Original Article

CORRELATION OF BLOOD LEVETIRACETAM LEVEL WITH CLINICAL SYMPTOMS AFTER VALIDATION BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY IN EPILEPTIC PATIENTS UNDER THERAPY

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ABSTRACT

Objective: To correlate blood levels of Levetiracetam with clinical symptoms after validating Liquid Chromatography–Tandem Mass Spectrometry method in epileptic patients on therapy.

Material and Methods: This Cross-sectional study was conducted at Toxicology Department, Armed Forces Institute of Pathology, Rawalpindi from July 2018 to December 2019. Blood samples were taken from 120 adult patients of both genders taking Levetiracetam. Samples were extracted with an efficient in-house extracting solvent and were transferred for analysis. Separation was achieved with Agilent Poroshell 120 EC-C18 column (2.1 x75mm, 2.7 micron). A Tandem mass spectrometry was utilized for detection and quantification of drug.

Results: Among 120 patients, 58 (48.3%) were males and 62 (51.7%) were females. The average concentrations of Levetiracetam was 24.54 ±19.50 µg/ml. Mean age was 25.05±6.80 years. History of recurrent fits was present in 28(23.3%). Duration of intake showed 45(37.5%) for less than five months, 65 (54.2%) for five months to one year and only 10 (8.3%) had taken it for more than one year without monitoring. Drug levels was < 10 µg/ml in 28 (23.3%) patients and 10 (8.34%) were having toxic levels ≥ 41 µg/ml in blood. RFTs and LFTs were deranged in 3 (2.5%) patients. Levetiracetam level was significantly (p-value=0.007) different among patients with fit history and (p-value=0.003) among RFTs and LFTs groups while Test of ANOVA of Levetiracetam level showed significant p-value <0.001 in duration of intake groups. Method validation showed accuracy > 90%±10 in three calibrators with linearity from 4.0 to 17 µg/ml. Limit of detection and quantitation were 0.5 µg/ml and 2.0 µg/ml respectively. Precision interassay mean ± SD/CV% µg/ml was 12.9 ± 2.7/1.2 and intra assay was 12.4 ± 8.2/1.4.

Conclusion: Levetiracetam were highly correlated with clinical symptoms, duration of intake. Method validation showed significant precision, accuracy and applicable to patient taking this drug.

Key Words: Levetiracetam**,** Limit of quantitation (LOQ), Liquid chromatography-mass spectrometry mass spectrometry (LCMS/MS).

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INTRODUCTION

Levetiracetam (LEV) is among the recent antiepileptic drugs which showed up in the year 2000 [1]. A linear relationship exists between the dose and the serum levels of Levetiracetam but various studies and experiments have proposed that serum levels of the drug can be affected by a variety of factors [2]. Level for Levetiracetam in blood is challenging due to its methodology [3]. Pharmacokinetic parameters of Levetiracetam are not consistent and predictable so Therapeutic drug monitoring (TDM) is needed for proper dosing of this drug and to avoid toxic effects related to drug overdose [4]. Another reason for TDM is recurrent seizures which are due to inadequate intake, hence, is mandatory for clinical benefit and patient compliance [5,6]. TDM of Levetiracetam is also important in patients who have changes in the pathophysiological state that affects renal, hepatic status. Dose individualization is an important element in prescribing antiepileptic's [7]. It is typically achieved by trough level of drug monitoring of patients after achieving the steady state, which is typically after 4 to 5 half-lives [8]. In our setup clinicians have expressed the pertinent need to adapt treatment by antiepileptic drugs using TDM [9]. Therefore, we decided to validate an analytical method for accurate quantification of blood level of Levetiracetam by liquid chromatography tandem mass spectrometry (LC-MS/MS) for the effective monitoring of treatment in patients undergoing Levetiracetam therapy [13] as this drug level was not frequently, analyzed in most Pakistani laboratories so far and later, find its blood level correlation with clinical history.

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MATERIAL AND METHODS

This study was conducted in Toxicology Department, Armed Forces Institute of Pathology, Rawalpindi from July 2018 to December 2019.

Ethical approval was taken from Institutional Review Board (Cons-CHP-6/READ-IRB/19/1048). Adults of both genders were included in study while children, pregnant women and patients with chronic diseases were not included in the study. Blood sampling was taken at trough level (just before the next dose) from 120 subjects on Levetiracetam therapy after taking consent. Detailed history proforma was filled for each patient which contains time of sampling, duration of therapy and history of fits during therapy. Blood was also analyzed for serum urea, creatinine, bilirubin, Alkaline phosphatase (ALP), Asparate transaminase (AST) and alanine transaminase (ALT) by colorimetric/enzymatic methods on Advia 1800. ClinCal® calibrator (Serum Calibrator lyophilized for Levetiracetam Keppra® LOT 1026 (1mg/ml) was obtained by RECIPIE Chemicals Germany. Calibrator was prepared by adding 3.0 ml HPLC-water to the vial and mixing for 15 minutes and serial dilutions were prepared accordingly. ClinChek® control level LOT 1506 with concentration (11.0 -16.6) µg/ml were purchased from RECIPIE company Germany. Controls were also prepared according to the mentioned instructions. Deuterated analogues, THC-d3(1 mg/mL) was used as the internal standard (Cerilliant Corporation, USA). Methanol LCMS grade, Acetonitrile, formic acid and Ultrapure water from Millipore apparatus (Merck-Germany) was used in the study.

Blood sample was collected in serum separator tube. After centrifugation serum was separated for analysis. 100 ul of serum is treated with 900 ul of extracting solvent containing Acetonitrile: Methanol: 0.1% formic acid in ratio of 2:2:1. The sample was then incubated for 1 hour at room temperature followed by centrifugation at 10,000 rpm for 30 minutes. The supernatant was collected and filtered by 0.2-micron filter into GC vial and injected into the LC–MS/MS system. Poroshell 120 EC-C18 column (2.1 x75mm 2.7 micron) was used for the Chromatographic separation of the drug [10]. The volume dispensed by injector was 10µl and the column was maintained at a temperature of 40°C. Separation was achieved at a flow-rate of 0.5mL/min, using a isocratic gradient elution for 05 mins with (A) mobile phase consisting of 0.1%formic acid in water (60%), and (B) mobile phase consisting of 0.1% formic acid in Acetonitrile (40%). A 0.2 µ membrane filter was incorporated for the filtration of two components of the mobile phase and degassed by an ultrasonic bath for 3 minutes**.** Post run time was one minute. For quantitative analysis of Levetiracetam, the detector was functioned under multiple reaction monitoring (MRM) mode. For desolation and collision Nitrogen gas was used. Mass Spectrometry (MS) parameters and acquisition data are tabulated in Table-I.

Method for Levetiracetam detection was validated in terms of precision, accuracy, linearity, Limit of detection (LOD), Limit of quantification (LOQ), Carry over, interference studies and selectivity by finding of no response in 05 blank samples according to the recommended guidelines [11-12]. Statistical Package of Social Sciences v 21 was used for the analysis of data for both descriptive and inferential statistics by independent t-test and test of ANOVA considering $p<0.05$ as statistically significant.

Table-I: MS parameters for detection of levetiracetam in human blood.

Parameter	
Scan Type	MRM
Polarity	Positive
Gas Temperature	350°C
Gas Flow	10 L/min
Sheath Gas Temp	293°C
Sheath Gas Flow rate	3 L/min
Nebulizer pressure	45 Psi
Capillary Voltage	4000 V
Nozzle voltage	500V
Chamber current	0.19µA
Acquisition parameters	Precursor ion-171.01 Product ion-126.1, 69.1 Dwell-50 (for both product ion) Fragmentor- 70 (for both product ion) Collision energy-9 for 126.1 product ion and 15 for 69.1 product ion

RESULTS

Among 120 patients, 58 (48.3%) were males and 62 (51.7%) were females. The average concentrations of Levetiracetam was 24.54 ±19.50 µg/ml. Mean age was 25.05±6.80 years. Liver function tests (LFTs) and renal functions tests (RFTs) (Mean ±SD) showed {urea (mmol/l) 6.9±3.4, creatinine (umol/l) 101±9.8, bilirubin (umol/l) 12.0±3.2, ALT (U/L) 35±9.5, ALP (U/L) 195±14.2 and AST (U/L) 38±9.9}. On evaluation; 28(23.3%) had history of recurrent fits and 92 (76.7%) had no fit history. Duration of intake showed that 45(37.5%) had taken this medicine for less than five months, 65 (54.2%) for five months to one year and only 10 (8.3%) had taken it for more than one year without monitoring. Drug level was < 10 µg/ml in 28 (23.3%), (10 – 40 µg/ml) optimum drug levels i.e. within reference range in 82 (68.3%) while 10 (8.34%) patients were having toxic levels ≥ 41 µg/ml of Levetiracetam in blood. RFTs and LFTs were deranged (>upper reference value) in 3(2.5%) in patients who had taken drug for greater than one year without monitoring. Levetiracetam level was significantly (p-value=0.007) different among patients with fit history and (p-value=0.003) among RFTs and LFTs groups (within reference range and >upper reference value) while was insignificant (p value=0.918) in gender

group. Test of ANOVA of Levetiracetam level showed significant difference (p-value <0.001) in duration of intake groups. Baseline characteristics of study population was shown in (Table-II).

Table-II: Baseline characteristics of study population (n=120).

Parameters	Characteristics	p-value□
Gender n(%)		
Male	58(48.3)	0.918
Female	62(51.7)	
Fits n(%)		
No Fit	92(76.7)	0.007
Recurrent Fits	28(23.3)	
RFTs and LFTs n(%)		
Within reference range	117(97.5)	0.003
>upper reference value	3(2.5)	
Duration of intake n(%)		
<5months	45(37.5)	0.001*
5 months to one year	65 (54.2)	
>one year	10 (8.3)	

□p-value was calculated by independent t-test
*p-value was calculated by Test of ANOVA

Method validation of Levetiracetam showed calibration curve was prepared with five different concentration of calibrator (2.0, 4.0, 8.22, 16.4 and 32 µg/ml) spiked in blood samples in five replicates which showed accuracy of 76.0, 105.5, 105, 98.1 and 127 % respectively and linearity from 4.0 to 17 µg/ml. Limit of detection LOD was 0.5 µg/ml, limit of quantitation LOQ was 2 µg/ml and Analytical Measurement Range (AMR) was 17.2 µg/ml. Specificity and selectivity of Levetiracetam standard was optimize to the retention time and the specific quantification product ion. Chromatogram showing precursor and product ions of Levetiracetam and linear calibration curve with three calibrators whose accuracy were >90±10% were shown in Figure 1. Precision study was done with Levetiracetam control, 10 replicates were run each day for 07 days which showed inter assay precision mean ± SD/CV% µg/ml was 12.9 ± 2.7/1.2 and intra assay precision mean ± SD/CV% µg/ml was 12.4 ± 8.2/1.4. Carry-over <1% was accessed by running high concentration levetiracetam patient sample followed by low concentration in three replicates. Robustness, interferences with lipemia, icteric and haemolyzed samples were also analyzed on three samples and no interferences were found. Method validation characteristic of Levetiracetam was shown in Table-III.

Table-III: Method validation characteristic of levetiracetam in human blood on (LCMS/MS).

Expected concentration of Calibrator (µg/ml)	Average recovered concentration of calibrator (µg/ml)	Accuracy (%)	LOD (µg/ ml)	LOQ (µg /ml)	AMR (µg/ ml)
2.0	1.52	76	0.5	2.0	17.2
4.0	4.22	105.5			
8.22	8.63	105			
16.4	16.0	98.1			
32.0	40.64	127			

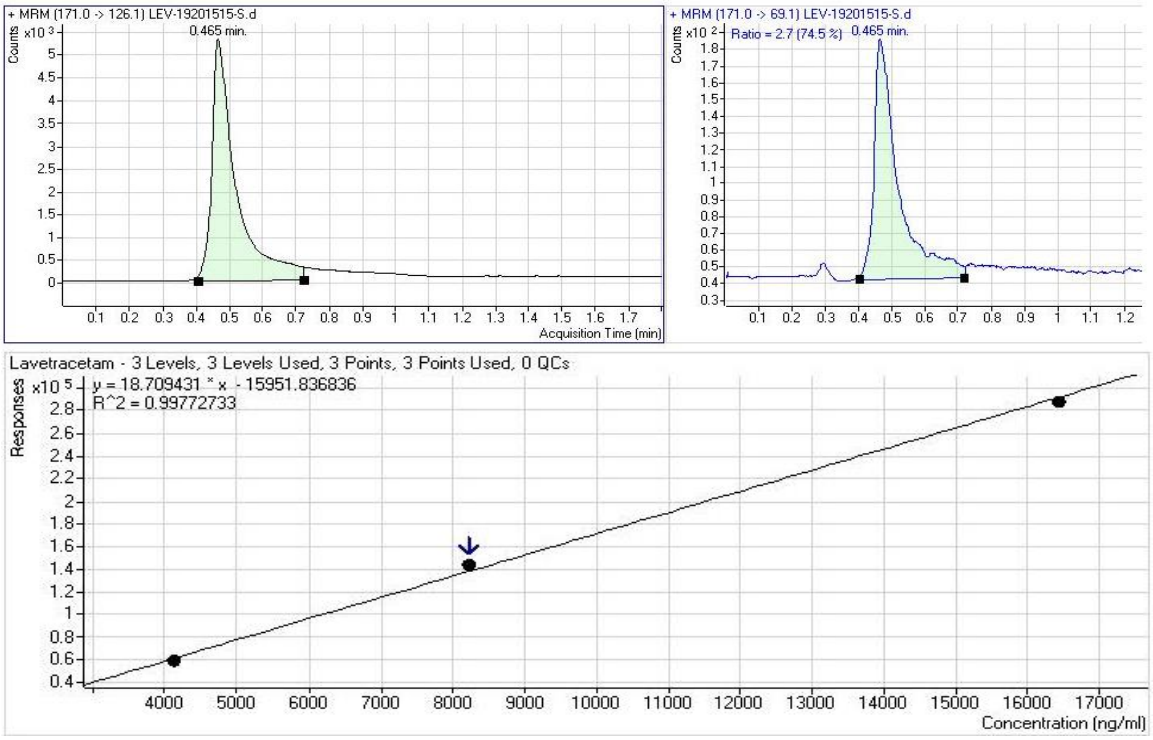

Figure-I: Chromatogram of Levetiracetam in human blood on LCMS/MS, linear calibration curve for Levetiracetam (n=3) in human blood using QQQ (LCMS/MS).

DISCUSSION

Our study showed that Levetiracetam method on LCMSMS was accurate, specific and applicable for monitoring as this drug shows significant difference in different clinical setting. The individuals who had history of recurrent fits, their Levetiracetam levels were also below therapeutic levels and their duration of intake was also less than five months. Due to either inadequate intake or non-compliance, steady state of this drug was not achieved in these patients most likely. Our study also showed that inadequate monitoring and continuous administration of this drug over one year led to development of toxic symptoms like deranged liver and renal function tests. These toxic symptoms mostly neglected due to poor awareness and non-availability of analysis in Pakistan. In the UK About 8% of [13] in contrast to our study where most patients were adults. Most patients had poor compliance and their levels were below therapeutic range showing inefficacy of this drug which was evident by their uncontrolled seizure history [11].

It is an effective second-generation drug which was approved in Europe in 2000 for adult use. Levetiracetam after oral administration is almost completely absorbed. Bioavailability of the drug is nearly 100 % [12]. Rise to peak levels in one hour and steady state concentration is maintained in two days if taken twice daily. Drug follows linear pharmacokinetics, time independent and dose proportional as evident by our study. In proper dosage this antiepileptic drug besides the advantage of controlling the seizure also has neuroprotective effects. Levetiracetam is known to cause harmful effects if not prescribed in proper therapeutic doses. It has a narrow therapeutic window because of which TDM becomes necessary. These were cardiotoxicity and renal impairments, commonly noted [13]. So, a strict surveillance is recommended for monitoring of treatment.

Different methods have been known for estimation of Levetiracetam in blood [14]. It includes Immunoassays, Capillary electrophoresis, GC-MS and LC-MS/MS technology [15] and our results were similar to these previous studies. Liquid chromatography tandem mass spectrometry (LC-MS/MS) became the reference method for analysis. The methodology of LC-MS/MS has transformed the area of bioanalysis and has radically appeared as a key instrumental element for the routine practice of laboratory medicine [16]. It has merit of a unique procedure for sample extraction and short analytical run. With the help of the creation of simultaneous calibration curves this technique is time saving, moreover the application is simple to blood samples from patients receiving antiepileptic treatment. In a study conducted by Karas *et al*, the authors have claimed to develop a method on HPLC-MS/MS with short analysis time and lower limit of quantitation (LOQ) i.e. 1.0 µg/mL with superior sensitivity [20-21]. Most of the steps involved in sample extraction in current study were in accordance with the guidelines and [22]. For appropriate extraction and quantitative analysis of Levetiracetam in blood, samples were treated with an efficient in-house extracting solvent containing ACN: Methanol: 0.1 % formic acid in water. Unlike previously, reported GC/MS methods, this method has the perk of simple sample preparation i.e. without time consuming derivatization. The emerging advancements in LC-MS/MS analysis for therapeutic drug monitoring open doors for a new era of personalized medicine and will broaden the horizon related to clinical judgement and dose individualization. This LC-MS/MS assay is proved suitable for clinical routine TDM in patients treated for epileptic or psychiatric disorders. It is also equally important for assessing drug levels in emergency cases after over dosage or intentional intoxication and inefficacy cases.

CONCLUSION

The described method utilizing LCMS technology allows detection and quantitation of Levetiracetam in human blood with significant precision and accuracy. Its levels were highly correlated with clinical history of fits, compliance and duration of its intake.

AUTHOR CONTRIBUTION

Muhammad Aamir & Safia Fatima: Devised the idea, analysis, acquire data and drafts manuscript.
Sehrish Naz, Zujaja Hina Haroon, Sobia Irum Kirmani & Muhammad Usman Munir: Contributed in data entry and contributed in manuscript reviewing.

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