3.2	0.397	0.983	0.414	1.043	4.2	6.0
RFM	8.310	19.500	8.714	20.144	8.798	20.014	1.0	-0.6	8.680	21.462	8.516	20.598	-1.9	-4.0
SRP	3.150	7.420	3.105	7.389	2.985	7.579	-3.9	2.6	3.134	7.907	3.073	7.796	-2.0	-1.4
SLA	2.540	5.740	2.450	5.957	2.525	5.928	3.1	-0.5	2.510	5.875	2.570	5.851	2.4	-0.4
TGB	0.063	0.151	0.065	0.150	0.063	0.151	-2.5	0.7	0.063	0.153	0.065	0.158	2.2	3.4
TPM	3.510	8.250	3.530	8.454	3.410	8.209	-3.4	-2.9	3.557	8.633	3.477	8.765	-2.3	1.5
VAL	22.900	51.800	21.838	51.768	22.203	50.408	1.7	-2.6	22.328	50.751	22.731	51.756	1.8	2.0
VGB	3.850	9.560	3.913	9.484	3.962	9.847	1.3	3.8	3.895	9.502	3.848	9.937	-1.2	4.6
ZNS	8.540	20.200	8.388	20.366	8.679	20.280	3.5	-0.4	8.360	20.243	8.733	21.024	4.5	3.9

Conclusion

A rapid, selective, linear, accurate, precise and cost effective method, which can be used routinely for the determination of the 28 AEDs in human serum, has been developed. This method has significant advantages in terms of clean and reproducible protein precipitation extraction procedure and a short chromatographic run time is only 6.0 min. The extraction method gave consistent and reproducible for AEDs from serum, with minimum interference and ion suppression. This validated method was successfully applied therapeutic drug monitoring of these antiepileptic agents in regular hospitals and reference laboratories for better therapeutic outcome.

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