

Identification and Quantitation Methods for Anti-Convulsant Drugs from Biological Matrix Relating to Forensic Cases

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Abstract

Context: Epilepsy, also known as “The Falling Sickness”, is a neurological disorder that occurs due to nerve cell disruption which results in improper signal transferring leading to seizures. Occurrence of abuse of Anti-convulsant drugs (ACDs) is increasing day-by-day, so the need for detection of these drugs due to their involvement in forensic cases.

Aims: In this research paper, Dispersive Liquid-Liquid Microextraction (DLLME) has been used for extraction of 4 ACDs-Lamotrigine (LMG), Oxcarbazepine (OXC), Valproic Acid (VA) and Topiramate (TPM).

Settings and Design: Ultrahigh Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS) was employed to analyze data utilizing multiple reaction monitoring (MRM) from biological matrix encountered in forensic and clinical scenarios.

Methods and Material: DLLME extraction method has been employed for isolation of 4 ACDs- LMG, OXC, VA & TPM from urine sample. Further quantification and analysis of drugs was employed by LC-MS/MS method.

Statistical analysis used: MS-Excel and GraphPad Prism softwares are used to calculate the linearity between the response and 6 different concentrations of standards taken for each drug.

Results: Linearity was obtained in the range of 5 – 200 µg/L for the targeted drugs. LOD and LOQ for the analytes were in the range of 7.5844 µg/ml to 12.1447 µg/ml and 22.9831 µg/ml to 36.8022 µg/ml. This method resulted in the highest recovery of 73 to 119 % for targeted drugs as compared to other published methods. The complete process followed ICH guidelines for optimization and validation of method.

Conclusions: A novel, sensitive and effective extraction method has been developed for concurrent extraction of 4 ACDs- LMG, OXC, VA & TPM from saliva sample. Values of LOD and LOQ obtained were lowest as compared to existing methods.

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Keyword: Epilepsy; DLLME, UHPLC-MS / MS; Analysis; Quantification.

Key Message: Anti-Convulsant Drugs, given for treating Epilepsy have been used for multidrug poisoning cases. New approach has been developed for simultaneous detection of these 4 drugs commonly found in such cases in forensic science - LMG, OXC, VA & TPM.



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INTRODUCTION

According to the World Health Organisation, Epilepsy has been characterized as fourth most common neurological disorder caused by repeating and unprovoked seizures resulting from sudden electrical disturbances in brain. The rate of epilepsy is increasing at an alarming rate causing around 50 million people to be affected by it. Developing countries are facing a rapid increment in epileptic cases with one patient in every 4-5 individuals being epileptic.

Anti-convulsant drugs (ACDs) or antiepileptic drugs, are the categories of drug prescribed by doctors to treat epilepsy. The commonly encountered ACDs can be further divided into first generation and second generation ACDs. Drugs that come under the former category are Valproic Acid, Carbamazepine, Phenytoin, etc and latter category are Lamotrigine, Oxcarbazepine, Topiramate some benzodiazepine. Also, the increasing cases of epilepsy directly impact the availability of these drugs in markets at commercial level.

Many papers have reported the cases related to overdosing of drug or self-intoxication using single or multiple drugs involving ACDs. Such cases are being reported from earlier years till recent days. In one case, a 28-year-old man was found dead due to overdosing of Valproic Acid. The concentration of valproate found in blood was 1050 mg/mL which was more than the toxic level.¹ Another case reported was of 41-year-old lady who was found dead at her home. Drug packages of TPM, CPM and FNP were found near the dead body. Further analysis showed high amount of topiramate (49 mg/L), more than therapeutic range in femoral blood, CPM (0.85 mg/L) & FNP in very low amount in peripheral blood. Higher concentration of TPM in association with CPM was found to be defined cause of death.² In another case report, a 64-year-old woman died by suicidal attempt taking MTZ, ELP and VA. The concentration of these drugs found in blood was 20.3, 65.5 and 417 mg/L; and was 17.0, 94.5 and 423 mg/L in urine which confirm the death due to intoxication of multiple drugs.³ One case reported was of intentional overdose of 2 new ACDs, mainly LMG and PGB. Death of the person was caused due to high amount of LMG in serum, 35.8 mg/L.⁴ In one of the fatality case, OXC was reported as the cause of death. Concentration of analyte found in femoral blood & gastric content was 66 mg/kg & 860 mg/kg which were found to be greater than the toxicity level responsible for the fatality of individual.⁵

There are some cases reported in which ACDs are responsible for causing many diseases such as Stevens-Johnson Syndrome⁶, Rhabdomyolysis⁷, Acute Live Failure⁸, Toxic Epidermal Necrolysis⁶ and many more.

Biological matrices play crucial role in forensic science as these allow the detection of drugs and their metabolites. There are many existing techniques for detection of these drugs in different biological matrices such as urine, plasma, blood, saliva, dried blood spot, *etc.* The process of detection of drugs starts with pre sampling, efficient extraction protocol followed by instrumentation analysis. Table 1 gives a brief regarding extraction method and instrumentation technique performed on different biological samples for confirming the presence of different types of ACDs.

A newly developed extraction method, DLLME, has been discussed in this paper which allows efficient extraction of 4 ACDs-LMG, OXC, TPM and VA simultaneously. DLLME is a miniature form of LLE techniques which was developed by Rezaee and other researchers. It utilizes extraction solvent, dispersing solvent and very low amount of sample for conduction of extraction process.⁹

Presently used extraction method is a ternary system-based method composed of biological samples, dispersing solvent and extraction solvent. Both dispersing and extraction solvents are injected into the biological sample using micro syringe leading to the formation of cloudy solution as extraction solvent disperse over the sample. Targeted analytes are absorbed by extraction solvent instantly after addition of solvent to the sample. Centrifugation process is performed leading to the sedimentation of extraction solvent with the targeted analyte at the bottom of tube. Sedimented products were then collected using micro syringe for further analysis.

The present research focus on optimization and validates a novel method combining both DLLME & LC-MS/MS for analysis of 4 ACDs from biological sample. Nevertheless, there is not much research for these ACDs. Present work will be niche for determination of mentioned ACDs from biological matrices. The utilized method was advantageous as it was simple in operation, fast, specific, highly efficient, environmentally benign, gave high recovery with employment of small amount of samples, extraction solvent and dispersing solvent.

MATERIALS AND METHODS

Chemical Reagent

Chemicals and reagents used in the process are Acetone, Chloroform, Methanol, Lamotrigine, Topiramate, Valproic Acid, Oxcarbazepine, Ammonium Format, Formic Acid, Distilled Water and Double distilled water were of analytical grade. Chemicals were obtained from Sigma Aldrich, St. Louis, MO, USA. The study was conducted at Amity

Institute of Forensic Sciences, Amity University, Noida, Uttar Pradesh, India.

Stock Solution

Methanol based stock solution of concentration 1mg/ml was prepared for all the procured ACDs (LMG, TPM, VA and OXC). Subsequently, working standard solutions of 6 calibrators (5, 10, 20, 50, 100 and 200 µg/L) were prepared by diluting stock solution in methanol.

Table 1: Developed methods and techniques for extraction and quantification of selected ACDs from different biological matrices

Analytes	Sample	Extraction Techniques	Instrumentation Techniques	References
VA	Blood	Direct Extraction	Gas- Liquid Chromatography	1
MTZ, ELP & VA	Blood & Urine	LLE	LC-MS/MS	3
TPM, CPM & FNP	Femoral blood, Bile, Kidney & Gastric Content	SPE	GC-MS	2
LMG	Heart blood, peripheral blood & liver	Alkaline extraction	EL-GC-MS	13
PMP & LMG	Human plasma	LLE	HPLC-DAD	14
CBZ, CBZ-10,11- epoxide, ECBZ, LMG, LVM, OXC, PTN, 4-hydroxyPTN & TPM	Urine	SPE	LC-MS-MS	10
ECBZA, OXC & CBZ	Plasma	SALLE	HPTLC	15
PTN, PNB, CBZ, CBZ-10,11- epoxide	Oral Fluid	PPE	HPLC-DAD	16
TPM	Plasma	LLE	Capillary electrophoresis with capacitively- coupled contactless conductivity detection	17
LVM, ZND, LMG, PLZ & PCP	Rat plasma and brain	LLE	HPLC	18
GBP, LMG, LVM OXC monohydroxy derivative & ZND	Serum	PPE	U-HPLC-MS/MS	19
CBZ, LMG, LVM & VA	Dried Blood Spot	PPE	LC-MS/MS	20
OXC, CBZ & ECBZA	Plasma or serum	Protein and Phospholipid precipitation	UPLC-MS/MS	21
VA	Human Serum	PPE	HPLC	11
PNB, PTN, CBZ & CBZ-10,11-epoxide	Human Plasma	SPE	HRMS/MS	22
LVM, LMG, ESX, FBM, RFD, ZND & monohydroxy CBZ	Dried Plasma Spots	Simple Precipitation	HPLC-UV	23
CBZ, LMG, OXC, PNB, PTN, CBZ-10,11-epoxide & LCBZ	Human Plasma	MEPS	HPLC-DAD	24
Levetiracetam & LMG	Blood	SPE	GC/MS	25
CBZ, CBZ-10,11- epoxide, GBP, LVM, lamhydroxy- derivative, PTN, TPM & VA	Human Plasma	PPE	LC-MS/MS	26
LMG & FBM	Human plasma	LLE	Reverse Phase HPLC	27
LVM, LMG, PNB, CBZ & CBZ- 10,11 epoxide	Dried Blood Spot	SPE	HPLC	28
OXC, CBZ, PNT & AZM	Human Plasma & Urine	MEPS	GC-MS	29
TPM	Plasma	SPE	GC-MS	30
VA	Saliva	SPE	LC-UV	31
ECBZA, ECBZ, OXC & R- LCBZ	Human Plasma	SPE	LC-MS/MS	32

Table Cont...

GBP, VBT & TPM	Human Plasma	SPE	HPLC-F	33
CBZ, OXC, ECBZA, CBZ-10,11-epoxide, 10,11-trans-dihydroxy-10,11-dihydro-CBZ & LCBZ	Human Plasma	SPE	HPLC-UV	34
OXC	Plasma	MEPS	LC-DAD	35
PTN, PNB, LMG & TPM	Human serum	SPE	Reversed Phase LC-MS/MS	36
OXC & 10- hydroxyCBZ	Plasma	LLE	LC-MS	37
TPM	Aqueous Solutions	SALLE	GC-MS	38
VA, 3-OH-VA, 4-ene-VA & 5-OH-VA	Human Plasma	SPE	LC-MS/MS	39

Abbreviations: VA: Valproic Acid; MTZ: Mirtazapine; ELP: Escitalopram; TPM: Topiramate; CPM: Citalopram; FNP: Flunitrazepam; LMG: Lamotrigine; PMP: Perampanel; CBZ: Carbamazepine; ECBZ: Eslicarbazepine; LVM: Levetiracetam; OXC: Oxcarbazepine; PTN: Phenytoin; ECBZA: Eslicarbazepine Acetate; PNB: Phenobarbital; ZND: Zonisamide; GBP: Gabapentin; ESX: Ethosuximide; FBM: Felbamate; RFD: Rufinamide; AZM: Alprazolam; LC-MS/MS: Liquid Chromatography- Tandem Mass Spectrometry; GC-MS: gas Chromatography- Mass Spectrometry; EI-GC-MS: Electron Ionisation GC-MS; HPLC-DAD: High Performance Liquid Chromatography- Photodiode Array “Detection” and “HPTLC”: High Performance Thin Layer Chromatography; U-HPLC-MS/MS: Ultra-High Performance Liquid Chromatography- Tandem Mass Spectrometry; UPLC: Ultra-performance liquid chromatography; HRMS/MS: High Resolution- Tandem Mass Spectrometry; HPLC-UV: HPLC- Ultra Violet Detection; HPLC-F: HPLC- Fluorescence Detection; LC-DAD: Liquid Chromatography- Photodiode Array Detection

Matrix

Preparation of biological matrix (Urine)

Simulated urine was prepared using 13 components which are mentioned in the table below (Table 2).

Table 2: Chemical Composition for synthesis of simulated urine

Compound	Chemical Formula
Urea	$\text{CH}_4\text{N}_2\text{O}$
Uric Acid	$\text{C}_5\text{H}_4\text{N}_4\text{O}_3$
Sodium Chloride	NaCl
Creatinine	$\text{C}_4\text{H}_7\text{N}_3\text{O}$
Sodium Sulfate	Na_2SO_4
Trisodium citrate dihydrate	$\text{NaC}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$
Potassium Chloride	KCl
Calcium Chloride	CaCl_2
Ammonium Chloride	NH_4Cl
Potassiumoxalate monohydrate	$\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$
Magnesium Sulfate Heptahydrate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
Sodium Phosphate Monobasic Dihydrate	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$
di-Sodium Hydrogen Phosphate Dihydrate	$\text{Na}_2\text{H}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$

DLLME Extraction

For DLLME procedure to take place, a glass tube with conical bottom was taken into which around 10 mL of spiked diluted sample was added. 960 μL

Acetone (Disperser Solvent) and 40 μL Chloroform (Extraction Solvent) was added to the tube. A cloudy solution appear immediately after adding both the solvents. The sample was then taken for centrifugation at 6000 rpm for about 6 minutes which resulted in sedimentation of organic phase to the bottom. Around 10 μL sedimented organic phase was taken for chromatographic analysis.

Instrumentation & LC-MS/MS Condition

Samples were extracted using DLLME, followed by quantitation using an Agilent 6470 platform LC-MS/MS. 5 mM Ammonium Format in 0.1% formic acid in water was taken as Mobile Phase A and 0.1% formic acid in 100% methanol was taken as Mobile Phase B. 10 μL of extracted sample was taken for analysis at column temperature of 55°C and gas flow rate of 6 L/ min using gradient method. The gradient of the mobile phases with time are given in table 3.

Table 3: Gradient of Mobile Phase A and B for chromatographic analysis

Time (in min)	A%	B%
0	95	5
0.5	95	5
10	60	40
12	40	60
15	5	95
16	5	95
18	90	10
20	95	5

In order to have effective information from various analyte peaks, the utilized method was operated in MRM mode.

Method Validation

The method used in this work was validated as per guidelines of ICH Tripartite (International Council of Harmonisation for Technical Requirements for Registration of Pharmaceuticals or Human Use Guidelines) Validation of Analytical Procedures: Text and Methodology (Q2(R1)).

The relationship between the response and concentration was measured in terms of area under the peak, also termed as linearity was determined by separate runs for all the 6 calibrations mentioned above. Weighted least square linear regression (WLSLR) was used for obtaining calibration curve along with other parameters such as slope, intercept and coefficient of regression (r^2). At the subsequent time, Relative standard deviation (RSD) was calculated for these parameters.

Limit of Detection (LOD) is the minimum amount of target analyte that can be detected using any method without any interference but cannot be quantified accurately. Limit of Quantification (LOQ) is the smallest concentration of the targeted analyte that can be measured by using particular method with accuracy and precision on the daily basis. LOD and LOQ were calculated for all the drugs at above-mentioned calibrations. As per ICH guidelines, accuracy must fall within the range of $\pm 20\%$ and precision should be below 20%.

Inter and intraday accuracy and precision were calculated. Inter-day parameters were assessed at 6 different concentrations (5, 10, 20, 50, 100 and 200 $\mu\text{g/L}$) for 3 consecutive days utilizing urine as biological sample. Subsequently, Intraday parameters were measured through 6 replicates for 3 concentrations, mainly lower, middle and upper concentration levels.

The absolute recovery for all the targeted analytes were calculated for above-mentioned 6 calibrations by comparing the analyte concentrations obtained after analysis with the amount of the analyte spiked in the biological matrix.

Matrix effect, in also defined as the interference of matrix on the extraction process and estimation of target analytes from the biological matrix. Matrix effect impacts the ionization ability of target analyte which either increases or decreases leading to the recovery ranges from 70 to 120%.

RESULTS AND DISCUSSION

Optimization of sample preparation: DLLME condition

Sample preparation and extraction procedure is an important part in quantitative analysis from biological matrix for targeted drug analysis. DLLME is an extraction procedure to separate targeted analytes from biological samples. It was developed for isolation of analytes from environmental samples and has been successfully employed for biological samples by researchers. DLLME, being simple, quick and reliable method was used to isolate 4 ACDs- LMG, OXC, VA & TPM from biological samples.

During the process, 10mL of diluted spiked sample was taken in conical bottom test tube. 1mL of acetone (disperser solvent) containing 40 μL of chloroform (extraction solvent) was injected into the test tube leading to the formation of cloudy solution. Sample was then centrifuged at 6000 rpm for 6 minutes resulting in formation of sediment at the bottom. A fraction (1 μL) of sediment was then taken using micro syringe for instrumental analysis.

In the present paper, DLLME procedure has been optimized based on various parameters, such as selection of extraction solvent, selection of disperser solvent, effects of volumes of disperser and extraction solvent, for achieving high and efficient recovery of targeted analytes. Various extraction solvents such as chloroform, dichloromethane and tetrachloroethylene were used along with different disperser solvents such as methanol, acetone and acetonitrile. Highest recovery for isolation of targeted drug was achieved when 960 μL of acetone as disperser solvent and 40 μL chloroform as extraction solvent were used.

Method validation

The presence of endogenous substances and other biological molecules were analyzed in the biological matrix. Matrix effect can occur in the form of Ion suppression which results in less signal intensity of targeted analyte and ion enhancement which leads to overestimation of drugs as compared to their true values. Chromatograms for the blank matrix sample and sample spiked with targeted drugs were studied as shown in fig.1. Retention times for LMZ, OXC, VA and TPM were at 10.003, 9.028, 7.452 & 11.995 minutes respectively. There was no indication of endogenous influence of the matrix and other biomolecules during the process.

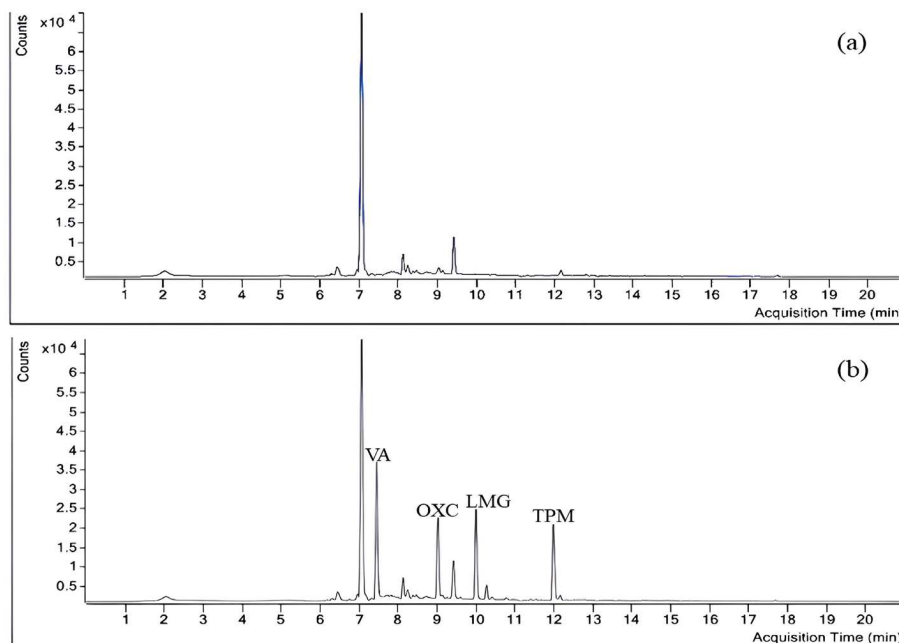


Fig. 1: Chromatogram of (a) Blank Matrix sample, (b) Spiked matrix with selected ACDs at 100 ppb

Interference Test: Quantification of 4 ACDs was done simultaneously. The data presented in table 4 shows that the presence of other drugs does not interfere with the detection of any particular drug as the recovery found to be same when a drug was extracted from the individual solution and a mixed solution with other drugs as well. This shows that simultaneous detection of LMZ, OXC, TPM and VA can be done in future analysis as well.

Linearity of the developed analytical method was observed for six concentrations (5, 10, 20,

50, 100 & 200 $\mu\text{g/L}$) in three replicates. Weighted Linear Regression Analysis was employed for obtaining calibration curve which shows a direct relation between the peak area and concentration of drug in biological matrix with coefficient of regression (r^2) ≥ 0.999 . Calibration curves for all the 4 drugs are shown in fig. 2.

Table 5 summarizes linear regression equations, value of coefficient of regression along with LODs & LOQs obtained for 4 analyzed drugs.

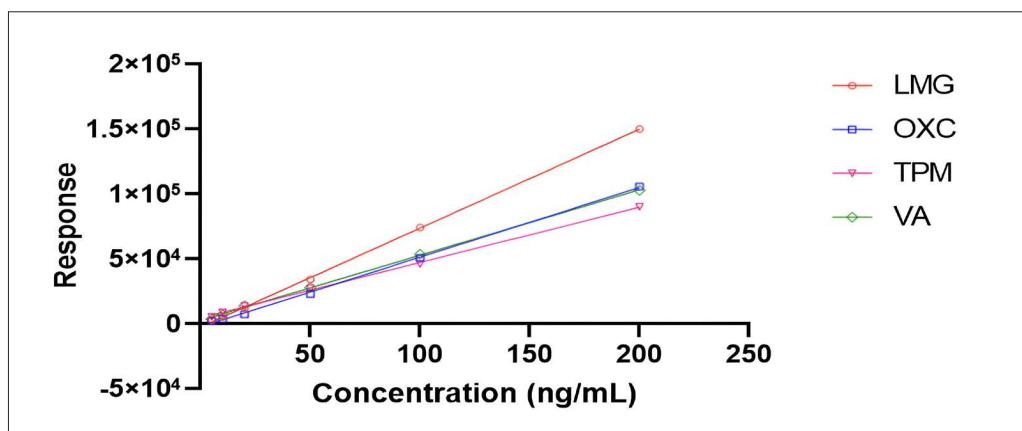


Fig. 2: Calibration curve of all the 4 drugs

As shown in table 6, LLOQ defined for all 4 drugs were defined as 5 $\mu\text{g/L}$, with suitable precision [in terms of coefficient variation (% CV)] and accuracy (in terms of % biasness). The LODs and LOQs

obtained for LMG, OXC, TPM & VA from the developed method are lowest as compared to the reported method by other researchers in the same biological matrix.

Table 4: Impact of Interference on recovery of individual drugs

Drug	Concentration (in µg/L)	% Recovery of drug (in individual solution)	% Recovery of drug (in combined solution of drugs)
OXC	5	123.556	123.556
	50	93.431	93.431
	200	102.86305	102.86305
LMG	5	119.112	119.112
	50	95.547	95.547
	200	101.46925	101.46925
TPM	5	73.01	73.01
	50	107.3964	107.3964
	200	98.6911	98.6911
VA	5	75.316	75.316
	50	101.3344	101.3344
	200	97.82335	97.82335

LC-MS/MS Conditions

LC-MS/MS conditions were improvised and applied to obtain uniform and well-balanced peaks in the chromatogram. Column temperature, mobile phases, solvent flow rate, pH of the buffer were analyzed in a way to obtain efficient and good results. Mobile Phase A used was a mixture of Ammonium Formate and Formic Acid as former narrow downs the peak and later helps in enhancement of the

peaks. For all four ACDs, the ESI-MS regulation was carried out in positive ion mode.

Chromatograms obtained after LC-MS/MS analysis for concurrent detection of 4 ACDs- LMG, OXC, TPM & VA at 6 different concentrations (5, 10, 20, 50, 100 & 200 µg/L) are shown in fig. 3. Retention times for LMG, OXC, TPM & VA are 10.003, 9.028, 7.452 & 11.995 minutes respectively.

Table 5: Linear Range, Linear Regression Equation, R², LOD, LOQ values for selected ACDs

Analyte	Linear Range (in µg/L)	Linear Regression Equation	R ²	LOD (in ng/mL)	LOQ (in ng/mL)
LMG	5-200	$y = 761.92x - 2649.4$	0.9996	7.58443	22.9831
OXC	5-200	$y = 536.75x - 2480.1$	0.9991	10.6875	32.3864
TPM	5-200	$y = 426.69x + 4415.6$	0.9989	12.1447	36.8022
VA	5-200	$y = 503.3x + 2406.4$	0.9996	7.73367	23.4354

By comparing the developed methods with the prior developed methods, it is found that the present method has various advantages involving novelty, utilization of low amount of samples and less time, more sensitivity and more efficiency for concurrent extraction of 4 ACDs - LMG, OXC, TPM & VA from biological matrices. Feng *et al.* in their paper isolated CBZ, CBZ-10, 11-epoxide, Eslicarbazepine, LMG, OXC, PHN, Hydrophenytoin & TPM from urine sample utilizing SPE followed by LC-MS/MS for the linear range from 50-5000 ng/mL. LOQs for PHN, Hydrophenytoin, Eslicarbazepine, OXC, LMG & TPM found to be 500 ng/mL and for CBZ and CBZ-10,11-epoxide, it was 50 ng/mL.¹⁰ As per

Zhao *et al.*, LOQ for VA was found to be 1 µg/mL after its extraction from serum using PPE-UPLC-MS.¹¹ LMG was quantified in whole blood for the “linear” range from 0.50-50.0 ug/mL using SPE followed by GC-MS. LOD and LOQ found for LMG were 0.15 ug/mL and 0.50 ug/mL.¹² LODs and LOQs in the proposed study were found to be less as compared to the values proposed by existing methods. Also, there are very limited methods developed for extraction and isolation of selected drugs from urine sample. The proposed method in this research can be used in forensic science laboratories as well as therapeutic drug monitoring for detection and quantification of selected ACDs from urine sample.⁴⁰⁻⁴⁵

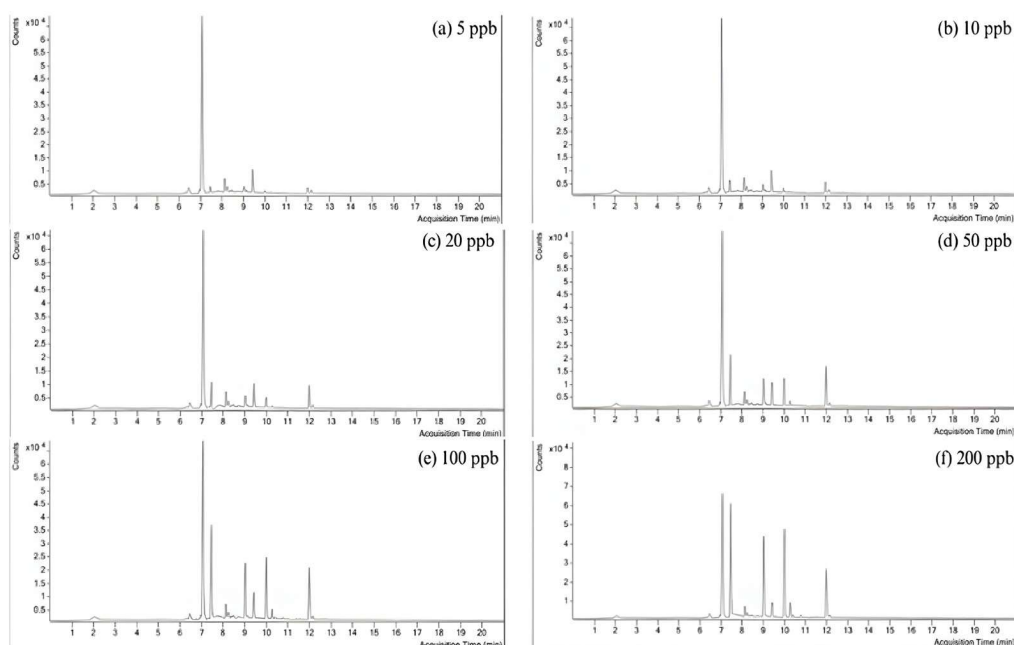


Fig. 3: LC-MS/MS Chromatogram for LMG, OXC, VA and TPM at concentrations (a) 5 ppb, (b) 10 ppb, (c) 20 ppb, (d) 50 ppb, (e) 100 ppb and (f) 200 ppb in biological matrix

Table 6: Recovery, RSD%, Interday and intraday precision for selected ACDs

Analytes	Conc. ($\mu\text{g/L}$)	Interday		Intraday	
		Precision (% CV)	Accuracy (% Bias)	Precision (% CV)	Accuracy (% Bias)
LMG	5	2.05	16.21	3.52	-7.44
	50	1.77	-5.21	4.3	-5.52
	200	1.03	0.027	0.34	-3.55
OXC	5	1.77	20.92	8.07	-7.6
	50	2.6	-8.36	0.93	-8.46
	200	0.89	1.61	1.46	-0.43
TPM	5	0.35	-26.63	4.52	-27.56
	50	3.02	4.57	1.64	-0.39
	200	1.2	-2.95	0.34	-1.35
VA	5	4.5	-23.22	1.95	-26.46
	50	1.69	1.8	2.82	-2.08
	200	1.05	-2.58	0.43	-1.67

Abbreviations: OXC: Oxcarbazepine; LMG: Lamotrigine; TPM: Topiramate; VA: Valproic Acid; CV: Coefficient of Variation

CONCLUSION

The main objective of this research was to develop an effective and sensitive method for detection and quantification of selected ACDs-LMG, OXC, TPM & VA from biological matrix. DLLME in combination

with LC-MS/MS has proved to be highly accurate and reliable method as lowest values of LOD and LOQ were achieved as compared to the existing methods. Values of LOD for LMG, OXC, TPM & VA were 7.58443, 10.6875, 12.1447 & 7.73367 ng/mL. The LOQ values for LMG, OXC, TPM & VA were 22.9831, 32.3864, 36.8022 & 23.4354 ng/

mL. Linearity between peak area and concentration were obtained for concentration ranging from 5- 200 µg/mL with recovery percentage varying from 73% to 123%. Developed method was found

to be cost-effective and reproducible which can be further performed on real samples for detection for selected ACDs with their application in forensic science laboratories and therapeutic drug analysis.

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